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NOVEL TECHNIQUES FOR THE *IN SITU* DETECTION OF BACTERIA ON SALAD LEAF SURFACES

By

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ABSTRACT

NOVEL TECHNIQUES FOR THE *IN SITU* DETECTION OF BACTERIA ON SALAD LEAF SURFACES

by Jennifer Warner

Bacteria such as *Escherichia coli* and *Salmonella* are capable of causing gastrointestinal illness if ingested with food. There is an urgent need within the food processing industry to improve the efficiency of both the sanitising processes used on fresh produce and the microbiological methods used to ensure its safety. The aims of this study were to develop novel microscopic methods to detect and quantify both indigenous leaf bacteria and viable human pathogens on salad leaves, without the need for recovery and culture, which may reduce the accuracy of quantification. The methods developed could then be used to quantify the number of indigenous and inoculated bacteria *in situ* on salad leaves as well as to identify factors affecting the quantity and spatial patterning of attachment. In addition, the efficacy of chemical biocides for the reduction of viable *Salmonella* on salad leaves was considered.

Episcopic Differential Interference Contrast (EDIC) microscopy coupled with epifluorescence was used to rapidly and non-destructively view the natural microflora *in situ* on spinach leaves. *Salmonella enterica* serovar Thompson was inoculated onto spinach leaves in order to observe spatial and temporal patterning of colonisation under differing conditions. *Salmonella enterica* serovar Typhimurium mutants defective in curli fimbriae production were used to assess the role of curli in attachment to abiotic surfaces and leaves. Viability determination of potential viable but non-culturable (VNC), sub-lethally stressed cells was performed to assess the effect of chemical wash treatments on *Salmonella* attached to fresh spinach.

The results obtained indicate that salad leaves are densely populated with naturally occurring bacteria; these are found predominantly in the margins between leaf epidermal cells as well as around leaf veins and stomata. Cells were present in complex three-dimensional aggregations, suggesting the presence of biofilms. Curli fimbriae were shown to be key in the attachment of *Salmonella typhimurium* to polystyrene but not to leaf surfaces. When *Salmonella thompson* was inoculated onto spinach leaves and then subjected to chemical washing in chlorine or Citrox, neither chemical was an effective biocide against the pathogen. Under-reporting of viable cell numbers by plate counting methods indicated that chlorine induced a viable but nonculturable (VNC) state amongst *Salmonella*.

These findings have important implications for the sanitisation of salads; both stomatal penetration and the formation of biofilms could protect enteric bacteria on leaves from chemical and mechanical disinfection strategies. If human pathogens are able to spread to salad leaves from contaminated soil, irrigation water or directly from animal faeces, and actively penetrate the interior of the leaves, then the microbiological safety of ready-to-eat salads cannot be guaranteed. This study also suggests that current methods for detection of pathogenic bacteria in foods following processing may be under-reporting the threat to consumers due to the induction of the VNC state amongst pathogenic bacteria.

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DECLARATION OF AUTHORSHIP

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications (see Appendix 3)

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LIST OF ABBREVIATIONS

16S	Small ribosomal subunit
3D	Three-dimensional
AFM	Atomic force mocroscopy
AI-2	Autoinducer 2
AIP	Autoinducer peptide
ANOVA	Analysis of variance
AO	Acridine Orange
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBSRC	Biotechnology and Biological Sciences Research Council
CCD	Computer-controlled digital
CFA	Colony-forming antigen
CFDA	Carboxyfluorescein diacetate
CFP	Cyan fluorescent protein
CFU	Colony-forming unit
CLSM	Confocal laser scanning microscopy
CRI	Congo Red indicator
CTC	5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DIC	Differential Interference Contrast

DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DVC	Direct viable count
EDIC	Episcopic Differential Interference Contrast
EF	Epifluorescence
EHU	Environmental Healthcare Unit
EPS	Exopolysaccharide
ESEM	Environmental scanning electron microscopy
GFP	Green fluorescent protein
HSL	Homoserine lactone
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
LB0	Luria-Bertani agar without NaCl
MAP	Modified atmosphere packaging
NCTC	National Type Culture Collection
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPFM	Pink-pigmented facultative methylotroph
QS	Quorum sensing
R2A	R2 agar
R2B	R2 broth
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SEM	Scanning electron microscopy
ssDNA	Singe-stranded deoxyribonucleic acid
TGGE	Temperature gradient gel electrophoresis
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UV	Ultraviolet
UV-B	Ultraviolet B (medium wave)
VNC	Viable but nonculturable

GLOSSARY OF TERMS

Aggregate	Mass of bacterial cells in close proximity to one another
Bacilli	Rod-shaped bacteria
Biofilm	Three dimensional matrix of microorganisms and secreted exopolymers attached to a surface
Community	A group of organisms of more than one species sharing a defined area
Cuticle	Waxy layer secreted onto the surface of a leaf
Endophyte	Bacterium capable of multiplying inside the leaf interior spaces
Endosphere	The interior or subsurface region of a plant leaf
Enteric	Associated with the intestine
Enterobacteriaceae	Taxonomic family of Gram-negative bacteria including may pathogens such as *Salmonella* and *Escherichia coli*
Epidermis	Surface cell layer of a plant leaf
Epiphyte	Bacterium capable of multiplying on the surface of a leaf
Gastroenteritis	Infection of the gastrointestinal tract resulting in vomiting, diarrhoea and sometimes fever
Methylotrophic	Able to utilise C_1 compounds as a food source
Phyllosphere	The plant leaf when considered as a bacterial habitat (encompasses both the phylloplane and endosphere)
Phylloplane	The plant leaf surface as a microbial habitat
Planktonic	Free living bacterial existence, not attached to any sort of surface

Population	Collection if individuals of the same species within a defined area
Quinolone	Family of antibiotics with bactericidal activity through inhibition of the enzyme DNA gyrase
Quorum sensing	Density-dependent chemical signalling in large populations of bacteria
Rhizosphere	The soil environment, when considered as a bacterial habitat
Serovar	Bacterial strain with possessing a particular combination of cell surface antigens
Stoma	Leaf gas exchange pore (plural ‘stomata’)
Trichome	Hair or hair-like structure on plants
Wettability	The degree to which water or other liquid will spread evenly across a surface

CHAPTER 1

INTRODUCTION

1.1 Overview

The turn of the twenty-first century has brought with it a growing human obesity crisis in the developed world. The current generation of children in the United States of America (USA) are estimated to be the first generation in over two centuries with a lower life expectancy than their parents, due to health problems associated with being overweight (Olshansky *et al.* 2005). High profile anti-obesity initiatives have been promoted by Health Agencies in the USA and Europe, such as the 'five-a-day' concept suggesting a minimum of five fruit and vegetable items are consumed each day as part of a healthy diet (Hancocks 2003).

In busy, consumer-driven societies there is a demand for fresh produce to be available all the year round, and for it to be convenient to obtain and prepare (Hampson *et al.* 2009). Consequently, a new generation of partially processed and 'ready-to-eat' fruit and vegetable products have become widely available in supermarkets. Unfortunately, the increase in such products is thought to have brought with it an increase in foodborne illness due to the responsibility of washing fresh produce becoming transferred from the consumer to the producer. A number of high-profile outbreaks of foodborne illness have been linked to pre-packaged, ready-to-eat salad products in both the USA and Europe, including incidences of the bacterial pathogens *Salmonella* (Health Protection Agency 2005) and *Escherichia coli* strain O157:H7 (Centers for Disease Control 2006b) infections. Interestingly, in the United Kingdom (UK), there are on average ten times the number of *Salmonella* infections per year compared to *E. coli* O157:H7 (Wong *et al.* 2004). A number of notable recent outbreaks of foodborne illness associated with the consumption of fresh produce are given in Table A.1 in Appendix 1.

In a time of increasing dietary awareness, the task the food industry currently faces is to satisfy increasing demand for processed salad products without compromising consumer safety. Recent outbreaks demonstrate that consumer safety from pre-packaged salad products cannot be guaranteed using current production and processing practices, whilst current research into the proliferation of human pathogens on leafy produce has so far been fragmented and narrow in its focus. This investigation was therefore initiated to try and address some of the fundamental questions relating to bacterial life on a leaf, before attempting to apply that knowledge to prevent and treat pathogen contamination of leafy produce intended for human consumption.

1.2 Bacterial life on surfaces

1.2.1 Bacterial surface attachment

Attachment to a surface confers a number of advantages for bacteria over existence in a planktonic (free-living) state; these include protection from environmental stresses and an increased opportunity for gene exchange. Surface attachment affects nutrient availability to bacteria, as organic surfaces may provide leached material, or a submerged surface may be continually washed by flowing water carrying nutrients in solution (Donlan 2002). Molecular interactions may cause nutrients to adsorb onto the surface itself, creating a concentration gradient relative to the surrounding environment. Water flow across the surface can carry away metabolic waste products, the build-up of which can alter local pH conditions and slow bacterial growth (Zhang and Bishop 1996). In multi-species bacterial communities, one organism's waste may be another's food source; for example, methylotrophic bacteria are capable of metabolising C_1 compounds produced as waste by other bacteria (Chistoserdova *et al.* 2009). Surface attachment makes this process more efficient by bringing different species into consistently close proximity, allowing some species convenient access to the metabolic waste products of other species.

Bacteria are capable of producing a variety of cell surface structures which improve motility, attachment and adhesion (Figure 1). Bacterial surface attachment is generally regarded as a two-stage process: initial, reversible association with a surface followed by irreversible adhesion. Reversible association with a surface is thought to be the result of non-specific interactions including electrostatic interactions, hydrophobicity and Van der Waals forces (van Loosdrecht *et al.* 1987). By contrast, irreversible attachment is thought to be largely mediated by cell surface appendages such as those in Figure 1. Understanding the role of individual cell surface structures is difficult as many bacteria possess several different types of surface appendage. In addition, as the production of many surface structures appears to be inducible rather than constitutive, the evidence for the role of any specific attachment factor tends to be controversial. For example in *E. coli*, the role of type I pili or fimbriae, which are short filamentous structures has at times been determined to be important in surface attachment (Cookson *et al.* 2002), whilst in other studies deemed irrelevant (Rivas *et al.* 2007). Flagella, long filamentous appendages involved in motility, have similarly been observed to play at times an important role in attachment (Bouttier *et*

al. 1997) whilst in other studies appearing unnecessary (Rivas *et al.* 2007). Clearly the production and utilisation of cell surface structures and their role in cell surface attachment is a complex and highly regulated process responding to a variety of exogenous conditions and signals.

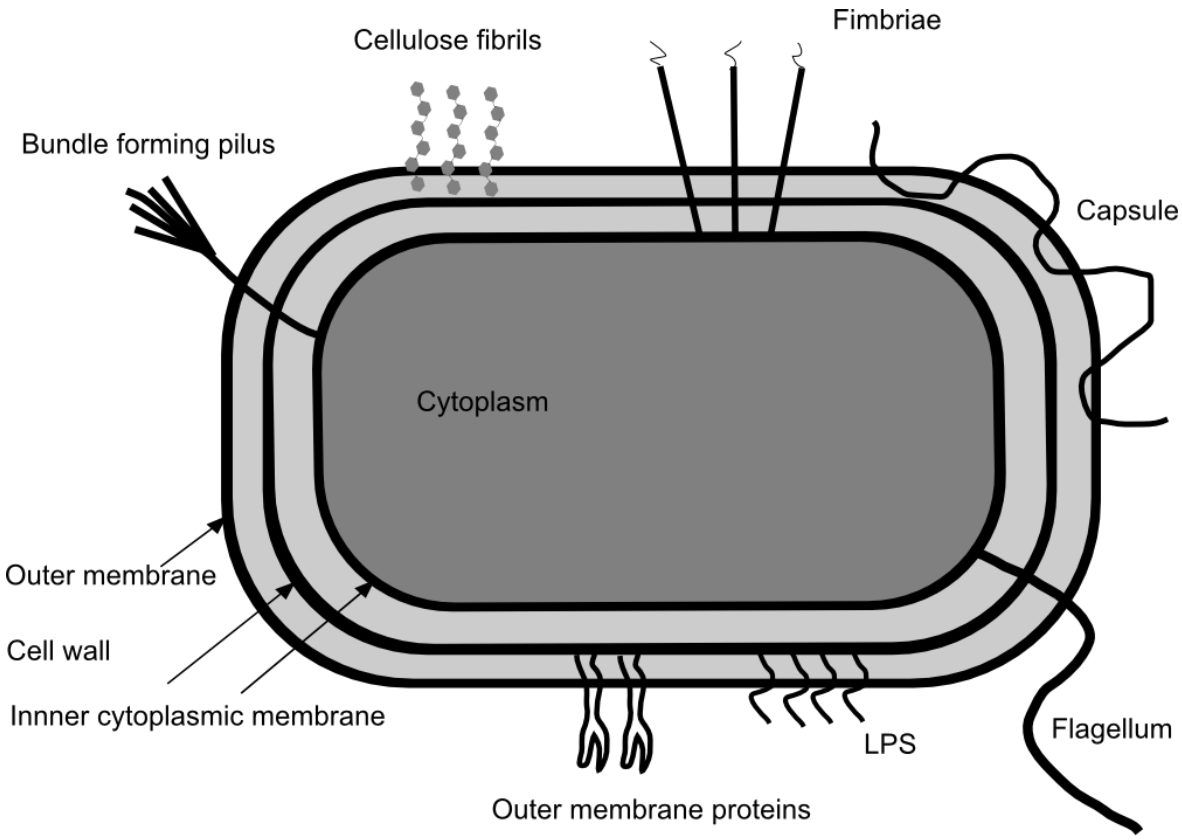


Figure 1: Schematic representation of bacterial surface attachment factors. Adapted from Matthews (2006)

1.2.2 Biofilms

1.2.2.1 What is a biofilm?

Bacteria on surfaces are able to exist at extremely high densities and frequently form complex three-dimensional assemblages bound together by secreted exopolymer material (Donlan 2002). This is composed predominantly of polysaccharide but also contains proteins, lipids and often extracellular DNA (Flemming *et al.* 2007). This has been described as a heterogeneous mosaic architecture (Walker *et al.* 1995). The complete assemblage is referred to as a biofilm. Biofilm formation by bacteria has specific phenotypic requirements which may be species dependent, such as the requirement for type IV pili on the cell surface in *P. aeruginosa* (O'Toole and Kolter 1998). Genotypic variation within a given strain also alters the ability to form biofilms if the variation occurs in one or more genes associated with secretory mechanisms or cell surface receptors (Drenkard and Ausubel 2002). A mature, mixed species biofilm is a highly complex but ordered three-dimensional structure (Figure 2).

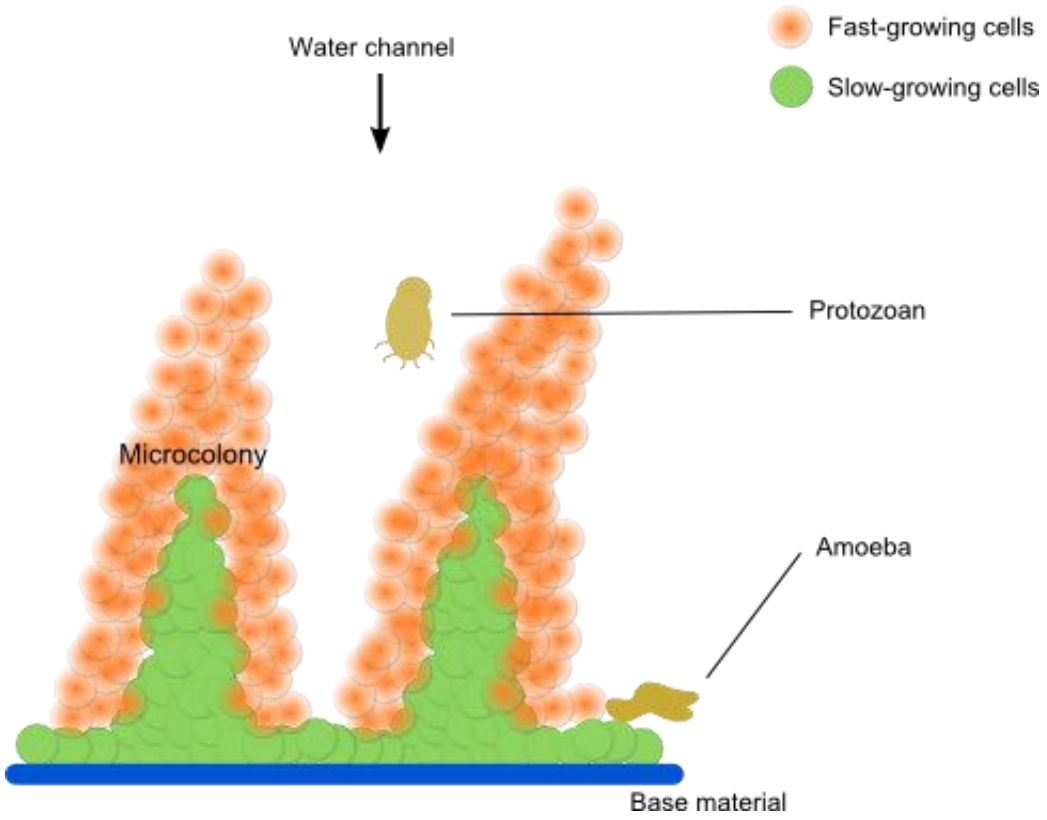


Figure 2: Structure of a mixed species environmental biofilm. Adapted from Keevil (2003)

Intact biofilms protect the bacteria within them from a variety of stresses, depending on the particular environment. For example inside a water pipe, attached bacteria are subjected to shear stress from the water flow (Percival *et al.* 1999), whereas on a leaf ultraviolet (UV) radiation and desiccation are two of the most prominent stresses (Lindow and Brandl 2003). The high resistance of biofilms to chemicals is economically important for many different industries and of great importance to the field of medicine, where biofilm formation has been linked to antibiotic resistance in pathogenic bacteria (Drenkard and Ausubel 2002).

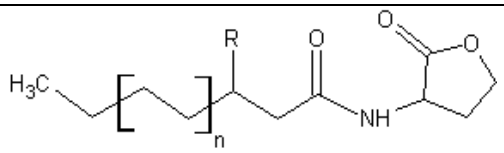
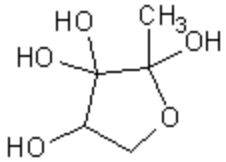
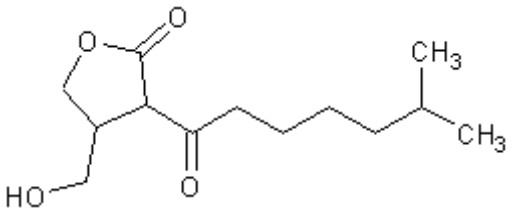
1.2.2.2 Biofilm structure

The exopolysaccharide (EPS) matrix component of a biofilm is what facilitates the formation of complex three-dimensional (3D) structures and is what differentiates a

biofilm from an unstructured aggregate. The complex bonding within a biofilm matrix is important in maintaining structural integrity which is key to resisting detachment from the surface by chemical (Simoes *et al.* 2005) and mechanical means (Branda *et al.* 2005). Staining with fluorescent conjugated lectins can be used to visualise the exopolysaccharide component directly (Neu *et al.* 2001). Tight binding of bacteria within this matrix means that although solitary bacteria do often detach from the biofilm, the majority of lost biomass occurs when large sections of the biofilm break off but do not disintegrate, which is the type of detachment that usually follows mechanical shear stress (Stoodley *et al.* 2001).

Signalling molecules are employed to great effect within the tissues of multicellular organisms to facilitate a coordinated response to an internal or external stimulus. For example, neurotransmitter substances such as acetylcholine facilitate the transduction of a nerve impulse between adjacent neuronal cells in animal species possessing neural networks(Alberts 2002). In plant species, nitric oxide and hydrogen peroxide are both thought to be involved in whole-plant responses to a wide variety of biotic and abiotic stress, including pathogen challenge and extremes of temperature (Neill *et al.* 2002b). Signalling molecules are typically recognised by specialised receptors spanning the target cell membrane, which relay the signal onwards to induce changes in cellular synthesis and metabolism as required (Alberts 2002). The assembly of a bacterial biofilm is a coordinated process, initiated in a density-dependent manner by the transfer of diffusible chemical signals between nearby bacterial cells, a process known as quorum sensing (QS). QS is a form of cell-cell communication in bacteria, facilitated, as in multicellular tissues, by the release and recognition of diffusible chemical signalling molecules, known as autoinducers (Antunes and Ferreira 2009). It is distinct from other forms of cell-cell signalling as it occurs in a density dependent manner. QS is generally used to mediate coordinated expression of a particular phenotype, for example initiation of biofilm formation through initiation of matrix production in surface-associated bacteria (Donlan 2002). In Gram-negative bacteria, autoinducers generally take the form of acyl homoserine-lactose (HSL) molecules (Table 1), which pass freely in and out of cells across the cell membrane. By contrast, Gram-positive autoinducers are typically autotinducer peptides (AIPs; Table 1) which are extracellular and bind to receptors on the cell surface.

Table 1: Bacterial quorum sensing molecules. Adapted from He and Zhang (2008).

Signal	Structure	Representative organism(s)
N-acyl homoserine lactone (AHL)		*Pseudomonas aeruginosa* (n = 4, R = O) Vibrio harveyi (n = 2, R = OH)
Autoinducer 2		*Salmonella typhimurium*
γ-Butyrolactone (autoninducer peptide)		*Streptomcyes griseus*

Although the majority of autoinducers appear to function primarily as intraspecies signals, one notable exception demonstrates that bacteria are able to facilitate interspecies crosstalk. Autoinducer-2 (AI-2) has been identified in 55 species of bacteria, including Gram-negative and Gram-positive species (De Keersmaecker *et al.* 2006); it has been

demonstrated to be involved in the organisation and pathogenicity of mixed-species dental plaques (Frias *et al.* 2001). AI-2 signalling has been observed in a number of enteric pathogens, affecting flagellar motility and the ability to form biofilms (Xavier *et al.* 2007). The ability of bacteria to coordinate metabolic activity, spatial organisation and even virulence in a multicellular manner using QS is likely to greatly enhance survival in competitive mixed species communities and hostile environmental conditions.

1.2.2.3 Biofilm composition

Biofilms can be artificially grown using a single bacterial strain in closed systems where all environmental conditions and stresses can be experimentally manipulated (Dibdin and Wimpenny 1999). This level of control means that the different dynamic forces at work on a biofilms, such as reproduction, shear stress and detachment are all known quantities and so the overall growth of the biofilm can be predicted using computer modelling (van Loosdrecht *et al.* 2002; Wagner *et al.* 2002; Marsh 2005). Predictions made using computer models, when compared to direct measurements of cultured biofilms, often correlate well (Kreft *et al.* 2001; Hunt *et al.* 2003); however their conclusions are still of limited relevance to naturally occurring biofilms not least because they struggle to simulate heterogeneous 3D structures.

A natural biofilm will rarely be composed of a single strain, and will be subject to a wider range of fluctuating stresses than an artificial biofilm; the fluctuation of stress levels can be more lethal than the stress itself. Consequently, modelling experiments are of limited use when compared to studies conducted on naturally occurring environmental biofilms. Environmental biofilms are composed of a number of bacterial species co-existing, resulting in complex mutualistic interactions (Wagner *et al.* 2002; Marsh 2005). They are frequently sites of horizontal gene transfer, providing a key source of genetic variation on an asexually reproducing population. For example, transfer of specific resistance genes within a biofilm leads to the development of a highly resilient communities which cannot be treated by antibiotics (Ohlsen *et al.* 2003).

1.2.3 Commercial implications of surface-associated bacteria

Biofilms can form in industrial water systems and cause microbially induced corrosion, a form of biofouling (Beech 2004). Solid surfaces in contact with water frequently become sites of biofilm formation. Biofilms can physically impede water flow by narrowing pipes and channels; in addition, they can harbour pathogens which resist disinfection whilst contained within a biofilm, then subsequently detach and re-contaminate treated water (Lund and Ormerod 1995); in addition, dead biomass released from bacterial biofilms can raise the nutrient concentration of solutions, promoting further microbial growth (Laspidou and Rittmann 2002). Metabolic processes occurring within biofilms can produce corrosive secondary metabolites which damage the surface on which they are situated; for example, sulphate-reducing bacteria generate hydrogen sulphide, which precipitates metal ions from surfaces, slowly replacing the metal surface metal sulphide compounds (Rao *et al.* 2005). Biofilm EPS can promote the adhesion of corrosion products to the surface, though their toxicity may limit further microbial growth. Leaching of metal compounds into water systems has serious implications for both public health, in the case of potable water systems, or biodiversity, if wastewater effluents are contaminated.

Biofilms have also been shown to play a role in a number of chronic infections, predominantly because of their enhanced resistance to antibiotics (del Pozo and Patel 2007). For example *Pseudomonas aeruginosa* biofilms can cause chronic infection of the lung in cystic fibrosis sufferers (Moreau-Marquis *et al.* 2008), whilst enteropathogeneic *E. coli* biofilms are responsible for recurrent urinary tract infections (Salo *et al.* 2009). In addition to resisting antibiotics, biofilms are also more resistant to host defences than their planktonic counterparts (Kharazmi 1991). Successive cycles of antibiotic therapy only serve to exacerbate the problem by selecting for resistance within the bacterial population. Programmed release of cells from a resistant biofilm allows infective cells to travel to other sites within the body, producing acute infections (Ehrlich *et al.* 2005). Whilst these infections may respond to antibiotic treatment, unless the resistant biofilm population is targeted, there is a high likelihood of recurring symptoms.

1.2.4 Key techniques for *in situ* analysis of bacterial surface life

In Section 1.2.2.1 the complex, ordered structure of bacterial biofilms was discussed. Microscopy techniques have been instrumental in elucidating bacterial surface colonisation

and the architecture of biofilms through *in situ* observation (Keevil 2003). Simple light microscopy allows the study of biofilms on transparent surfaces such as glass and Perspex, through transmission of light through the surface and sample. Opaque surfaces, however, require more complex imaging techniques. For example, Confocal Laser Scanning Microscopy (CLSM) uses lasers to excite fluorophores in fluorescent dyes and stains applied to a sample; high-resolution, 3D images can be produced, and subsurface structures can be detected (Pamp *et al.* 2009). However, the technique widely regarded as both expensive and time-consuming; in addition, the ability to resolve biofilm structure relies on careful choice of specific fluorescent dyes to stain EPS components (Staudt *et al.* 2004). By contrast, Scanning Electron Microscopy (SEM) produces detailed images at higher magnification than CLSM, and does not require the application of fluorescent dyes. SEM has been used to visualise biofilms in a number of different systems, including household surfaces (Rayner *et al.* 2004) and industrial systems (Arnold and Bailey 2000). The use of SEM for the study of biofilm architecture is, however, limited by the stringent drying and fixation required prior to sample analysis. These preparatory procedures are can transform a hydrated matrix into a flattened layer (Keevil 2003). Environmental Scanning Electron Microscopy (ESEM) is a modification of the technology which does not require complete dehydration; however, excess water must still be aspirated prior to imaging and this may still destabilise the biofilm matrix (Priester *et al.* 2007). As a consequence, both SEM and ESEM can produce artefacts during sample preparation which may potentially be misinterpreted as genuine features of biofilm architecture.

Episcopic Differential Interference Contrast (EDIC) microscopy is an emerging technique in the field of biofilm microbiology. A modification of Nomarski Differential interference Contrast (DIC) optical technology, EDIC microscopy reflects light off a surface rather than transmitting it through a sample (Keevil 2003). Heterogeneity in the plane of the sample alters the path of the returning light, producing areas of contrast and giving the resulting image a pseudo-3D appearance. Uneven, curved and opaque surfaces can all be visualised using EDIC microscopy, which can be coupled with epifluorescence (EF) to allow the use of fluorescent stains and dyes. Non-contact objective lenses allow the visualisation of fully hydrated biofilm matrices without the risk of artefact generation through fixation, dehydration or crushing with coverslips. EDIC/EF has been successfully used to study contamination of a variety of surfaces including surgical instruments (Lipscomb *et al.* 2007) and drinking water pipes (Wilks and Keevil 2006). Figure 3 represents an EDIC

microscope with attached digital camera and computer system for software-assisted image capture.

Figure 3: EDIC microscope with attached camera and computer system.

1.3 The plant leaf surface as a bacterial habitat

1.3.1 Leaf surface physiochemistry

Plant leaf surfaces are coated in a thick cuticle which consists largely of secreted waxes, predominantly cutin. This layer is strongly hydrophobic, as it is designed to minimise water loss from the leaf tissue (Nawrath 2006). The exact composition and crystalline structure of the cuticle varies with plant species, as does the overall thickness (Wagner *et*

al. 2003). The wax crystal structure of a leaf will affect water and air currents at the leaf-atmosphere interface; this will affect how hospitable that leaf surface is for bacteria colonisation and growth (Beattie 2002).

The physiochemical properties of the cuticle affect epiphytic bacteria in two ways: firstly the hydrophobicity affects the energetics of the attachment process, and secondly it affects nutrient availability to colonists. Thicker cuticles with a high proportion of waxes in their composition can block the majority of nutrients from leaching out of the leaf epidermis onto the surface (Beattie 2002). They also have low wettability, meaning that water falling on the leaves stays in tightly formed droplets rather than spreading over the surface. Bacteria which colonise areas of leaf between the droplets will suffer greater desiccation stress than neighbouring cells which are in contact with surface water (Marcell and Beattie 2002). Virulent plant pathogens are able to alter leaf surface permeability by a variety of means including mechanical penetration, as is typical amongst the fungal pathogens, or biochemical weakening of the cuticle and cell wall structures (Schreiber *et al.* 2005). The cuticle thickness is heterogeneous according to the type of plant cell it covers; for example the cuticular layer is relatively thin around stomatal guard cells as there needs to be freedom of movement to allow stomatal closure (Andrews and Harris 2000; Nawrath 2006). Many plant species produce a thicker cuticle on the upper leaf surface than the lower surface (Leigh and Coplin 1992); this is likely to be because heat from the sun makes the lower surface more prone to water loss through transpiration which is largely blocked by the cuticular layer.

1.3.2 Leaf surface topography

The leaf interior is encased on both surfaces by an epidermal cell layer, which is responsible for production of the leaf cuticle (Gniwotta *et al.* 2005). Epidermal cells interlock to form a continuous layer with deep margins between individual cells. The epidermis of an undamaged leaf is impenetrable save for the many air pores or stomata dotted across the surface; these are vital for gas exchange during photosynthesis and respiration (Taiz and Zeiger 2002) but, when open, provide direct access to the leaf interior for microorganisms (Underwood *et al.* 2007). Depending on the plant species, stomata may be above, in line with, or below the plane of the leaf surface. Veins provide the most topographical variation on a leaf; major veins such as the leaf midrib are often visibly

raised relative to the plain the leaf surface. The margins between vascular elements are often deeper than epidermal cell margins and the join between epidermis and vein is also frequently forms a deep recess, as can be seen in Figure 4.

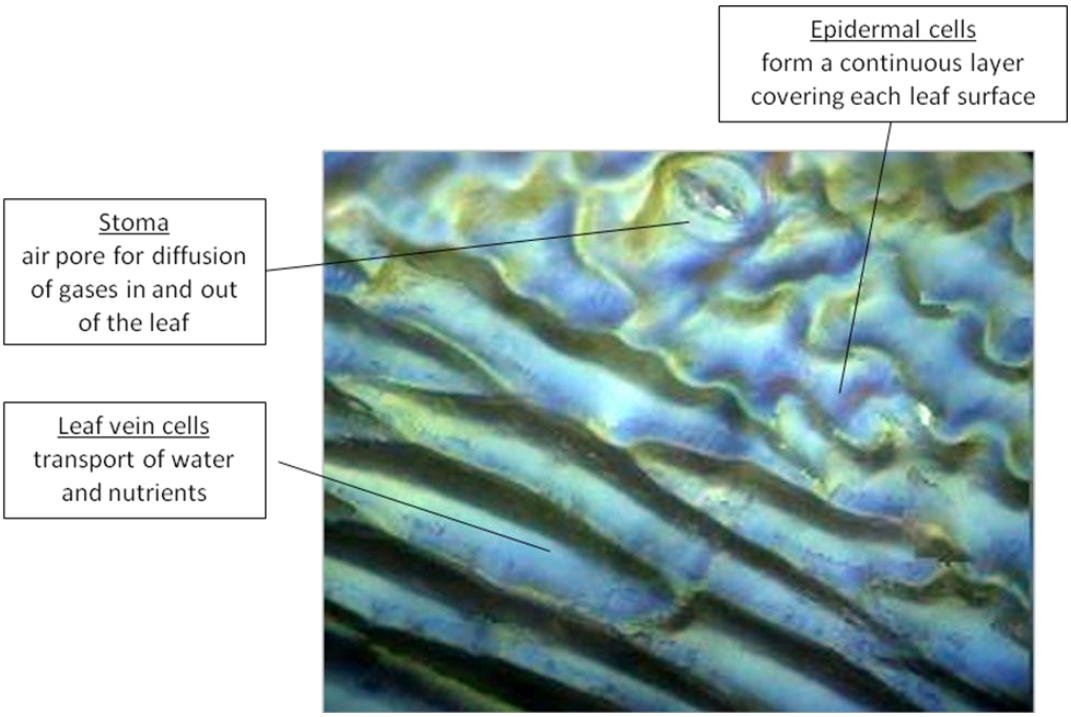


Figure 4: Image of a rocket leaf showing the three major anatomical features commonly visible on the surface of dicotyledonous plants.

1.3.3 Environmental conditions on a leaf

With regards to the ability of a plant surface to support bacterial populations, one of the most important characteristics is the limited availability of water. Plants have evolved to minimize water loss to the surface and consequently surface water is typically only available from external sources such as rainfall, condensation or irrigation (Beattie 2002). This means that water levels on leaf surfaces are both spatially and temporally heterogeneous, as water droplets do not persist on the surface for long before either running off or evaporating (Wagner *et al.* 2003). It has been demonstrated that leaves

grown in elevated atmospheric humidity support higher bacterial numbers (Kinkel *et al.* 2000), strongly suggesting that water availability is a key factor which limits population growth.

Nutrient availability is also very limited on a leaf surface. Nutrients occur as infrequent 'hotspots' originating from either external applications including fertiliser and bird faeces, or via nutrient escape from wounded or senescing leaves. Bacterial colonisation events are well spread over the surface of a leaf but survival and population growth are strongly linked to localised nutrient sources (Leveau and Lindow 2001). This means that the localised availability of nutrients, whatever their origin, have a direct effect on the spatial patterning of bacterial growth in the phyllosphere.

Another stress encountered by phyllosphere epiphytes is ultraviolet (UV) radiation from the sun. A number of bacteria produce pigments which confer protection from UV (Jacobs *et al.* 2005). The pink-pigmented facultative methylotrophs (PPFMs) and the fluorescent Pseudomonads are both examples of UV tolerant bacteria commonly found on plants. However, whilst phyllosphere community structure alters markedly in response to UV-B irradiation (Kadivar and Stapleton 2003), the less tolerant species are not eliminated completely, suggesting that protection can come from factors other than actively induced defences.

Most bacterial species have phenotypically distinct strains with varying levels of epiphytic fitness. Mechanisms which confer an advantage colonising one surface may not always do so on another. For example, genotypes of *Salmonella* giving the best fitness colonising the human gastrointestinal tract do not confer the same advantage in the phyllosphere (Brandl and Mandrell 2002), which is unsurprising given the considerable differences in the two environments.

1.3.4 Diversity of life on a leaf

Ercolani (1991) identified 25 different bacteria to genus level or lower from olive leaves using recovery and growth on selective agar media. On average, *Pseudomonas syringae* accounted for half of the total bacteria found on the samples. The plant pathogens *Xanthomonas campestris* and *Erwinia herbicola* were also abundant, with the remaining bacterial species each accounting for 5% or less of the community. The *Pseudomonas*

species frequently dominate phyllosphere communities, such as *P. fluorescens*, on strawberry plants (Krimm *et al.* 2005), while other fluorescent Pseudomonads dominate on grasses (Behrendt *et al.* 2003). One commonly detected species, *P. fluorescens* is not harmful to plants (Lindow and Brandl 2003); neither is the soil bacterium *P. putida* which is also often found on leaves . *P. syringae* however is one of the most virulent bacterial plant pathogens (Dulla *et al.* 2005), which is likely to account for its dominance of the phyllosphere. *P. aeruginosa* is of relevance to human consumption as it is able to infect not only plants but also humans (Plotnikova *et al.* 2000). The closely related species *Xanthomonas campestris* is also a virulent plant pathogen able to attain very high population numbers on leaves (Jacques *et al.* 2005). *X. campestris* excretes very large quantities of exopolysaccharide which is used as a food thickener or emulsifier under the name 'xanthan gum' (García-Ochoa *et al.* 2000).

The *Erwinia* species are economically important necrotrophic plant pathogens responsible for a variety of blights, soft rots and other tissue necroses which particularly affect vegetable bulbs and tubers (Barras *et al.* 1994). The genus *Erwinia* is a member of the family Enterobacteriaceae, whose members also include many key human gastrointestinal pathogens, the presence of which on the surfaces of leafy food crops is discussed in Section 1.5.

Far less abundant but still of interest are the growth-promoting colonists referred to as the pink-pigmented facultative methylotrophs or PPFMs (Madhaiyan *et al.* 2005). As their name suggests these bacteria are able to utilise C_1 compounds where available, an unusual trait in bacteria. This characteristic and their distinctive pink pigmentation are both key survival advantages enabling them to avoid starvation and UV damage in the phyllosphere (Omer *et al.* 2004).

1.3.5 Routes of colonisation

The phyllosphere is a very fragmented habitat, as individual leaves are spatially separated from one another; attached only by leaf stems. Contamination requires direct contact with a source of inoculum, with or without the aid of a vector. With some recent fresh produce-associated food poisoning outbreaks the source was clearly traceable back to contaminated irrigation water which had deposited pathogenic bacteria onto the leaves (Brackett 1999; Solomon *et al.* 2002). Other outbreaks, however, may have no obvious source.

Rhizosphere and phyllosphere bacterial populations often contain a number of similar species of microorganism (Berg *et al.* 2005; Zachow *et al.* 2008). Interestingly, as the leaves of many plant species do not actually touch the soil, an explanation is needed for the transfer of bacteria from soil to leaf. It is thought that at least some colonisation of leaves takes place during germination when the emerging hypocotyl pushes through contaminated soil, and that bacterial populations spread over the entire plant surface during growth (Solomon *et al.* 2002). One recent study postulates that the nematode *Caenorhabditis elegans* may be responsible (Kenney *et al.* 2006). The study demonstrates the migration of the worms towards *Salmonella*-inoculated bovine manure, and from there towards fruit and vegetables, which subsequently tested positive for the pathogen. This may well be the case in the field where fruits and vegetables touch the soil, but may not explain the contamination of aerial plant surfaces. Another study has implicated slugs as vectors for *E. coli* O157; this strongly suggests that they have the potential to carry many other bacterial species. Slugs are able to move over the entire above-ground region of a plant and deposit viable bacteria not only in their faeces but also from the slime they secrete which can be found on their entire body surface (Sproston *et al.* 2006).

Deposition of bacteria onto the leaf surface does not guarantee successful colonisation. Bacterial mortality is highest immediately following attempted colonisation; Leveau and Lindow (2001) found that following inoculation, although bacteria were initially ubiquitous across leaves, within a matter of hours viable bacteria could only be found in clusters near high concentrations of nutrients. Monier and Lindow (2003) noted that survival past this initial critical period is much higher for bacteria found in aggregates than for solitary bacteria.

1.3.6 Bacterial attachment to plant tissue

Bacterial interactions with the rhizosphere have been studied extensively in commercially important crop plants, as plant-microbe interactions in the soil can be a major determinant of plant fitness and overall crop yield (Morgan *et al.* 2005). By contrast, bacterial attachment to aerial plant surfaces is less well understood; trends in attachment behaviour must be inferred from a small number of discrete and highly focussed studies. Bacteria can be deposited on aerial plant surfaces via wind or water splashes, yet in the hours following deposition they become tightly attached to plant tissue, resisting mechanical detachment

(Morris *et al.* 1997). Bacterial emigration from leaf surfaces appears to be at its highest when leaves are at their warmest and driest, suggesting that non-specific forces such as hydrophobicity or cell surface charge may be key determinants of attachment (Lindemann and Upper 1985), though many other interactions are also thought to be involved (Figure 5).

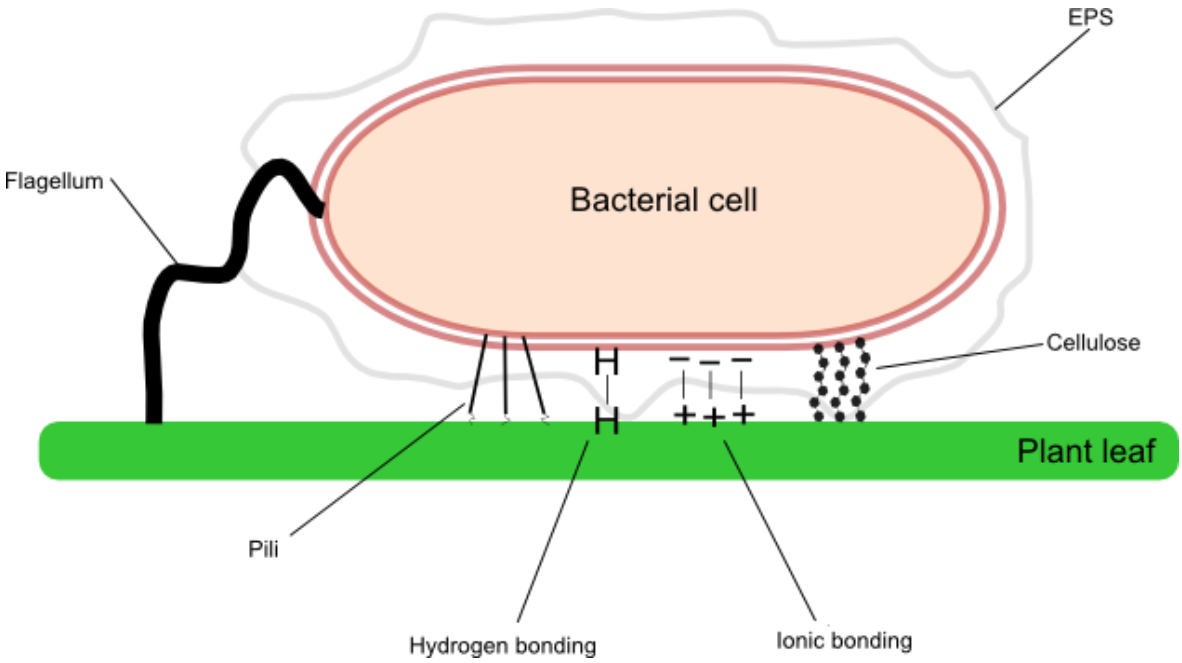


Figure 5: Factors likely to be involved in bacterial attachment to plant leaf surfaces.

A number of studies have described biofilm formation on leaf surfaces, though at present the frequency of their occurrence on plant phylloplanes is unclear. AHL signalling has been observed *in vitro* in a number of epiphytic plant species (Quiniones *et al.* 2005; Karamanoli and Lindow 2006); in addition, QS mutants of *Pseudomonas syringae* have been shown to possess a reduced ability to survive on phylloplanes (Quiniones *et al.* 2004). Cell surface adhesins are thought to play a role in the colonisation of roots, and may therefore also be involved in attachment to leaves (Smit *et al.* 1987).

It is evident that the genetic basis of bacterial attachment to phylloplanes is not well understood, not least because of the differences between individual bacterial and plant

species. Virulence factors have been implicated in both plant pathogens and non-native species such as *Salmonella* spp., though plant pathogens can show considerable epiphytic fitness on plants outside their host range (O'Brien and Lindow 1989). Further studies exploring a broader range of epiphytes are needed to try and identify common strategies for phyllosphere colonisation.

1.3.7 Leaf-associated biofilms

Although biofilms are well characterised on abiotic surfaces, the evidence produced so far for their presence on leaves has been inferred from a series of observations rather than direct visualisation of attached biofilms. By comparison, there has been far more interest in studying microbial associations with plant roots and as a result the evidence for biofilms associated with roots is stronger (Molina *et al.* 2003), and includes more direct visualisation using SEM. Many key features of microbial biofilms have been observed on the surfaces of plants, such as aggregation, attachment and the production of exopolysaccharide. For example, the production of exopolysaccharide is induced in a density dependent manner in *P. syringae* via QS (Quiniones *et al.* 2005), which is indicative of biofilm formation. A few studies have used microscopy to directly observe bacterial aggregates on the leaf surface (Morris *et al.* 1997; Morris *et al.* 1998; Carmichael *et al.* 1999; Monier and Lindow 2005b). Efforts have been made to quantitatively determine the proportion of epiphytes existing in a solitary versus aggregated state (Charkowski *et al.* 2002; Monier and Lindow 2005b), however they encountered a high degree of variation between samples.

Many attempts to study biofilms on leaves are centred around inoculating a plant with a pure culture of a bacterial strain of interest. The bacteria are given an incubation period of around a week to establish biofilms, which are then studied by techniques such as SEM (Jacques *et al.* 2005). There is concern that the conclusions drawn from such studies are of limited value since bacterial immigration of a single strain in isolation is highly unlikely to occur in the environment, and also that the incubation times are insufficient to allow the formation of a biofilm that would survive the dehydration and fixation steps required for electron microscopy (Morris *et al.* 2002).

The presence of biofilms on leaf surfaces seems to provide an answer to the question of how this hostile environment can support such an abundance and diversity of bacterial life.

Monier and Lindow (2005a) concluded that if new bacteria landed on an existing bacterial aggregate, their chances of survival were greatly increased relative to landing on an unoccupied area of leaf. This suggests that the aggregate either increases the availability of nutrients or confers protection from UV stress; in fact it is probably a combination of both factors. Aggregation has been shown to facilitate survival on leaves (Monier and Lindow 2003; Jacobs *et al.* 2005) and also induce nutrient leakage from solid synthetic media (Porro *et al.* 1997).

The observation that biofilms exist on leaves raises as many questions as it does answers. Little is known about the comparative composition of the solitary and aggregated populations on leaves in terms of either their phylogeny or their phenotypic diversity. Biofilms in other systems are sites of horizontal gene exchange (Barkay and Smets 2005) and this is also thought to occur on leaves (Bailey *et al.* 2002); however the degree to which this takes place is unclear. There is also the question of the extent to which the presence of a biofilm alters the physiochemical environment around it. Unlike a sheet of stainless steel or a water pipe, a plant leaf is a dynamic surface capable of detecting and responding to bacterial attachment. Plants have a variety of inducible defence responses against pathogens (van Loon *et al.* 2006), some of which are more specific than others, and a number of these may be activated in response to non-pathogenic bacterial attachment. Plants are known to be able to produce compounds which mimic the *N*-acyl HSL class of bacterial QS signal molecules in response to bacterial HSL signalling (Bauer and Mathesius 2004). Currently however, the mechanism of detection and synthesis is unknown, as is the effect on bacterial colonists.

1.3.8 Host-colonist interactions

Successful plant pathogens have two separate, interchangeable life strategies. The first is tolerance strategy, where little or no population growth takes place and genes designed to resist environmental stress are switched on. This strategy is thought to be used when conditions are not favourable for pathogenesis, such as when a bacterium lands on a non-host plant (Hirano and Upper 2000). Penetration of the leaf allows access to extensive intercellular spaces where bacteria are shielded from UV and desiccation stress, and there is a greater availability of nutrients. This environment can support endophytic (leaf

interior) population growth within a leaf whose surface conditions are too severe for the epiphytic (leaf surface) population to increase.

The second survival strategy is pathogenesis of the host. Many bacteria are necrotrophic pathogens, meaning that they obtain their nutrients by causing cell death and nutrient leakage in the host (Porro *et al.* 1997; Marco *et al.* 2005). Biotrophic pathogens are more subtle, and some can produce analogues of plant hormones which induce nutrient leakage without causing tissue death, such as indoleacetic acid produced by *Erwinia herbicola* (Brandl *et al.* 2001).

1.4 Methods used in the study of plant-associated bacteria

1.4.1 Culture

Before the advent of molecular techniques, culture-based bacterial detection method dominated published studies of the phylloplane. For example, Ercolani (1991) used culture media and biochemical tests to isolate and identify more than sixty separate bacterial species living on the leaves of olive trees. The development of selective culture media for the specific detection of species of interest has greatly aided environmental microbiology, as outside the laboratory bacteria exist in complex communities from which target organisms must be distinguished. Selective or semi-selective media have been used in the isolation of bacterial plant pathogens such as *Xanthomonas campestris* (Laurent *et al.* 2009) and *Pseudomonas* spp. (Wilson et al 1999); human enteric pathogens can also be isolated and quantified from plant tissues using selective media (Lin *et al.* 1996; McMahon and Wilson 2001).

To circumvent the need for stringent for selectivity, some studies have used surface-sterilised leaves to reduce or eliminate background microflora (Wilson *et al.* 1999; Sabaratnam and Beattie 2003). Surface sterilisation inactivates background microflora so that plant and bacterial responses are more defined during processes such as bacterial attachment. However, such studies do not reflect actual events in nature as background microflora modify the phylloplane environment through metabolic processes, pathogenicity and induction of host responses.

The value of culture media for the isolation and quantification of leaf-associated bacteria is limited primarily by the efficiency of recovery and the suitability of culture conditions. At present little published data exists on the efficiency of bacterial recovery from phylloplanes, though a number of different methods including manual shaking, homogenisation and sonication have been used (Morris *et al.* 1997; Morris *et al.* 1998). Although culture conditions can often be optimised for well characterised bacterial species, the use of selective media may not be suitable for analysis of phylloplane community diversity due to the likelihood of nonculturable or stressed organisms being present. Consequently, recent studies of phylloplane communities have utilised novel, culture-independent methodologies (Saito *et al.* 2007).

1.4.2 Molecular techniques

The gene encoding the small or 16S subunit of ribosomal RNA (rRNA) in bacteria is a valuable indicator of phylogeny. Most phylogenetic groups have one or more unique sequences within the 16S rRNA, referred to as oligonucleotide signature sequences. These sequences have been determined for many bacterial species, and can be aligned with rRNA sequences determined by experimentation in order to identify unknown bacteria. 16S sequences can also be used to create profiles or 'fingerprints' of entire bacterial communities. The Polymerase Chain Reaction (PCR) can be used to amplify all of the 16S rRNA-encoding genes present in a mixed sample of bacteria.

Separation of the PCR products using temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) generates a banding pattern or fingerprint representative of the bacterial diversity present within the original sample because of the signature sequences mentioned previously. Both electrophoresis techniques are used for improved separation of PCR products that are too similar in size to be separated on a conventional agarose gel (Osborn and Smith 2005). PCR-DGGE has been applied to a number of studies in phyllosphere community profiling. Its most popular use is as a 'before and after' comparison tool to analyse community-wide effects of a particular stress or treatment. For example, when maize plants were exposed to UV-B stress, it was discovered that variations in UV tolerance among epiphytic species caused an increase in species diversity following UV-B treatment (Kadivar and Stapleton 2003), suggesting that

the dominant bacterial species were most severely affected. Another study used the technique to compare the bacterial diversity on tobacco leaves dry-cured for different periods of time, and found that community structure altered very little for the first 6 months of drying (Zhao *et al.* 2007). Bands excised from gels can be sequenced to identify prominent taxonomic groups within a community (Yang *et al.* 2001). This is useful if a few species have superior stress tolerance relative to their neighbours, as it is a first step towards indentifying common mechanisms of survival. Statistical analysis, such as the Shannon index, can be used on banding patterns to assess the differences in diversity between two or more samples (Ibekwe and Grieve 2004).

1.4.3 *In situ* observation

1.4.3.1 Fluorescent stains and dyes

A viability assay can be designed using a general nucleic acid stain to enumerate total bacteria and a selective stain for either live or dead bacteria. For example propidium iodide (PI) can only penetrate damaged cell membranes and so will only be visible in dead bacteria. The stains 5-carboxyfluorescein diacetate (CFDA) (Wang *et al.* 2005) and 5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC) stain only live cells (Yu *et al.* 1995) as the original compounds are non-fluorescent, requiring active metabolic machinery to generate fluorescent products.

Direct fluorescent staining of bacteria on leaves is in its infancy. Many studies have instead preferred to detach individuals and clusters of bacteria into solution and then collect them onto filters which can be stained directly. Bacteria in solution or on inorganic surfaces can be readily visualised using fluorescent nucleic acid staining with any one of a number of readily available stains; examples include Acridine Orange, 4'-6-diamidino-2-phenylindole (DAPI) and the SYTOX family of dyes. Staining intact plant surfaces presents more of a challenge as the plant cells themselves contain nucleic acids, potentially resulting in very high background fluorescence if they become stained by the dyes. Plants contain many photosynthetic pigments which are strongly autofluorescent over multiple wavelengths, particularly in the red region of the visible spectrum (Lang *et al.* 1991).

1.4.3.2 Green Fluorescent Protein (GFP)

The discovery that Green Fluorescent Protein (GFP), first isolated from the jellyfish *Aequorea victoria*, can be expressed stably and constitutively in a variety of hosts was a landmark in molecular and cell biology. Complex fusions coupling GFP to a variety of genes of interest allow spatial and temporal localization at any scale from whole-organism to subcellular localisation (Prasher 1995). For microbiologists, the ability to clone GFP into bacteria using plasmid transformation gives an enormous amount of flexibility to control expression by manipulating features of the plasmid construct, including the type of promoter or the selectable markers used. For example, instead of creating bacteria that constitutively express the protein, an inducible promoter can be used to create bioluminescent bacteria which only fluoresce in the presence of the substrate necessary to induce the promoter; this may be a specific carbohydrate or other small metabolite. In microbial ecology it is more usual to opt for a stable, constitutive expression of GFP as it gives highly fluorescent bacteria that can be inoculated into an environment and subsequently tracked both spatially and temporally in real-time. This is particularly useful for complex environmental samples such as foods or sewage sludge, where the dense and diverse microflora already present potentially make recovery and culture difficult (Stevens and Jaykus 2004). GFP-expressing bacteria and CLSM together enable microbiologists to visualize the behaviour of bacteria in biofilms and even track horizontal gene transfer (Christensen *et al.* 1998; Moller *et al.* 1998).

Few published studies exist which have used GFP to directly study the behaviour of enteric bacteria on leaves. Those that are available have concluded that enteric bacteria attach preferentially to cell margins, wounds and around stomata (Morris *et al.* 1997; Wilson *et al.* 1999; Monier and Lindow 2004) – observations which support those made using other fluorescent staining methods (Morris *et al.* 1997; Morris *et al.* 1998). SCLM has also provided the first conclusive evidence that enteric pathogens are capable of penetrating the leaf interior, as GFP bacteria have been imaged in the sub-stomatal cavities of coriander leaves (Brandl and Mandrell 2002).

1.5 Control of pathogens within the food industry

1.5.1 Sanitisation of produce-handling environments

An increase in reported cases of fresh produce-associated gastrointestinal disease outbreaks is perhaps an inevitable consequence of an increase in production and consumption. Contamination of fresh fruits and vegetables can occur during the growing process, from sources such as infected seed (Cooley *et al.* 2003), manure (Ingham *et al.* 2004) or irrigation water (Solomon *et al.* 2002). Postharvest processing practices are also capable of causing contamination if equipment, workers or surfaces are unsanitary. Therefore stringent monitoring of the entire production chain in order to minimise health risks to consumers.

Control measures within the processing environment include operating a cold chain, whereby all of the handling and storage processes from harvest to sale take place in refrigerated conditions in order to suppress microbial population growth (Rediers *et al.* 2009). The advantage of operating a cold chain is that both spoilage microorganisms and pathogens are prevented from proliferating by reduced temperatures, so shelf life is maximised.

Food processing environments frequently feature surfaces and equipment made predominantly from stainless steel (Boulane-Petermann 1996). Many bacteria have been shown to survive and proliferate on stainless steel (Kusumaningrum *et al.* 2003; Wilks *et al.* 2005); biofilm development has also been observed (Silagyi *et al.* 2009). Surfaces submerged in water, such as washing tanks, are more prone to colonisation by biofilms. Where such structures form, bacteria are more resistant to chemical disinfectants than their planktonic counterparts (Joseph *et al.* 2001; Augustin *et al.* 2004), and may be sources of bacterial release back into previously decontaminated water (Batté *et al.* 2003). Automated produce washing lines increase the risk of produce cross-contamination via such processes if the wash water is recirculated, as often happens to reduce water consumption in food processing plants (Hilborn *et al.* 1999; Gil *et al.* 2009).

1.5.2 Sanitisation of fresh produce

A great many studies have been undertaken in recent years evaluating the efficacy of different reagents and processes for the sanitisation of fresh produce. The justification for

widespread use of chemical biocides within the produce-processing industry comes from studies such as those by Beuchat *et al.* (2004) and Gonzalez *et al.* (2004) demonstrating a 2-3 log reduction in pathogen numbers using biocides, compared to approximately a 1 log reduction from washing in water alone. Currently sodium hypochlorite is the most commonly used sanitiser for fruit and vegetable crops worldwide; in aqueous solution it dissociates according to the following equation:

$$NaOCl + H_2O \rightleftharpoons HClO + NaOH$$

HClO, or hypochlorous acid, is a powerful oxidising agent, thought to inactivate bacteria through oxidative damage and disruption of the bacterial cell membrane (Gil *et al.* 2009). Solutions are most effective at high pH (≥ 11)(Estrela *et al.* 2002); alkaline conditions with fewer hydrogen ions alter the above equilibrium in favour of HClO, which possesses much greater biocidal activity than NaOCl - the more prevalent chlorine compound at lower pH values. Hypochlorite is the preferred chlorine donor for biocidal solutions as it is safer to store and dissolves more readily than chlorine gas (Gustin 2005). Hypochlorite washing of leafy salad crops has been shown to produce on average a 1-log reduction in coliform bacteria, the standard indicator of food and water quality (Soriano *et al.* 2000). Similar reductions were obtained for leaf associated *Listeria monocytogenes* (Beuchat *et al.* 2004) and *E. coli* O157:H7 (Foley *et al.* 2004). Regrettably, the use of chlorine-based sanitisers within the food industry poses a risk to produce handling workers; Chlorine reacts with organic matter to produce unwanted by-products including trihalomethanes and chloramines, which are dangerous both because of their acute toxicity at high concentrations (Hery *et al.* 1998) and also their potential to cause cancer (Komulainen 2004). Consequently, safer sanitisers are continually being sought in an effort to eliminate this risk.

Ozone has long been licensed for the treatment of drinking water (Camel and Bermond 1998); its oxidising activity is greater than that of chlorine and decomposes in less than one hour to produce oxygen, a harmless by-product. Ozone is generated for industrial use by the application of high voltage to gaseous oxygen, before being dissolved in water to produce an aqueous solution as required. Unfortunately, whilst ozone is a potent oxidising

agent, it is difficult to dissolve in water; undissolved gas bubbles cause tissue discoloration and damage where they come into contact with plant material (Sihota 2008).

Other chemical wash treatments include peracetic acid and hydrogen peroxide. Like chlorine and ozone, these are oxidising agents capable of inactivating bacterial cells through disruption of the cell membrane. However, both are much weaker oxidising agents than chlorine (Penna *et al.* 2001), the consequence of which is only weak biocidal activity against produce-associated bacteria (Hajduk and Surówka 2005; Ruiz-Cruz *et al.* 2007). Alternative sanitisers proposed for use on fresh produce and evaluated at laboratory scale include plant essential oils; however, bactericidal activity was found to be both species specific and temperature-sensitive (Smith-Palmer *et al.* 1998; Hammer *et al.* 1999). In a small number of cases, essential oils were also found to adversely affect produce quality (Gutierrez *et al.* 2008). Such studies indicate that in order for the concept to be used on an industrial scale, a mixture of several key essential may be necessary to maximise effectiveness against a range of pathogens and environmental conditions.

Several studies have indicated that combining two or more sanitisation treatments can produce synergistic effects which reduce microbial contamination to a greater degree than any single biocide. For example, ozone and organic acids display synergistic activity against *E. coli* O157:H7 and *Listeria* on lettuce (Yuk *et al.* 2006), whilst, chlorine and irradiation show a synergistic biocidal effect against *E. coli* on coriander (Foley *et al.* 2004).

1.5.3 Packaging

In addition to the use of sanitisation treatments, microbial growth on fresh fruits and vegetables can be suppressed by the use of appropriate packaging and storage conditions. In Section 1.5.1 the benefits of low temperatures in food preservation were discussed; to maximise product shelf like, produce is frequently not only chilled but also encased in specialised Modified Atmosphere Packaging (MAP)(Church and Parsons 1995). The ability to control the gas composition inside sealed packaging to create MAP helps to suppress the growth of aerobic pathogens and spoilage bacteria by the removal of most or all available oxygen inside the packaging (Allende *et al.* 2004). MAP utilises a known gas composition at the time of sealing the package, but no further control of the atmosphere is employed (Cutter 2002). Consequently, metabolic processes associated with cells in the

foodstuff itself, as well as any resident microflora, may alter the atmosphere inside the packaging; this must be taken into account when selecting the gas mixture to be used.

A number of studies have assessed the role of MAP in suppressing bacterial growth on pre-packaged fresh fruit and vegetable products. In general, increasing nitrogen and carbon dioxide (CO_2) concentrations and lowering oxygen relative air produces a bacteriostatic effect (Lee and Baek 2008), though this can be overcome if the initial microbial load is sufficiently high (King *et al.* 1991) or the temperature becomes raised (Jacxsens *et al.* 2002). Nonetheless, in combination with cold chain production and surface sanitisation of produce prior to packaging, MAP is an important factor in extending product shelf life without compromising quality or safety.

1.5.4 Preventative measures

Given that pathogen reduction by post-harvest sanitisation techniques rarely exceeds around 2-log (Gil *et al.* 2009), prevention of contamination events is widely regarded as central to safe produce production (Beuchat and Ryu 1997). If pathogens can be prevented from entering the production chain at their source, less reliance on chemical biocides is required. Seed sterilisation can be used to reduce the contamination of aerial plant surfaces which would otherwise be present at the time of germination; this technique has also been shown to reduce the incidence of commercially significant plant diseases in crops. Following germination, growing crops must then be protected from exogenous sources of bacterial cells, two of the most prevalent being manure-based fertiliser and irrigation water (Solomon *et al.* 2002).

Contaminated irrigation water has been implicated in a number of high-profile outbreaks of foodborne illness (Centers for Disease Control 2006d; Soderstrom *et al.* 2008). The quality of irrigation water taken from rivers is strongly influenced by upstream land use; bacteria present livestock faeces can be washed into streams and rivers and be carried many miles in a viable state before water is removed and applied to crops.

Improper composting of animal manure can lead to contamination of leaf surfaces from the soil. Composting of manure is utilised to stabilise organic nutrients and reduce odour as well as inactivating pathogenic microbes and weed seeds. Elevated temperatures inside large manure piles can be tolerated by thermophilic bacteria, yet are lethal to many enteric

pathogens (Lung *et al.* 2001). however, careful temperature monitoring and turning of manure is required to ensure that all parts of the pile are exposed to elevated temperatures (Pell 1997). Following the application of contaminated manure to soils, a number of vectors have been proposed for transmission of bacteria to plant leaves; these include splashing following irrigation (Solomon *et al.* 2002) or invertebrate movement from organisms such as slugs (Sproston *et al.* 2006) or nematodes (Kenney *et al.* 2006). Contamination of both animal manure and irrigation water can be largely prevented by careful microbiological monitoring prior to application. In addition, stringent monitoring of livestock health may help to reduce pathogen entry into the agricultural system (Pell 1997).

1.5.5 Quality control

1.5.5.1 Methods for monitoring food safety

Conventional isolation methods using selective microbiological media are widely used for pathogen monitoring within the food industry (de Boer and Beumer 1999). Colony counting of diluted samples can be used to quantify microbial load, whilst a variety of biochemical tests can be used to identify presumptive pathogens. In the UK, the Health Protection Agency provides standard methods, specifying recommended media and incubation conditions for individual pathogens, such as those for *Salmonella* (Health Protection Agency 2007a). Allowing for sample preparation, dilution and plating, colony growth, counting and possible biochemical testing, conventional methods can take several days to give a result. In addition, production of media and sample preparation are labour intensive processes, though automated plating and colony counting techniques can reduce the time taken to process samples. Biochemical testing kits such as the API system (BioMérieux, France) supply all the reagents to provide rapid and convenient serotyping of isolated bacterial colonies.

Alternative detection and enumeration technologies that are used, albeit in a less widespread manner include the adenosine triphosphate (ATP) bioluminescence assay, in which the firefly enzyme luciferase is applied to a sample; subsequent luminescence of luciferase in the presence of ATP is used to assess viable microbial load (Aycicek *et al.* 2006). ATP bioluminescence has a detection limit of 10^4 bacterial cells required to produce a positive signal (Davidson *et al.* 1999), which may present a problem for bacteria

with low infective doses. In addition, at present ATP bioluminescence cannot distinguish between bacterial species (de Boer and Beumer 1999) and is therefore more suited to monitoring of total microbial load in situations such as production of sterile materials.

Epifluorescence microscopy can be used to quantify total microbial load recovered from a sample by collection of suspended microbes on a membrane filter and staining with a general nucleic acid stain (Morris *et al.* 1998). However, in order to distinguish between bacterial species, sequence-specific probe hybridisation is required, which is more time consuming and more technically difficult than simple nucleic acid staining (Tortorello and Reineke 2000). PCR-based detection has been successfully used in laboratory-scale studies for the detection of pathogens in fresh produce (Guo *et al.* 2000; Johannessen *et al.* 2002). However, costly reagents and low reproducibility of results between different laboratories have held back the uptake of molecular techniques on a large scale (Hill 1996). Clearly there are number of criteria which must be met in order for new technologies to replace conventional culture techniques; speed, cost and reproducibility of results being amongst the most critical for widespread use.

1.5.5.2 The Viable but Nonculturable (VNC) concept

Optimum conditions and culture media have been determined for the majority of commonly studied bacteria. When these are applied, a healthy, metabolically active population of the chosen species can be expected to produce good colonial growth. However, when the population has been subjected to injury or stress, those conditions may no longer produce growth. First impressions suggest that cell death has occurred; however it is now known that sublethal stress can cause sufficient physiological change in the bacteria to prevent growth in culture media. This is known as the Viable but Nonculturable (VNC) state (Byrd *et al.* 1991). In pathogenic bacteria, the relationship between culturability and cellular functions is complex; some stresses such as peracetic acid or chlorine treatment cause bacteria to become VNC but maintain virulence (Caro *et al.* 1999; Jolivet-Gougeon *et al.* 2006), whilst VNC status has previously been observed to be a reversible physiological state (Ravel *et al.* 1995; Mizunoe *et al.* 1999).

Selective media are commercially available to identify an enormous range of bacteria and protocols including an enrichment step have proved helpful in the recovery of sub-lethally stressed cells (Drysdale *et al.* 2004) which would otherwise be non-culturable and give

false negative results (Kolling and Matthews 2001). The time constraints imposed by experimental protocols can place limits on the accuracy of culture-based enumeration, as some stressed bacteria may not form colonies within the incubation period normally sufficient to detect that strain and will therefore not be counted (Kogure *et al.* 1979). In very mixed bacterial populations, slower-growing bacteria may have less space and nutrients available to them as a result of more dominant, faster growing species which would result in a count skewed in favour of a few dominant strains (Reasoner and Geldreich 1985).

Despite growing laboratory-based evidence that plate count methodologies alone are an inaccurate determinant of microbial load, they remain the cornerstone of pathogen monitoring in the food industry (de Boer and Beumer 1999). Consequently, if VNC cells are present they are likely to escape detection entirely using current quality control (QC) protocols; alternative, culture-independent technologies need to be adopted more widely to allow detection of VNC pathogens.

1.5.5.3 What defines bacterial cell death?

As discussed in Section 1.5.5.3, bacterial cell viability had been widely regarded being directly reflected by cultivability. Colony counting on agar media, or enumeration in broth via Most Probable Number or optical density, have formed the backbone of most microbiological studies to date. Increasingly, however, awareness of the VNC state is forcing researchers to turn to other definitions of cell viability and find techniques to support these. In the context of laboratory-based research, the definitions of cell death are operational rather than conceptual; philosophical concepts regarding death may not plausibly be evaluated within a laboratory setting (Kell *et al.* 1998). Having determined an operational parameter for cell death, it is then possible to select a technique which distinguished between cells which meet the chosen assumption of death and cells which do not.

Cell cycling and reproductive potential can be assessed without the requirement for the formation of visible colonies or turbidity. Elongation of viable cells in the presence of quionolone and yeast extract has previously been used to infer viability (Kogure *et al.* 1979); cells which do not respond to the treatment are presumed to be unable to go through a normal cell cycle and thus be nonviable. The limitation of cultivability and cell

elongation, which depend on reproductive capacity, is that the period of observation limits the ability to assess viability. A time period must be selected in which it can be stated that the cells under study could reasonably be expected to have multiplied (Kell *et al.* 1998). There is no guarantee that cells which have not multiplied may have done so if observed for longer, and thus may not be truly dead.

Membrane integrity has also previously been regarded as key to bacterial viability (Duffy and Sheridan 1998; Giao *et al.* 2009). Membrane-impermeant dyes such as propidium iodide are excluded from intact cells but can stain those with compromised membranes. Alternatively, the accumulation in cells of the dye Rhodamine 123 relies on an energised plasma membrane for active uptake. Cells with damaged membranes may meet the criteria for nonviability, such positive propidium iodide staining (Boulos *et al.* 1999), when there is no guarantee that the membrane damage is lethal to the cell.

Rhodamine 123 is also indicative of a cell's metabolic activity, as its uptake into cells is ATP-driven and this requires cell respiration to occur. Tetrazolium salt reduction using compounds such as CTC, which yields a fluorescent product when metabolised, can also indicate active cell metabolism (Corich *et al.* 2004). Like cultivability, the success of such an assay depends on the period of observation; a cell which fails to metabolise the substrate within the duration of the experiment is automatically presumed nonviable, so care must be taken when using such techniques on slow growing or heavily stressed cells (Kell *et al.* 1998).

The degredation of RNA and later in dead cells present an opportunity for viability assessment by analysis of cellular nucleic acid content.A wide variety of nucleic acid stains are commercially available, though care must be taken with regard to specificity as some have affinity to DNA or RNA only, whilst others will bind to both. Quantification of fluorescence is perhaps best achieved using flow cytometry, but samples can be assessed visually for comparative purposes using standard fluorescence microscopy.

Whichever technique for viability assessment is preferred, the ability of bacterial cells to change state from a culturable to nonculturable phenotype and back again suggests that death cannot be inferred by the failure to meet a single criterion for viability.

1.6 Aims and objectives

The overall purpose of this investigation has been to use novel imaging techniques to allow *in situ* study of phylloplane-associated bacteria. The ability to directly visualise leaf surfaces is utilised to remove quantitative inaccuracies, inherent in classical microbiological techniques, which use recovery of bacteria and subsequent culturing. The use of EDIC microscopy for this purpose is proposed due to the potential for rapid, non destructive imaging of surfaces and its previous use in the visualisation of biofilms (Keevil 2003). Following development of the necessary techniques, it was intended that EDIC imaging of the phylloplanes of leafy salad crops could be used to answer a number of key questions relating to both native leaf microflora and immigrant human enteric pathogens whose presence may pose a risk to human health.

The first aim of this investigation was to examine intact communities of microorganisms *in situ* on plant surfaces using EDIC microscopy with epifluorescence. Plant leaves are very responsive to the environment and so the techniques used to view them during this study have been optimised to be as rapid and non-destructive as possible to avoid producing images depicting artefacts of the experimental procedure as frequently happens when using techniques such as SEM.

Direct imaging of the leaf surface allowed bacterial colonists to be viewed in their natural spatial context relative to one another and to the topography of the leaf surface itself. A key objective of this work has been to develop a method of distinguishing genuine bacterial cells from inorganic debris on the leaf surface using molecular staining techniques. Chapter 3 presents a novel method sensitive enough to allow quantitative assessment of the indigenous phylloplane populations on different salad leaves, involving development of a staining protocol to allow the efficiency of traditional recovery and culture techniques for measuring total bacterial load to be assessed. The possibility of developing a bacterial viability assay suitable for use directly on leaves is explored in Chapter 3, in order to better understand the proportion of bacterial colonists which were viable and whether spatial arrangement affected survivorship.

In order to place in context the novel techniques applied to phylloplane microbiology during this investigation in context, the second aim of this study has been to directly compare *in situ* microscopy with conventional recovery and culture methodologies. In

Chapter 4, EDIC/EF microscopy techniques developed during the study have been used to quantify total bacterial load on a variety of salad leaf species for comparative purposes and also to assess the efficiency of the Pulsifier as a tool for bacterial recovery from the phylloplane.

Having discovered fundamental information about the autochthonous microbial communities and their distribution on salad leaf surfaces, the third aim of this investigation is to consider the process of phylloplane colonisation by the enteric pathogen *Salmonella*. With the aid of GFP reporter technology, EDIC/EF microscopy is proposed in Chapter 5 as a technique to analyse spatial patterns of colonisation relative to key leaf topographical features and resident microflora. The molecular mechanisms of attachment to phylloplanes are explored using a selection of *Salmonella* mutant strains deficient in genes involved in the curli biosynthesis pathway.

Finally, in Chapter 6 this investigation will attempt to answer the question of how to effectively sanitise produce intended for human consumption so that it could be marketed as 'ready-to-eat' without posing a risk to human health. The widely used biocide sodium hypochlorite has been evaluated alongside a novel sanitiser for the decontamination of fresh spinach leaves contaminated with *Salmonella*. Biocidal activity is assessed using both standard plate count and microscopic analyses in order to determine the total viable and total culturable *Salmonella* populations recovered from leaves, with the aim of inferring the VNC population size resulting from sanitisation processes. The effects of chemical biocides on the appearance and physiology of the leaves themselves will also be considered.

CHAPTER 2

GENERAL METHODOLOGY

2.1 Leaf material

Fresh baby salad leaves used in this study were provided by Vitacress Salads Ltd. Unless otherwise specified, all baby leaves were harvested from field-grown salad crops of no more than six weeks of age. Where possible, leaf material of UK origin was used, but due to the limitations of the growing season, material from other countries was also used, predominantly Spain and Portugal.

2.2 Bacterial strains and culture media

Salmonella enterica serovar Thompson (hereafter *Salmonella thompson*) strain RM2311 containing plasmid pWM1007 and strain RM2313 containing plasmid pWM10012 were kindly provided by R. Mandrell at the United States Department of Agriculture. The plasmid pWM1007 incorporates the gene encoding the *Aequorea victoria* GFP protein while pWM10012 incorporates the gene for CFP, the cyan derivative of GFP (Miller *et al.* 2000). Both plasmids contain genes required for replication along with a gene encoding kanamycin resistance (*kan*), which acts as a selectable marker for expression of the fluorescent proteins.

Salmonella enterica serovar Typhimurium (hereafter *Salmonella typhimurium*) strains each lacking a different gene involved in the curli biosynthesis pathway were kindly provided by F Norel at the Institut Pasteur in France. Mutant strains contained a chloramphenicol resistance gene (*cm*) to provide phenotypic selection. Strains each lacking a functional copy of one the genes *crl*, *rpoS* or *csgB* were used in the study, alongside both a double mutant lacking *crl* and *csgB* and the wild type parent strain, *S. typhimurium* ATCC 14028. All of the bacterial strains used in the study are listed in full in Table 2.

Table 2: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
Salmonella thompson		
RM2311	RM1987(pWM1007)	(Brandl and Mandrell 2002)
RM2313	RM1987(pWM1009)	(Brandl and Mandrell 2002)
Salmonella typhimurium		
ATCC 14028	Wild type	American Type Culture Collection
ATCC*rpoS*	ATCC 14028 Δ*rpoS*::*cm*	(Robbe-Saule *et al.* 2006)
ATCC*crl*	ATCC 14028 Δ*crl*::*cm*	(Robbe-Saule *et al.* 2006)
ATCC*csgB*	ATCC 14028 Δ*csgB*::*cm*	(Robbe-Saule *et al.* 2006)
ATCC*crlcsgB*	ATCC 14028 Δ*cr*l Δ*csgB*::*cm*	(Robbe-Saule *et al.* 2006)
Plasmids		
pMW10	Km^r Mob^+ repB lacZ oriV	(Wosten *et al.* 1998)
pWM1007	Km^r; pMW10 Δ*lacZ*V[(T1)4-Pc-*gfp*-T1]	(Miller *et al.* 2000)
PWM1009	Km^r; pMW10 Δ*lacZ*V[(T1)4-Pc-*cfp*-T1]	(Miller *et al.* 2000)

Salmonella strains were stored at -80^{o}C on Protect^{TM} beads (Fisher, UK) in accordance with the manufacturer's instructions and were routinely sub-cultured in Tryptone Soya Broth (TSB; Oxoid, UK) supplemented with 50 μg/ml kanamycin (Sigma-Aldrich, UK) or 15 μg/ml chloramphenicol (Sigma-Aldrich, UK) where required for selection of mutant phenotypes.

Where *Salmonella* cells were recovered from environmental samples, such as leaf material, serial dilutions of the resultant suspension were made in phosphate-buffered saline (PBS;

Oxoid, UK) and inoculated by spreading evenly onto Rambach agar (VWR, UK) plates. Rambach agar is a chromogenic medium for the selective isolation of *Salmonella* species from complex environmental samples (Rambach 1990). *Salmonella* species metabolise propylene glycol in the medium; resulting in a localised decrease in pH which precipitates Neutral Red from within the medium, producing a characteristic red colony colour (Figure 6). *E. coli* O157:H7 NCTC 12900, which lacks the Shiga-like toxin genes, was used as a quality control organism to confirm the selectivity of each batch of Rambach agar produced. *E. coli* produces blue colonies whereas *Salmonella* forms pink-red colonies. *Salmonella* colonies were enumerated after growth at 37 ^{o}C for 24 h.

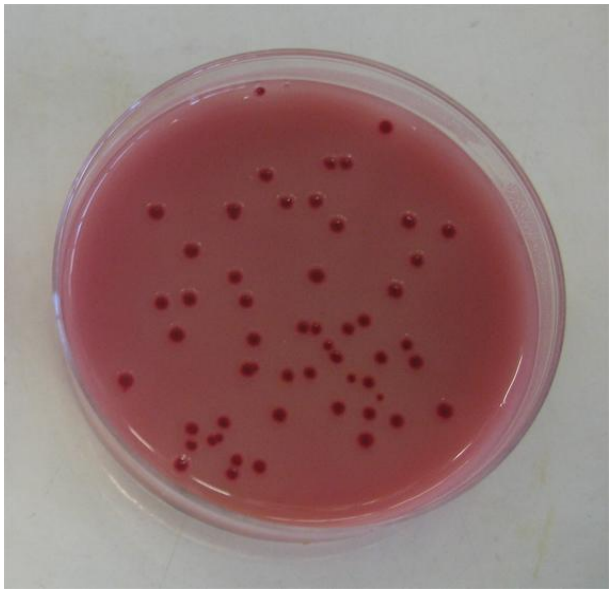


Figure 6: Salmonella thompson *colonies grown on Rambach agar, producing the red colony colour characteristic of* Salmonella *strains grown on this medium.*

Following recovery from leaves, total bacterial samples were grown on R2A agar (Oxoid, UK) at 22 ^{o}C for 4 days. R2A is a low nutrient medium used in potable water analysis (Reasoner and Geldreich 1985) with an extended period of incubation designed to allow slow-growing bacteria to form visible colonies and also time for development of coloured colonies in pigmented bacterial species (Figure 7); these conditions are also designed to

help resuscitation of sub-lethally stressed bacteria. Details of the manufacturers of the culture media used throughout this investigation are given in Table 3.

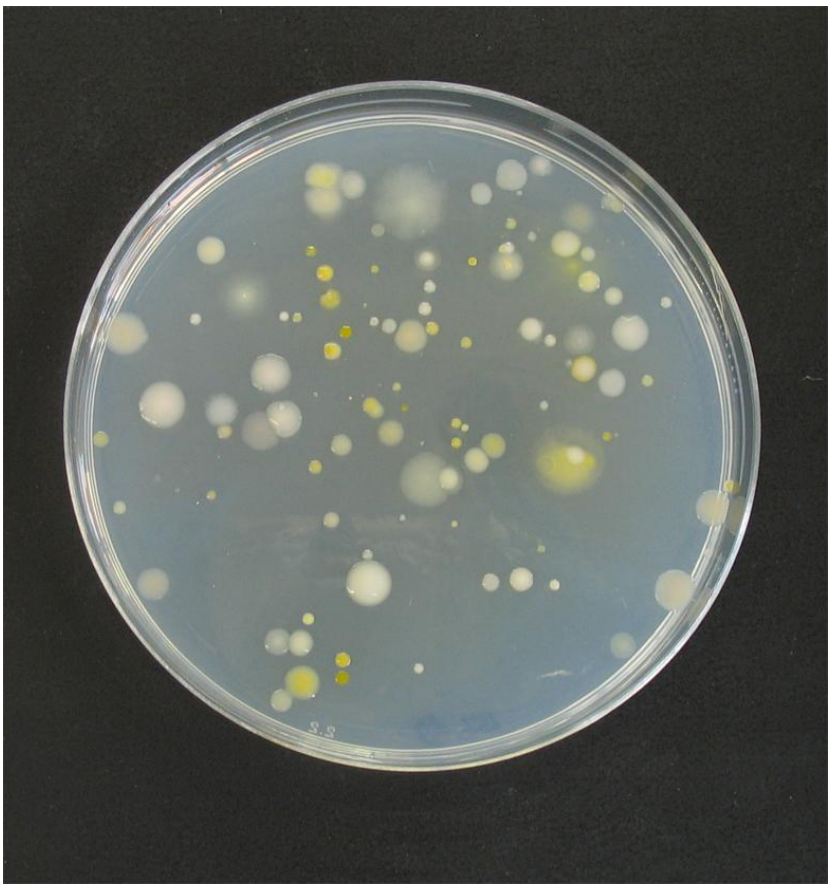


Figure 7: Total culturable heterotrophs recovered from spinach leaf surfaces, grown on R2A medium to allow pigment development and improve detection of slow-growing cells.

Table 3: Culture media used in this study. Formulations of custom-made media are given in Appendix 2.

Name	Abbreviated name	Source
Agars		
Congo Red indicator agar	CRI	Custom-made
Luria-Bertani agar	LBA	Merck
Luria-Bertani agar without NaCl	LB0	Custom-made
R2A medium	R2A	Oxoid
Rambach agar	-	Merck
Tryptone soya agar	TSA	Oxoid
Broths		
Colony-Forming Antigen broth	CFA	Custom-made
Luria-Bertani broth	LBB	Merck
R2 broth	R2B	Custom-made
Tetrathionate broth	-	Oxoid
Tryptone soya broth	TSB	Oxoid

2.3 Preparation of standard inocula

Standard inocula were used to experimentally contaminate leaf material and additionally as starter cultures for biofilm formation. Stationary-phase cultures of *Salmonella* strains were grown from frozen bead stocks by transferring a single bead into TSB supplemented with antibiotics where appropriate. Cultures were incubated at 37 ^{o}C with shaking at 200 rpm for ~20 h prior to use.

Cell numbers in overnight cultures were determined by pour plate counts of serial 1 in 10 dilutions of the culture, in which 1 millilitre aliquots of the dilutions were mixed with 20 ml cooled sterile molten (45 ^{o}C) TSA then allowed to solidify before incubation for 24 h at 37 ^{o}C and subsequent enumeration of colonies. Cultures were then diluted to 1-5 × 10^{6} CFU/ml in sterile distilled water (dH_2O) to form the inocula. Cell numbers in the inoculum were then verified using the same pour plate count method.

2.4 EDIC microscopy of leaf surfaces

Episcopic Differential Interference Contrast (EDIC) microscopy was used to study the surfaces of spinach (*Spinacia oleracea*), rocket (*Eruca vesicaria*) and watercress (*Nasturtium nasturtium-aquaticum*) leaves in order to evaluate key features of the leaf surface, along with the quantity and localisation of bacterial colonization.

Conventional Differential Interference Contrast (DIC) microscopy uses a prism to produce two parallel polarised beams of light which simultaneously hit a sample. Shape, thickness and composition variably influence the paths of each light beam (Figure 8). The interference of the two beams with one another after having passed through the sample produces a pseudo-three dimensional image. EDIC microscopy build upon this principle, using a light source and prism located above the microscope stage, to produce an image from episcopic light refracted by the surface of the sample (Figure 9). EDIC microscopy is of particular use in surface microbiology as it allows for the direct visualisation of bacterial colonisation *in situ* on opaque materials such as plastics, metals and also tissue surfaces (Walker and Keevil 1994; Keevil 2001). The microscope system used in this investigation was custom designed by Best Scientific Ltd (UK) to combine EDIC microscopy with epifluorescence.

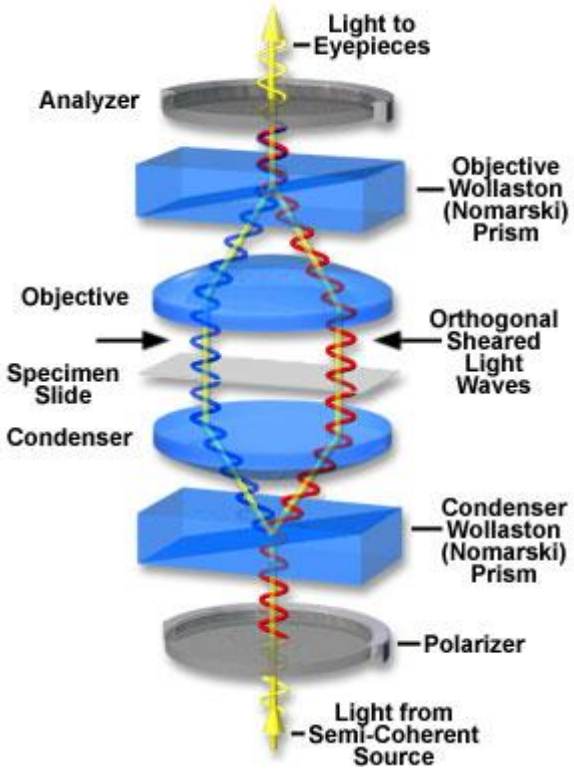


Figure 8: Schematic diagram of the optical light path in conventional DIC microscopy.(Olympus 2009)

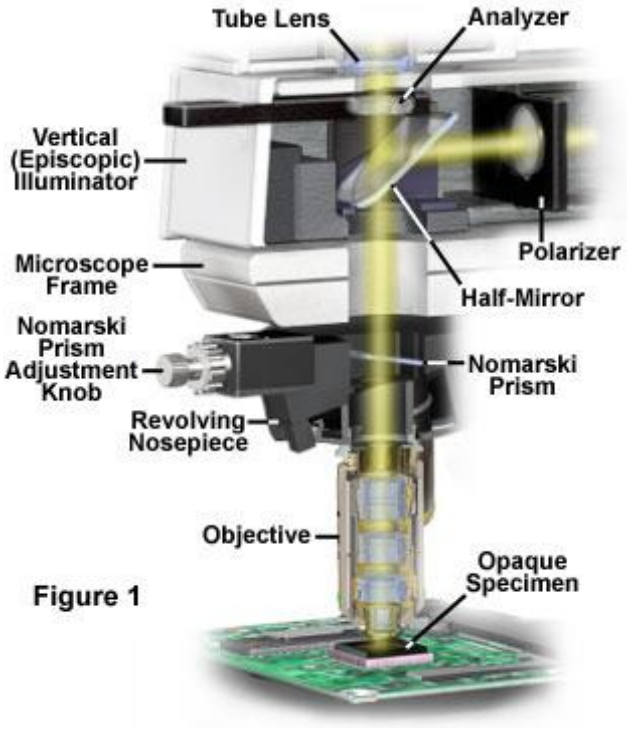


Figure 9: Diagram of the optical path in EDIC microscopy of an opaque sample. (Nikon 2009)

Leaf pieces measuring approximately 1 × 1 cm were aseptically cut and placed onto sterile glass slides. In order to stabilise the leaf surface for microscopy 100 µl of 1.2 % (w/v) cooled molten (45 ^{o}C) water agar was first placed onto the glass slide; leaf pieces were then gently pressed into the agar (Brandl and Mandrell 2002). Leaf pieces were then examined under the EDIC microscope at both 500× and 1000× magnification; long working distance lenses were used as they do not require the use of coverslips or oil. Multiple image capture in the *z*-plane using a computer-controlled digital (CCD) camera (Roper Industries, UK) was employed to study selected regions with particularly uneven topography, thus allowing a greater portion of the field of view to be brought into focus for analysis.

2.5 Scanning electron microscopy (SEM) of rocket leaves

SEM of rocket leaves was performed using a method adapted from Gniwotta *et al.* (2005). Unwashed baby rocket leaves were dried at 22 ^{o}C for 72 h, following which 5 × 5 mm

pieces were cut and mounted onto circular aluminium "stud" sample mounts. Samples were sputter-coated with gold palladium to an average depth of 20 nm. Leaf samples were then viewed using a Hitachi S800 scanning electron microscope; images were captured using an attached CCD camera.

2.6 Use of nucleic acid stains in conjunction with EDIC/EF microscopy for *in situ* visualisation of phylloplane bacteria

A number of different fluorescent nucleic acid stains were tested for their ability to selectively stain bacteria *in situ* on leaf surfaces. The stains used and their associated characteristics are listed in Table 4. All staining solutions were prepared in dH_2O and filter-sterilised using a syringe-mounted membrane filter of 0.2 µm pore size prior to use. Working solutions were prepared from frozen stock solutions on the day of use. Details of the preparation of staining solutions, staining times and fluorescence filter sets used are given in Table 2.3.

Leaf samples were prepared and mounted on slides in an identical manner to that described previously for EDIC microscopy (Section 2.4). Stains were applied directly to the leaf surface in 50 µl aliquots using a Gilson style pipette, incubated in the dark for the appropriate period of time and rinsed with sterile dH_2O. Each sample was left to dry for approximately 30 min prior to viewing using EDIC/EF microscopy with the appropriate fluorescence filter sets. Where quantitative analysis was required, a total of at least 200 bacterial cells per sample were enumerated from a minimum of 10 fields of view using an eyepiece counting grid. A calibrated graticule slide was used to verify the sample area viewed through the counting grid to enable bacterial numbers to be expressed per unit area. The requirement for at least 200 cells to be enumerated has become a convention in microbiology, as it has been determined to optimise the 95 % confidence intervals assuming that the data follows a Poisson distribution. Whilst increasing the number of cells counted per filter may reduce the variability of the data, increasing the sample volume analysed through use of replicate filters is widely perceived to be a better sampling strategy (Kepner and Pratt 1994). Consequently, duplicate leaf disks were produced for each leaf sampled. Where 10 fields of view failed to yield a count of 200 cells or more, extra fields of view were examined until the total was reached. As such occurrences were very rare, the sampling strategy was not altered for all samples under study.

Table 4: Characteristics of nucleic acid stains used in this study

Name	Manufacturer	Specificity	Concentration of working solution (μM)	Staining time on leaf surfaces (min)	Fluorescence filter set used
Acridine Orange	Fisher	ssDNA, dsDNA, RNA	6.63	5	FITC, TRITC
4',6-diamidino-2-phenylindole (DAPI)	Sigma	dsDNA	28.56	20	DAPI
SYBR Green I	Invitrogen	dsDNA	*	20	FITC
SYBR Green II	Invitrogen	RNA	*	20	FITC
SYBR Gold	Invitrogen	ssDNA, dsDNA, RNA	*	20	FITC
SYTOX Orange	Invitrogen	dsDNA	5.00	20	TRITC
BacLight LIVE/DEAD Assay					
Component A (SYTO-9)	Invitrogen	ssDNA, dsDNA, RNA	5.01	30	FITC
Component B (Propidium Iodide)	Invitrogen	ssDNA, dsDNA, RNA	30.00	30	TRITC

* Formula weight proprietary to Invitrogen and kept confidential. Working solution was 1/10000 dilution of the supplied stock solution.

2.7 Recovery of bacteria from the phylloplane using the Pulsifier

Bacteria were recovered from leaf surfaces using a Pulsifier (Microgen Bioproducts Ltd; Figure 10) for culture-based analyses. Leaf samples were processed according to standard method ISO 7218 (International Organisation for Standardization 2007); samples weighing 25 g each were added to 225 ml sterile PBS in a stomacher bag (1:10 w/v) and pulsified for 30 s; the resulting diluent was then decanted into a sterile container for further analysis. In addition, the Pulsifier was used for analysis of individual leaves. A single leaf was added to 50 ml sterile PBS in a small stomacher bag and pulsified for 30 s. The diluent was decanted as described previously. Individual leaves and 25 g leaf samples were retained after pulsification when required for microscopic analysis.

Figure 10: The Pulsifier (Microgen Bioproducts 2003).

2.8 Use of membrane filters for quantitative EDIC/EF microscopy of bacteria recovered from the leaf phylloplane

EDIC/EF microscopy was used for culture-independent enumeration of bacteria recovered from leaves. Bacterial suspensions obtained by pulsification of leaves were stained using either DAPI or SYBR Green I using the final concentrations and staining times given in Table 2.3. Following staining, 1 millilitre aliquots of each sample were vacuum filtered through black, 25 mm 0.2 µm pore Nuclepore® track-etched polycarbonate membranes using vacuum filtration. Black membranes were chosen in order to reduce background fluorescence during microscopy.

Membranes were viewed using EDIC/EF microscopy with appropriate filter sets (see Table 2.3). For enumeration of bacteria, fields of view were photographed using the microscope's attached CCD camera. Stained cells in each image were identified and enumerated manually; the average number of cells present on the filter and hence per millilitre of suspension) was then calculated. A minimum of ten fields of view containing a combined total of at least 200 cells were analysed for each filter examined.

2.9 Determination of total culturable heterotrophs by R2A plate count

Total culturable heterotrophic bacteria were determined by growth on R2A medium (Oxoid, UK). Individual unprocessed baby salad leaves were pulsified as described in Section 2.7. Serial 1 in 10 dilutions of the resulting suspension were produced using sterile PBS; 200 µl aliquots of each suitable dilution were then inoculated by spreading onto triplicate R2A agar plates. Plates were incubated at 22 °C for 4 days, after which the number of colonies per plate was counted. In order to maximise the accuracy of the colony count, only counts of between 20 and 200 colonies per plate were enumerated.

2.10 Growth of static biofilms in 6 well plates

2.10.1 Mixed phylloplane isolates

Total phylloplane bacteria were recovered from fresh baby spinach leaves into sterile PBS as described in Section 2.7 and used to grow static biofilms. Following pulsification of each sample, 1 millilitre aliquots were taken from the resulting bacterial suspension and put into separate wells of a 6 well plate (Greiner Bio-One, Germany). Half strength R2

broth ($R2B_{50\%}$) was added in 2 ml volumes to each biofilm well to give a total culture volume of 3 ml. Biofilms were grown in static cultures at 22 ^{o}C for 7 d; following this, the culture media was gently aspirated and replaced with 3 ml of fresh $R2B_{50\%}$ at 48 h intervals.

Cells were removed from the wells by vigorous scraping with a sterile pipette tip and suspended in 50 ml sterile PBS. Resulting suspensions were pulsified for 30 s; control (unpulsified) suspensions were also prepared. One millilitre aliquots were then stained with 28.56 μM DAPI solution for 15 min and inoculated onto membrane filters (see Section 2.8) for qualitative evaluation of biofilm break-up by the Pulsifier. Filters were viewed using EDIC/EF microscopy at 1000× magnification using a DAPI filter set.

2.10.2 *Salmonella* strains

Starter cultures of *Salmonella thompson* GFP and *Salmonella typhimurium* strains were cultured as described in Section 2.3. Cultures were enumerated by TSA plate count and diluted to approximately 1-5 × 10^4 CFU/ml. One ml aliquots were added to each well of a 6 well culture plate; half strength CFA medium ($CFA_{50\%}$, custom-made) was added to each well to give a final culture volume of 3 ml. Plates were incubated in static culture at 28 ^{o}C for 48 h prior to analysis.

Salmonella biofilms grown in 6 well plates were stained and examined using EDIC/EF microscopy. The supernatant from each well was gently removed by aspiration and the remaining biofilms rinsed twice with sterile PBS. One millilitre of 1/10000 × concentration SYBR Gold solution was added to each well and the plates were incubated in the dark for 15 minutes. The staining solution was rinsed from each well using sterile dH_2O and the biofilms allowed to dry for 20 min at room temperature. Biofilms were viewed at 1000× magnification using a FITC filter set. Six images were captured per well of each 6-well plate; the percentage surface coverage was determined by automated image analysis (see Section 2.17).

2.11 Comparison of curli production in *Salmonella* mutant strains using CRI plates

CRI plates were used to qualitatively determine curli production amongst the *Salmonella* strains used in this study. During growth on CRI plates, curli-expressing strains bind

Congo Red from the culture medium and produce red colonies; non curli-expressing strains produce white/cream colonies.

Overnight cultures of wild-type *Salmonella typhimurium*, its four curli synthesis mutants and *Salmonella thompson* GFP were prepared as described in Section 2.3. Each strain was inoculated onto CRI plates by streaking 1 µl of broth culture using a sterile loop, incubated at 28 ^{o}C for 72 h and then photographed using a Canon A95 digital camera. In order to ensure expression of the mutant phenotypes, CRI agar used for curli mutant strains was supplemented during production with 15 µg/ml chloramphenicol.

2.12 Quantitative Congo Red binding assay

Quantitative determination of curli production in *Salmonella* strains was performed using a modified form of an assay described by Gophna *et al.* (2001). Overnight cultures of *Salmonella typhimurium*, its four curli synthesis mutants and *Salmonella thompson* GFP were prepared as described in Section 2.3. Cultures were diluted with sterile TSB until their absorbance, determined using a spectrophotometer (Ultrospec II, LKB Biochem), was 0.90 at 620 nm (OD_{620}).This had previously been determined by TSA plate counts to give samples containing 1-2 $\times 10^{10}$ CFU/ml.

One millilitre aliquots of the diluted cultures were placed into sterile microcentrifuge tubes and pelleted by centrifugation at 8000 × *g* for 5 min. Supernatant from each sample was gently removed by aspiration and the cell pellets resuspended in 1 millilitre of 0.002 % (w/v) aqueous Congo Red. Samples were incubated at room temperature for 10 min before being pelleted by centrifugation at 8000 × *g* for 5 min. The OD_{500} of the supernatant from each sample was determined against a background of fresh Congo Red solution in order to determine the reduction in Congo Red levels as brought about by the binding of the stain to curli fibres present on *Salmonella* cell surfaces.

2.13 Quantifying attachment of *Salmonella* cells to individual spinach leaves

2.13.1 *Salmonella thompson* GFP

In order to determine the efficiency and spatial patterning of attachment, single unprocessed baby spinach leaves were suspended vertically in 125 ml of bacterial

inoculum consisting of 1-5 × 10^6 CFU/ml *Salmonella thompson* GFP in sterile distilled water (Figure 11). Sterile PBS was substituted for sterile distilled water in some samples for comparison of its effect on bacterial attachment to leaves. Leaves were incubated at 22 °C for 24 h; control samples of the inoculum with no leaf were also included to assess the level of inoculum growth during the incubation period. Leaves were then removed, 1 cm leaf discs were cut with a cork borer and analysed microscopically. For each leaf, one disk of the upper surface and one of the lower surface were prepared. Bacteria were counted directly using the EDIC microscope fitted with a FITC filter set to facilitate visualisation of GFP fluorescence. A minimum of 10 fields of view per disk were counted to give a total of at least 200 bacterial cells.

Figure 11: A single baby spinach leaf suspended in an inoculum of ~5 × 10^6 CFU/ml; Salmonella thompson GFP. Planktonic bacterial cells have equal access to the upper and lower leaf surfaces during inoculation.

2.13.2 *Salmonella typhimurium* strains

Leaves were inoculated as in section 2.13.1 using inocula containing 1-5 ×10^6 CFU/ml *Salmonella typhimurium* wild-type or one of its curli-expressing mutants. Following

inoculation, leaves were removed from the inoculum, rinsed by gently dipping in sterile dH_2O and then pulsified individually as described in Section 2.7. Serial 1 in 10 dilutions of the resulting suspension were plated onto Rambach agar. Serial dilutions of the inoculum used on each leaf and also the control inoculum were also plated onto Rambach agar after the incubation period to determine the approximate number attached to the leaf. All Rambach agar plates were incubated at 37 ^{o}C for 24 h before the colonies were counted.

2.14 Survival of *Salmonella thompson* on spinach leaves treated with chemical wash solutions

2.14.1 Preparation of wash solutions

All chemical wash solutions were prepared in batches of 1 L by dilution to the appropriate concentration in sterile dH_2O. Sodium hypochlorite solutions were prepared from Haz-Tab tablets (Inverclyde Biologicals); one Haz-Tab was dissolved in 1 L of water to produce a stock solution containing 2500 parts per million (ppm) free chlorine. This stock was further diluted to produce working solutions of 20, 50 and 100 ppm free chlorine. The stock solutions were kept for no more than one week; working solutions were prepared on the day of use. The chlorine concentration of all working solutions was verified prior to use with the aid of Merckoquant® chlorine test strips (Merck, USA).

Citrox solutions were prepared by direct dilution of Citrox 14W disinfectant solution (Citrox Ltd, UK) in sterile dH_2O. Solutions of 0.175 %, 0.35 %, 0.525 % and 0.7 % (all v/v) were prepared on the day of use. An organic antifoaming agent (BC Antifoam 86/013; Basildon Chemicals, UK) was added to the solutions at a ratio of 125 µl per 0.175 % Citrox present. Control solutions containing Antifoam in the same concentrations, but no Citrox, were also prepared.

2.14.2 Washing process

Leaf samples each weighing 25 g were placed into separate Stomacher bags each containing 225 ml of the chemical wash solution being evaluated. Samples were then washed by vigorous manual agitation of the bag for 2 min, following which the wash

solution was decanted. Sterile PBS (225 ml) was then added to each bag and samples were pulsified for 30 s. One millilitre aliquots of each resultant suspension were inoculated onto black membrane filters as described in section 2.8. As the *Salmonella thompson* cells expressed GFP and fluoresced green under epifluorescence microscopy, no staining was required prior to analysis using EDIC/EF microscopy.

Culturable *Salmonella thompson* cells were quantified by Rambach plate count as described in section 2.2. During processing of hypochlorite-washed leaf samples, a semi-selective pre-enrichment step was included to improve detection of stressed cells (Health Protection Agency 2007b). Serial 1 in 10 dilutions of the pulsified diluent were inoculated onto 45 mm 0.2 µm cellulose nitrate membrane filters in 10 ml aliquots. Filters were placed onto sterile media pads soaked in Tetrathionate broth supplemented with 20 µl/ml filter-sterilised iodine iodide solution (5 g iodine, 6 g potassium iodide, in 20 ml dH_2O; both Sigma-Aldrich, UK) and 20 µg/ml novobiocin (Sigma-Aldrich, UK). These were incubated at 37 ^{o}C for 24 h before the membrane filters were transferred onto Rambach agar plates and incubated as described in section 2.2. Red colonies indicating *Salmonella* cells on the filters were then enumerated.

2.15 Cell elongation assay for determination of Direct Viable Count

Direct Viable Counts (DVC) were performed using a modification of the method originally described by Kogure *et al.*(1979). One millilitre aliquots of recovered cell suspension were filtered through 25 mm 0.2 µm Isopore^{TM} track-etched polycarbonate membranes (Sigma-Aldrich, UK) in order to distinguish viable, metabolically active *Salmonella* without the use of plate counts. A minimum of 3 filters were prepared per wash treatment. Membranes were placed onto media pads soaked in 50% R2 broth ($R2B_{50\%}$) supplemented with 10 µg/ml pipemidic acid (Sigma-Aldrich, UK), incubated at 22 ^{o}C for 16 h and then examined by EDIC/EF microscopy using a FITC filter set. Elongated and non-elongated green-fluorescing cells were defined visually and then counted separately in a minimum of 10 randomly selected fields of view ensuring a total of 200 bacterial cells were counted per filter.

2.16 Image processing and analysis

EDIC/EF based image capture and, where required, *z*-plane scanning, was performed with the aid of Image-Pro Plus software v5.0.2.9 (MediaCybernetics). Image-Pro Plus was also used to perform automated analysis of static biofilm surface coverage where required by comparison of colours versus black pixel numbers on images of stained biofilms. Fluorescence images with a high degree of background colouration were processed using Adobe Photoshop CS2 (Adobe Systems Inc.) to decrease the background fluorescence. In order to ensure accurate comparison, the degree of background reduction was kept identical for all images examined. Adobe Photoshop was also used to increase the contrast of the pseudo-3D images created by EDIC microscopy for qualitative analysis; this was performed in order to allow for better visualisation of the heterogeneity in leaf topography.

2.17 Statistical analysis

Experimental procedures were performed independently a minimum of three times in order to ensure sufficient data for statistical analysis. Analyses were performed using the SigmaStat 3.5 (Systat Software Inc.) software package.

CHAPTER 3

EVALUATION OF EDIC MICROSCOPY FOR THE STUDY OF INDIGENOUS BACTERIA IN THE SALAD LEAF PHYLLOPLANE

3.1 Introduction

In situ detection of bacteria on surfaces including stainless steel and plastics is well documented (Azevedo *et al.* 2006; Rivas *et al.* 2007). Advanced microscopy, combined with molecular staining techniques, allows quantitative and specific detection of bacteria and bacterial biofilms. However, methods for *in situ* detection of bacteria in the salad leaf phyllosphere are less advanced; a plant leaf is living material and so represents an entirely different kind of surface, one capable of reacting not only to its bacterial colonists, but also to the conditions of sample preparation. In addition, the variety of photosynthetic pigment compounds contained within a leaf emit autofluorescence across much of the red region of the visible light spectrum (Lang *et al.* 1991).

The imaging techniques presented in this chapter form part of the first published investigation into the use of EDIC microscopy to study bacteria on the phylloplane (Warner *et al.* 2008). Several documented examples exist of EDIC microscopy being used in rapid and non-destructive examination of microbial biofilms on various surface types (Surman *et al.* 1996; Keevil 2003); the first aim of this investigation was to fully evaluate the potential of EDIC microscopy to study phylloplane bacteria. Direct study of the leaf surface allows bacterial localisation to be examined in relation to key leaf topographical features such as stomata, trichomes and veins. A second aim was to directly demonstrate the presence of intact biofilms *in situ* on leaves; there are currently few published studies claiming to have done so. In this study EDIC microscopy was used to examine bacteria on the surfaces of salad leaves; this allowed bacterial localisation to be considered in relation to key topographical features of the leaf surface. The final aim of this work was to find a staining protocol which could be used to enumerate bacteria present on leaves without the need for recovery, thus allowing their spatial localisation to be considered quantitatively. Three commonly used bacterial staining techniques were evaluated for their potential to quantitatively distinguish bacteria from the associated debris found on leaf surfaces: the first two, Acridine Orange and DAPI, are widely used and inexpensive fluorescent nucleic acid-binding dyes. The third was the BacLight LIVE/DEAD bacterial viability assay. BacLight combines the nucleic acid dye SYTO-9 with the membrane impermeant dye Propidium Iodide to produce a quantitative analysis of bacterial viability using techniques such as fluorescence microscopy or flow cytometry. Also evaluated were three members of

the family of SYBR molecular cloning dyes: SYBR Green I, SYBR Green II and SYBR Gold.

3.2 Materials and Methods

Details of the materials and methods used in this study are described in Chapter 2. Fresh baby leaves of spinach, rocket and watercress were examined using EDIC microscopy; rocket was also selected for SEM because of its successful dehydration prior to mounting and coating. A number of different nucleic acid stains were tested on the surfaces of spinach leaves; spinach was selected due to its year-round availability and also long sample lifespan under episcopic light, which was conferred by its robust leaf physiology relative to the other leaf species investigated. Stains considered suitable for quantitative *in situ* detection of bacteria were validated for specificity when used to stain phylloplane bacteria.

3.3 Results

3.3.1 EDIC microscopy of leaf surfaces

Fresh salad leaf surfaces were examined in order to try to identify bacterial contamination and relate it to key topographical features of the leaf. EDIC microscopy at 500× and 1000× magnification, using lenses with a long working distance, was used to examine the surfaces of fresh leaves without need for fixation or coverslips. Spinach, rocket and watercress leaf surfaces were observed directly using the EDIC bright field channel of the microscope. Figures 12 - 14 show typical micrographs obtained by direct examination of salad leaves using this method.

Surface contamination was frequently observed in the margins and junctions between leaf epidermal cells and also more centrally on the leaf cell surfaces (Figure 12). This contamination had many different morphotypes; some bodies were of an appropriate size and shape to be bacterial cocci or bacilli (Figure 12A, B). Other, larger bodies of material had a structured three-dimensional form, either granular (Figure 12C) or more slimy (Figure 12D) in appearance. Figure 12B was captured during examination of a visibly senescent leaf; the pale region is one of many that lacks the swollen appearance and

definition of individual leaf cells, and is likely to be a region of localised cell death on the leaf. Notably, this region also has many particles on the surface which resemble solitary bacterial cells; these appear to be present at a higher concentration in the senescent region than on surrounding leaf cells.

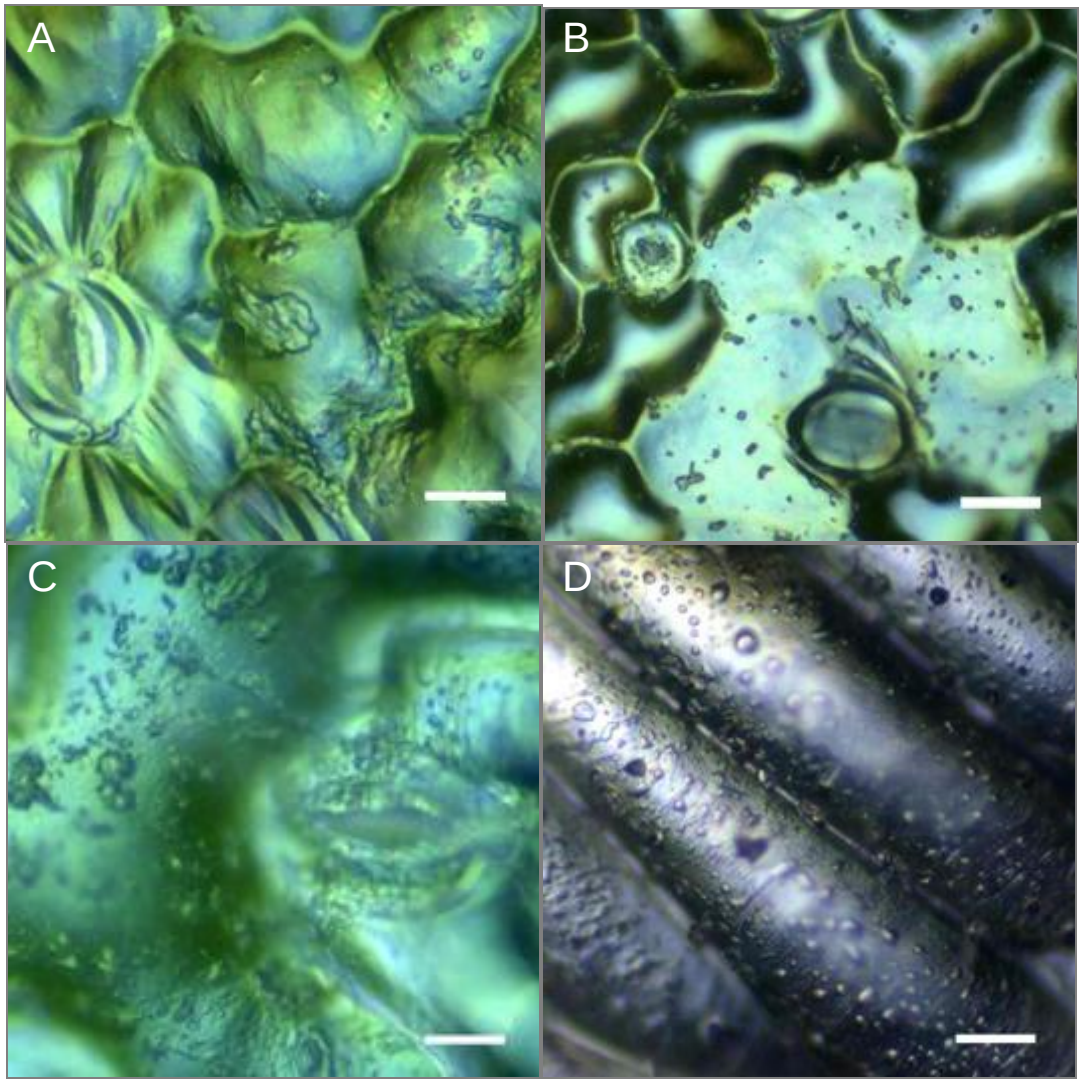


Figure 12: EDIC micrographs of salad leaf surfaces: watercress (A), rocket (B), spinach (C) and iceberg lettuce (D). Surface contamination is visible in epidermal cell margins (A), in regions of leaf senescence (B) and on the surface of epidermal cells (C and D). Bar = 10 µm.

The vascular system of the leaf species used in this study provided a topographically distinct region for study relative to the surrounding epidermis; leaf vascular elements take on the form of long parallel cells, with deep intercellular margins (Figure 12). Major veins typically appeared raised above the plane of the leaf surface. Material observed in these regions, like that identified on the leaf epidermis, was observed in the form of discrete particles (Figure 13B, C) or larger aggregations (Figure 13A, D). Heavy contamination, such as that apparent in Figure 13D, was observed to have formed a continuous layer of material of a more amorphous nature, covering extensive regions of the leaf surface. As before, some material was observed to be granular (Figure 13A, C) whilst other samples presented a more slimy appearance (Figure 13B, C).

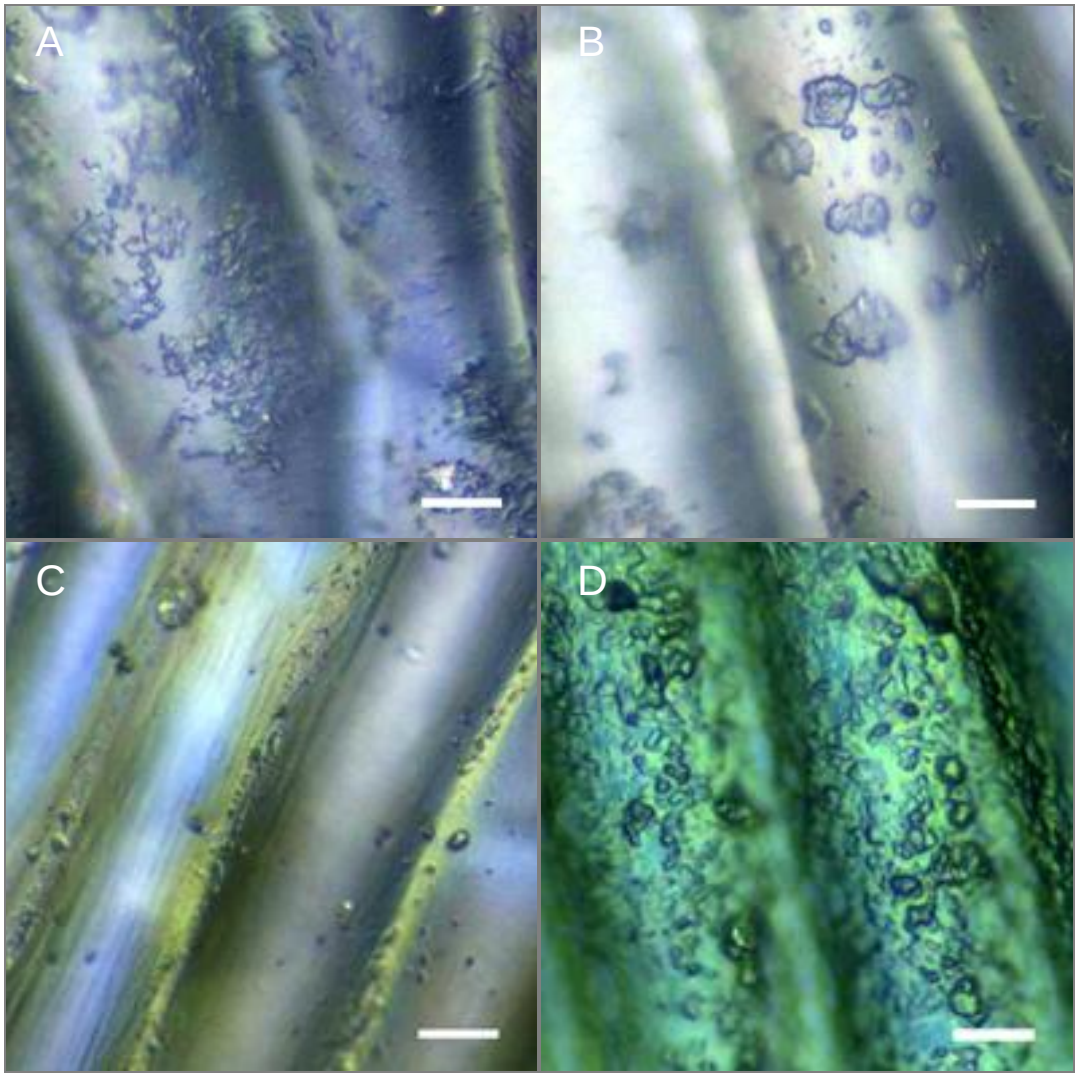


Figure 13: EDIC micrographs of leaf veins belonging to spinach (A and B) and rocket (C and D) leaves. Surface contamination levels vary between dense (A and D) and sparse (B and C) coverage of the leaf cells by foreign material. Bar = 10 µm.

Leaf surfaces possess numerous stomatal openings, which were observed in both open and closed states during this study. Figure 14 shows representative EDIC micrographs of stomata from fresh baby spinach and rocket leaves. Topographically, stomatal guard cells may be on the same plane as the leaf surface, however on the leaves examined in this study they were more commonly observed to be either sunken or raised relative to the plane of the surrounding epidermal cell layer. Based on the observations within this study, stomatal position did not seem linked to leaf species, as all three arrangements were apparent on all leaf types studied. Younger leaves seemed to have a generally higher number of stomata found to be flat on the leaf surface than older, more senescent leaves. Overall, however, a great deal of variation was observed between individual leaves and as such the trend was not a strong one.

Figure 14A shows a stomatal opening containing a single aggregate of material, which appears to be protruding out of the opening and above the plane of the surrounding leaf surface. From this image alone it is unclear as to the nature of the material, regardless it should be noted that it does not have the characteristic angular crystalline morphology normally seen when viewing soil particles; in addition, from its appearance this material could potentially be could be a bacterial aggregate. The size and position of the aggregate suggest that its presence would likely impede normal stomatal closure. Similar material was also frequently apparent on stomatal guard cells (Figure 14B). As observed on leaf veins, heavier surface contamination often seemed to take on a slimy appearance (Figure 14C), and formed a continuous layer covering the guard cells and extending into the intercellular junctions of the surrounding epidermis. Figure 14D is noteworthy as it depicts what is likely to be fungal contamination of the leaf. Putative fungal hyphae can clearly be seen emerging from the stomatal opening; this would be typical of the latter stages of the life cycle of a colonising mould. This type of feature was observed very infrequently, and only associated with spinach leaves.

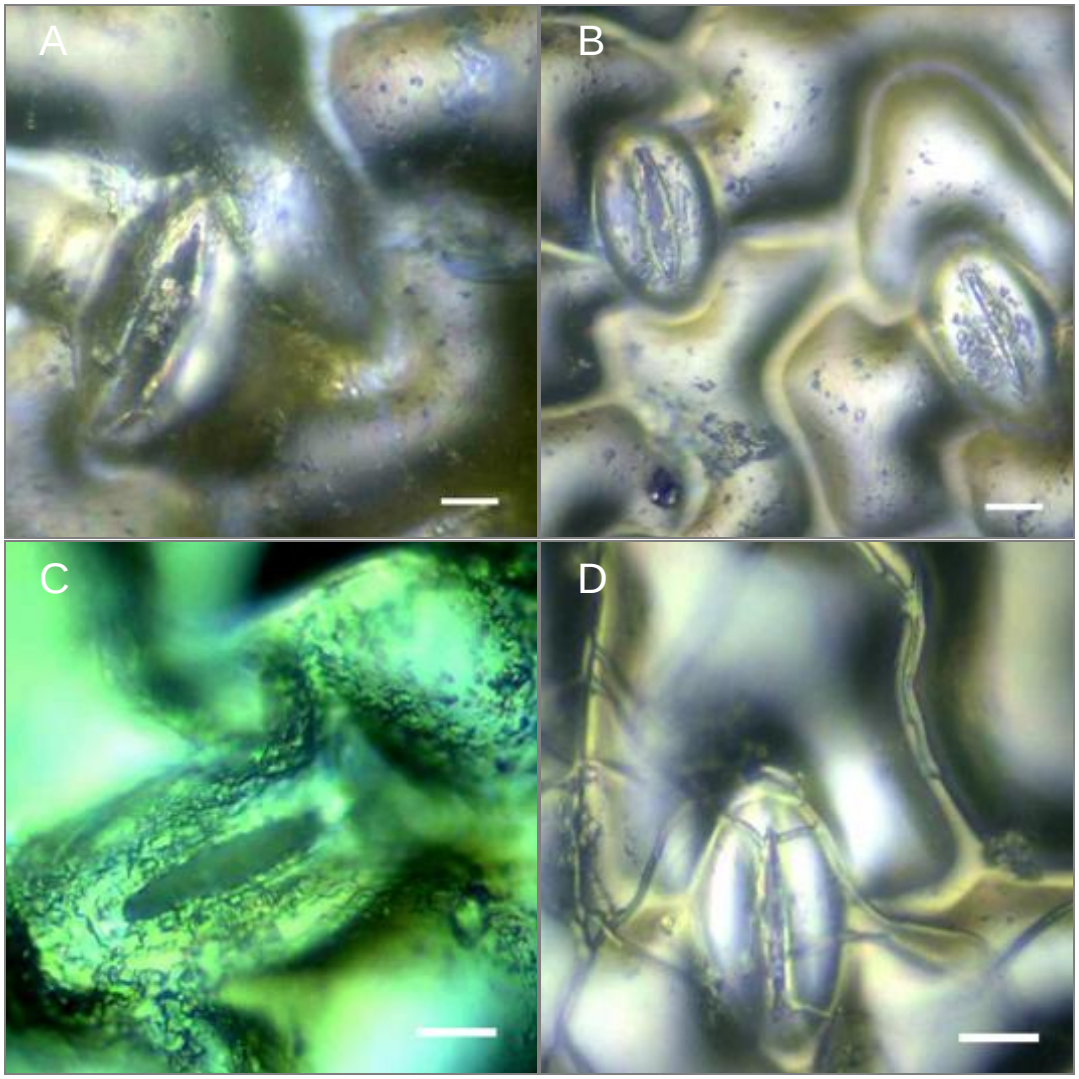


Figure 14: EDIC micrographs of spinach (A, C and D) and rocket (B) leaf surfaces. Note contaminating material inside the stomatal opening (A) and covering the guard cells (B and C). Putative fungal hyphae are shown emerging from a stoma in panel D. Bar = 10 µm

Stomatal openings are key route for the exchange of gases, gaseous chemical signals and potentially also microorganisms between the interior and exterior of a leaf (Underwood *et al.* 2007). Therefore, EDIC microscopy was also used to compare the number of stomata present on the upper and lower surfaces of spinach leaves. The data presented in Figure 15 clearly indicate that the lower surface of the leaf was observed to possess a greater number of stomata than the upper surface, a difference that was found to be statistically significant (paired *t*-test; $p < 0.01$, d.f. = 9). However, the stomatal numbers on the lower surface show a greater degree of variation than the corresponding values for the upper surface. It is

interesting to note that despite this significant difference, there are still markedly high numbers of stomata on the upper leaf surface, which is more vulnerable to environmental stresses such as water loss through transpiration.

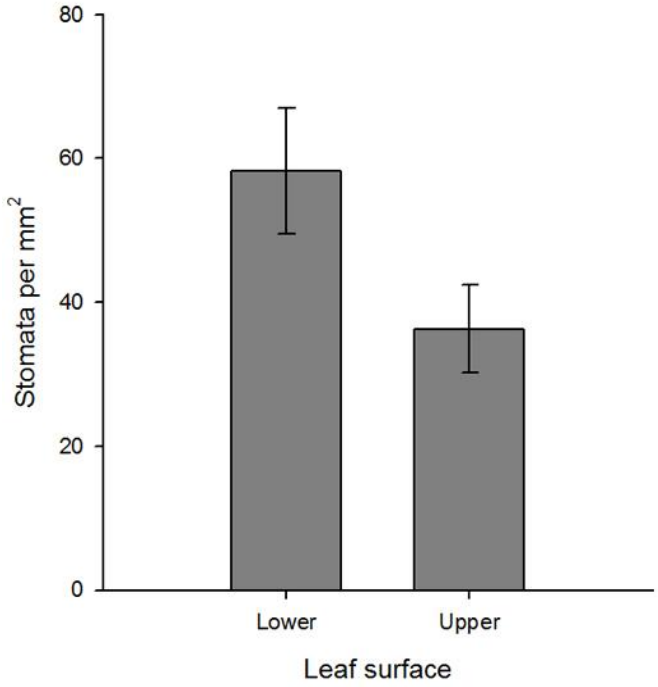


Figure 15: Average numbers of stomata per mm^2 on the upper and lower surface of spinach leaves. Error bars represent ± standard error of the mean.

3.3.2 Comparison of EDIC and SEM for viewing the phylloplane

Baby rocket leaves were chosen for comparison of EDIC and SEM microscopy techniques as they were found to be sufficiently thin for dehydration prior to SEM, but robust enough to allow extended EDIC viewing without wilting. The two techniques were evaluated relative to one another in terms of ease of sample preparation, ease of imaging and quality of results obtained.

Figure 16 shows an SEM micrograph of a rocket leaf; structures of a size appropriate to be bacillar and coccoid bacterial cells can be seen on the leaf surface. The magnification of the image, 8000×, was by no means highest magnification attainable using the technique,

however, higher magnifications tested were found to be unsatisfactory, providing no useful topographical reference points with which to aid in interpretation of the images produced. EDIC microscopy was limited to 2000× magnification, with 1000× producing the most satisfactory balance of detail and topographical context.

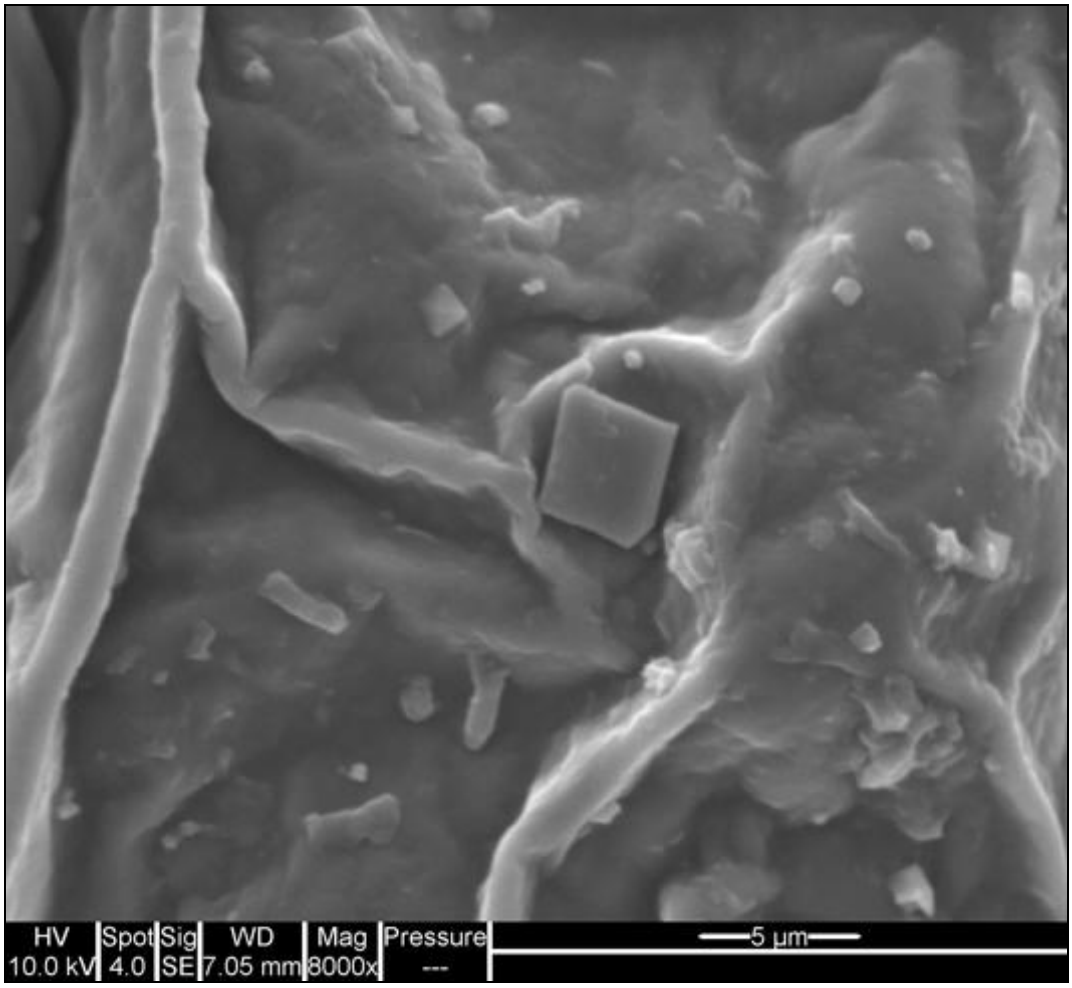


Figure 16: SEM micrograph of a rocket leaf.

3.3.3 Combining EDIC with epifluorescence to view total bacteria

3.3.3.1 Acridine Orange

Acridine Orange was evaluated for its ability to stain nucleic acids contained within phylloplane contamination and thus distinguish cellular material from inorganic debris. EDIC/EF microscopy was used to view the stained leaf pieces. Acridine Orange has two distinct emission spectra when bound to either DNA or RNA; Figure 17 shows an Acridine Orange -stained leaf surface viewed using EDIC/EF with fluorescence filter sets appropriate for both bands of emission. Bacterial cells are more readily identifiable by their DNA-bound green fluorescence (Figure 17A) than their RNA-bound red fluorescence (Figure 17B). Both images show high levels of background fluorescence. In Figure 16B this is due at least in part to red fluorescence emitted by chlorophyll contained within the leaf. Green background fluorescence (Figure 17A) is observed at greater levels than that encountered on an unstained leaf and is therefore likely to be Acridine Orange staining of DNA within the leaf tissue itself. Background fluorescence at the levels encountered following Acridine Orange staining equalled the fluorescence of some of the stained bacteria, and therefore it cannot be said with certainty that all stained bacterial cells are visible as they may have become obscured by background fluorescence.

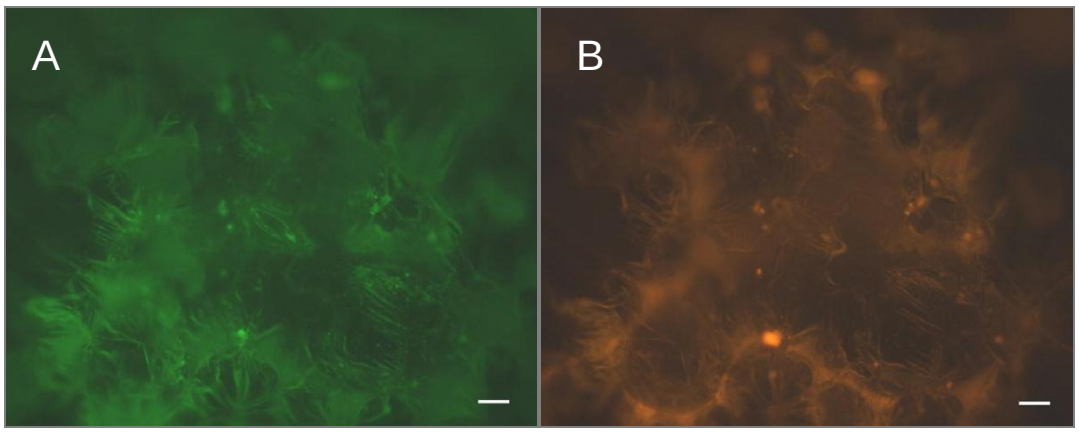


Figure 17: EDIC/EF micrographs of an Acridine Orange stained spinach leaf, illustrating the stain's characteristic dual fluorescence emission in both the green (A) and orange (B) regions of the spectrum. Bar = 10 µm

3.3.3.2 BacLight LIVE/DEAD® Bacterial Viability assay

In order to distinguish between live and dead bacterial phylloplane colonists, the BacLight LIVE/DEAD® Bacterial Viability kit was tested on fresh spinach leaves. Component A, the proprietary nucleic acid stain SYTO-9, emits green fluorescence when bound to DNA, and was viewed with an appropriate epifluorescence filter set. Figure 18A is a typical EDIC/EF micrograph of SYTO-9 staining on a spinach leaf, performed as part of the BacLight assay. No bacterial cells are distinguishable; there is a high degree of green fluorescence visible. As noted previously (Section 3.3.3.1), unstained spinach leaves emit low levels of green background fluorescence; these images therefore clearly indicate that SYTO-9 penetrates the spinach leaf interior. Fluorescence was observed to be localised around the cell wall region of leaf cells, suggesting that penetration of the stain is largely limited to the leaf apoplast.

Propidium Iodide (component B) enabled the visualisation of individual bacteria cells in the epidermal cell margins. High levels of red autofluorescence were a problem in some samples, but the degree to which this seemed to obscure the viewing of bacteria was extremely variable. More concerning was the readiness with which Propidium Iodide appear to produce localised non-specific staining, such as the large bright object in the lower left of Figure 18B. Such objects were often identified as crystalline soil particles by a combination of morphology comparison, assessment of their tendency to appear more yellow in colour than bacteria, and fluorescence under all fluorescence filter sets (indicating reflection of episcopic light).

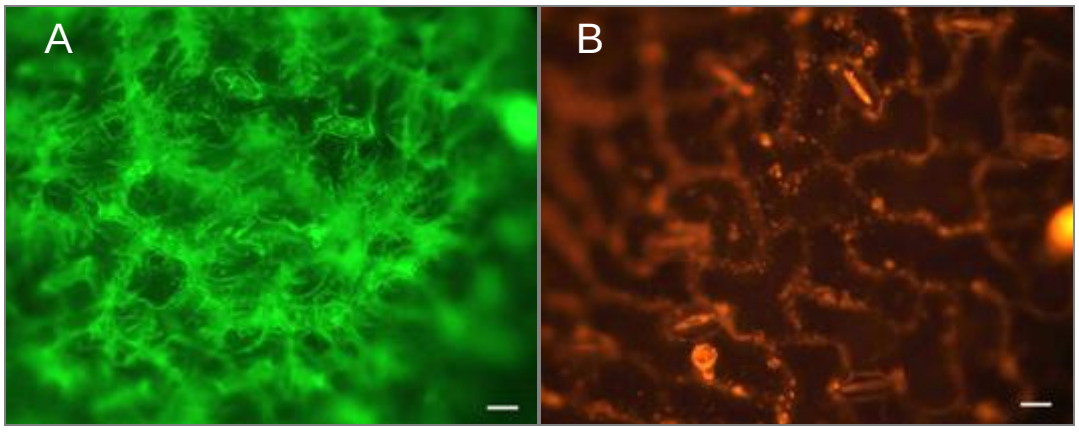


Figure 18: BacLight LIVE/DEAD Viability Assay performed on a spinach leaf, showing SYTO-9 staining (A) of total bacteria and Propidium Iodide staining (B) of dead bacterial cells only. Bar = 10 µm

3.3.3.3 DAPI

Spinach leaves stained using the cyan-fluorescent stain DAPI revealed individual bacterial cells readily distinguishable from the background material (Figure 19). Solitary cells, microcolonies and larger aggregations can all be distinguished. Crystalline soil particles on some samples (not shown) were faintly illuminated in blue, but the difference in colour relative to more cyan hue of DAPI, and the difference in fluorescence intensity made them readily distinguishable. Aside from the soil particles, the majority of debris visualised on the leaf surface was in fact DNA-containing cellular material, and the size and shape of the solitary microorganisms suggests a bacterial rather than fungal or protozoal origin. In some samples, particularly watercress, the nuclei of leaf epidermal cells were stained, a phenomenon not seen with any of the other nucleic acid stains tested; this occurred only infrequently and their size and location ensured these artefacts were distinguishable from surface microorganisms. Interestingly, rocket leaves became more autofluorescent in the blue region of the spectrum with age; this phenomenon was unique among the leaf types used in this study and prevented the use of senescing leaves in any experiments involving DAPI staining as the background fluorescence obscured stained bacteria. Regardless, the clarity of DAPI-staining of phylloplane bacteria demonstrated great potential for *in situ* quantitation.

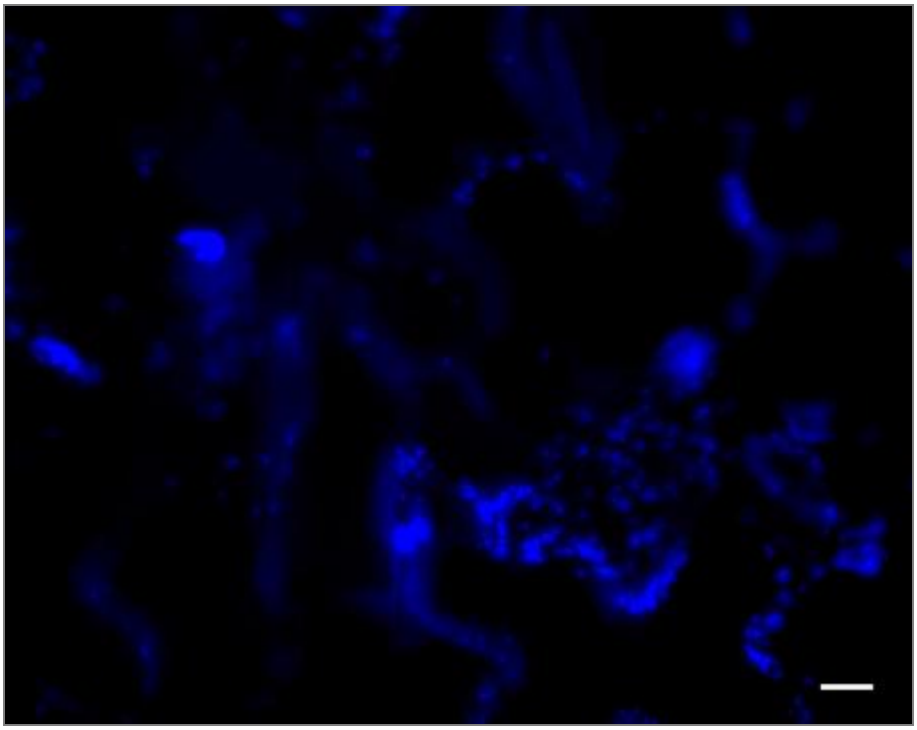


Figure 19: Epifluorescence micrograph of bacteria on a spinach leaf stained with the nucleic acid stain DAPI, which fluoresces cyan when bound to double-stranded DNA. Bar = 10 μm.

In Figure 20A, a large aggregate of cellular material is present on the surface of a spinach leaf; it is 41 μm in length at its longest point, and is situated in the centre of a leaf epidermal cell. DAPI staining (Figure 20B) confirms that the large aggregate and much of the surrounding material is cellular material containing DNA. The EDIC image appears to depict a three dimensional structure to the aggregate indicating that it is not a monolayer of cells but rather a microcolony typical of biofilm formation. Coverslips were not used during EDIC/EF microscopy; the sample viewed in Figure 20 was neither compressed nor crushed during sample preparation and can therefore be considered a true structural representation of the aggregate and not an artefact. Background fluorescence was low enough to allow epifluorescence micrographs of DAPI-stained cells to be overlaid onto the corresponding EDIC image of the leaf surface, as in Figure 20B.

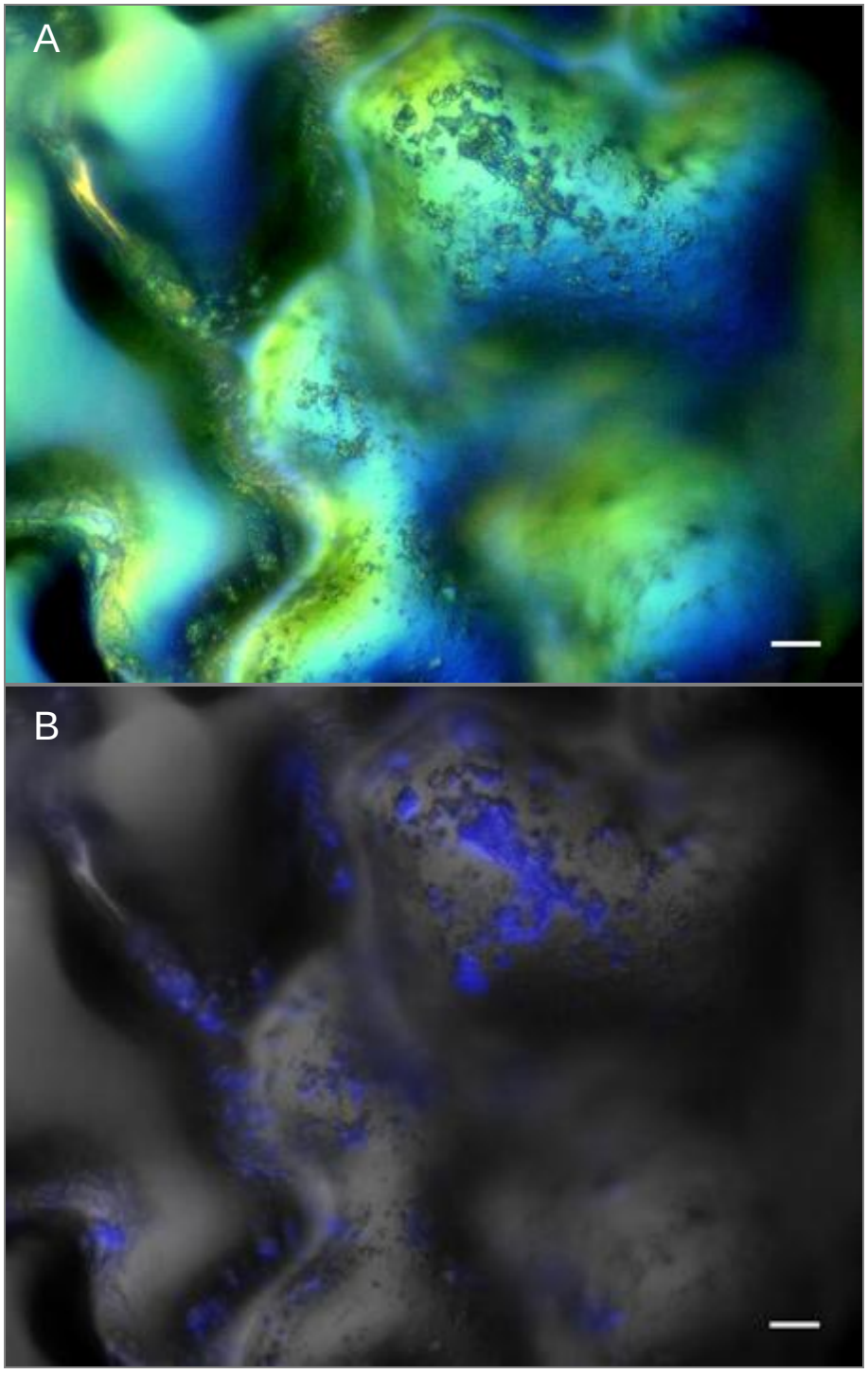


Figure 20: EDIC micrograph (A) of the lower surface of a spinach leaf, showing heavy bacterial contamination in the centre of an epidermal cell as well as in the margins. The same image converted to monochrome and overlaid with a fluorescence micrograph of the DNA stain DAPI (B) demonstrates that the majority of the debris on the leaf surface is cellular material in the form of microbial colonisation of the leaf. Bar = 10 µm.

3.3.3.4 SYBR dyes

The SYBR family of nucleic acid gel stains were evaluated as potential stains for the visualisation and quantitation of total bacteria *in situ* on the phylloplane. In addition, the dead cell stain SYTOX orange was evaluated as a potential viability determinant. Figure 21 shows representative EDIC/EF micrographs of spinach leaf pieces, each treated with one of the stains.

SYBR Green I, which stains dsDNA, produced good quality images with bright fluorescence and clear definition of individual bacterial cells (Figure 21A). Background fluorescence from non-specific binding was considered minimal. Solitary cells and microcolonies are clearly visible on the leaf surface; the quality could potentially allow cells to be enumerated from images of this quality. By contrast, SYBR Green II specific binding to ssDNA and RNA (Figure 21B), produced only faint fluorescence, which was often difficult to distinguish from the background. Non-specific staining of the edge of the stomatal opening gave the brightest fluorescence of any part of the sample. The fluorescence level and image clarity produced using SYBR Gold staining (Figure 21C) were greater than that produced by SYBR Green II, but not as intense as SYBR Green I. It is also important to note that the quality of the staining produced by application of SYBR Gold was less consistent than that produced by the other two SYBR stains tested; large regions of samples often failed to stain when other fields of view exposed clearly visible bacterial cells.

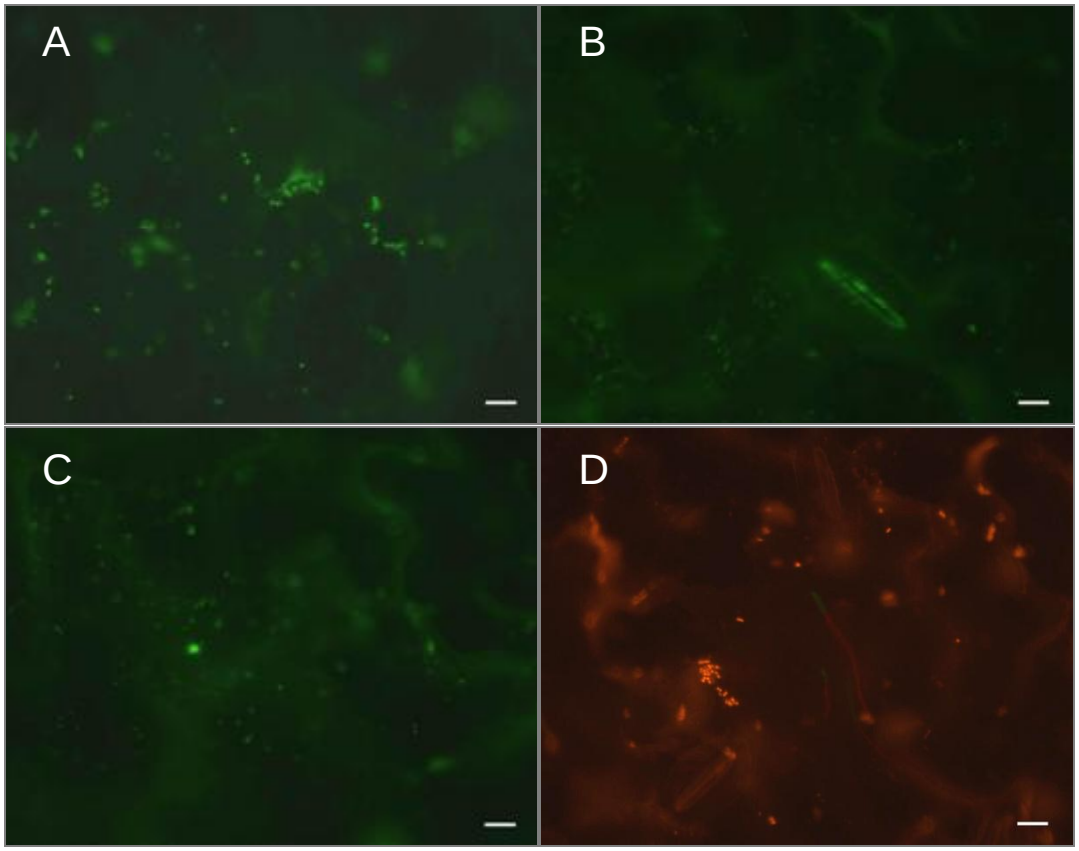


Figure 21: EDIC/EF micrographs of SYBR stains applied to fresh spinach leaves. Stains tested were SYBR Green I (A), SYBR Green II (B) and SYBR Gold (C), which all produced varying degrees of green fluorescence when interacting with phylloplane bacteria. SYTOX Orange (D) emitted orange fluorescence following penetration of bacterial cells with compromised membranes. Bar = 10 µm

The membrane impermeant nucleic acid stain SYTOX Orange is designed as a selective indicator of dead bacterial cells. Upon application to spinach leaves, it was possible to see intense orange-fluorescence illuminating a number of bacteria (Figure 21D). Fluorescence intensity was markedly higher than any of the other nucleic acid stains assessed in this study. However, as encountered during procedures involving SYBR Gold, the quality of staining was inconsistent across a single leaf sample; whereas some parts of the leaf produced staining such as that presented here, other regions appeared to lack staining effect, often emitting only faint and diffuse orange coloration. This mis-staining occurred too frequently for the lack of fluorescence to be the result of there simply being no dead cells; where mis-staining occurred it was frequently accompanied by several adjacent fields of view entirely devoid of fluorescing cells. Attempts were made to use counterstains

(SYBR Green I or DAPI); however this produced samples with unacceptably weak and diffuse orange fluorescence (data not shown).

3.3.4 Validation of DAPI for quantitative determination of phylloplane bacterial populations

DAPI staining was successful in visualising phylloplane bacteria and showed potential as a quantitative indicator of bacterial colonisation (Section 3.3.3.3); its binding specificity was therefore compared with SYBR Green I, which is regarded to have a higher binding specificity for DNA in bacteria (Weinbauer *et al.* 1998). Table 5 shows the average number of stained bacterial cells per ml of suspension following recovery from the leaf surface into suspension and subsequent staining with either DAPI or SYBR Green I. The use of DAPI produced a slightly higher average cell count than SYBR Green I, however, this was found not to be statistically significant (paired t-test $p > 0.05$). Counts obtained from DAPI-stained samples additionally presented a marginally higher degree of variation, as shown by standard deviation data.

Table 5: Comparison of the number of stained bacteria enumerated when suspensions of mixed phyllosphere bacteria recovered from spinach leaves were stained with either DAPI or SYBR Green I and visualised on filters using EDIC/EF microscopy. Values in parentheses indicate standard deviation from the mean of six filters per stain.

Stain	Stained bacteria per ml
SYBR Green I	$2.91(\pm 0.49) \times 10^6$
DAPI	$3.00(\pm 0.53) \times 10^6$

3.4 Discussion

3.4.1 Spatial patterning of bacterial colonisation on salad leaf phylloplanes

Under EDIC microscopy, the surfaces of all salad leaves examined exhibited marked levels of debris. Greatest concentrations of material were observed in the margins between epidermal cells, across the surfaces of veins and on stomatal guard cells. These observations are consistent with those made in previous studies using SEM (Carmichael *et al.* 1999; Rayner *et al.* 2004; Yadav *et al.* 2005). Unlike SEM, samples prepared for EDIC microscopy were not dehydrated prior to use; this resulted in the production of pseudo-3D images that provided a more accurate representation of the depth and morphology of any contamination present. Viewing fully hydrated leaf surfaces also shows how the water content of leaf epidermal cells produces the characteristic swollen cell centre and deep intercellular junctions, producing localised variation in exposure to environmental stresses such as drought, shear stress, and UV radiation. Examining fresh, undamaged tissue was also particularly important when making judgements relating to biofilm formation on leaves.

Bacterial aggregations were frequently observed on all leaves using EDIC microscopy. Aggregation has previously been reported on leaves (Fett and Cooke 2005; Jacques *et al.* 2005), and native aggregates are thought to aid the survival of subsequent immigrants (Monier and Lindow 2005a). Bacterial colonisation of cut leaf surfaces has been reported previously (Seo and Frank 1999; Solomon *et al.* 2002); nutrient leaching from the cut edges was thought to be the reason for preferential bacterial colonisation relative to intact leaf surfaces. On a senescent leaf, even the death of a single epidermal cell can encourage and increased density of bacterial colonisation relative to the surrounding areas. In an environment with such limited nutrient availability, any increase in nutrients may create a chemotactic gradient attracting colonists from surrounding areas.

Stomata are key leaf features as they provide a potential route for migration between the phylloplane and endosphere bacterial populations. The endosphere is important as a reservoir of pathogenic cells as well as a route for pathogenesis itself; endosphere populations can therefore be key determinants of host fitness (Kuklinsky-Sobral *et al.* 2004). The stomata observed in this study frequently possessed large quantities of

aggregated material in the stomatal openings and covering the guard cells; this often took the form of three dimensional structures or slimy deposits. As is commonly seen across the angiosperms, the leaf surfaces of spinach leaves were shown to possess significantly more stomata on the lower than the upper leaf surface.

Leaf veins also appeared to be sites of extensive colonisation; the vascular bundles carry water and nutrients and it is possible that this is a favoured site for survival due to leakage of these normally very limited resources onto the surface. The deep clefts between vascular elements are likely to offer increased protection from drought, ionising UV radiation and shear stress relative even to standard epidermal cell margins.

The size, three dimensional appearance and frequent occurrence of the slimy deposits observed on leaves examined in this study strongly indicated that such deposits could be biofilm or biofilm precursor formations. Previous studies have claimed to demonstrate the presence of biofilms *in situ* on plant aerial surfaces using SEM (Gras *et al.* 1994), CLSM (Carmichael *et al.* 1999), and epifluorescence microscopy (Morris *et al.* 1997), although these techniques do not readily visualise the exopolymer production characteristic of biofilm slime. Since the leaf surface is a dynamic and hostile environment, biofilms may play a vital role in the survival of epiphytic bacteria, offering protection from fluctuating environmental stresses (Molina *et al.* 2003; Morris and Monier 2003). Biofilms may also positively influence the survival of non-native bacterial immigrants, including human pathogens such as *E. coli* and *Salmonella*. Such alien immigrants, although adapted to very different types of host, can achieve high population numbers on leaves; the enteric pathogens *Campylobacter* (Brandl *et al.* 2004) and *Salmonella* (Brandl and Mandrell 2002) are two such examples. However, factors determining survival and population growth on the phylloplane are clearly complex; the insect pathogen *Bacillus thuringiensis* is unable to proliferate, instead rapidly commencing stress-induced endospore formation post inoculation onto leaves (Maduell *et al.* 2008).

3.4.2 Evaluation of EDIC, EDIC/EF and SEM as tools for phylloplane study

SEM imaging of the rocket leaf samples was quite rapid and straightforward to perform; however, by contrast the sample preparation was extensive, requiring 48 h drying time and 45 min sputter coating prior to actual imaging. By contrast, rocket leaves examined by EDIC microscopy were mounted on glass slides for convenience, but imaging would have

been possible without this step. EDIC images of leaves can be created within less than an hour of sample receipt, compared to 3 days for SEM. However, sample longevity becomes an issue when using EDIC microscopy as fresh, unfixed leaf tissue typically becomes unusable 3-5 hours after microscopy commences. Fixed and sputter-coated SEM samples do deteriorate in air due to oxidation of the coating, but long-term storage under a vacuum or inert gas is possible.

The absence of drying and fixation, combined with the use of non-contact objective lenses, all but eliminates the presence of artefacts on microscopy specimens. Artefacts are a common problem during SEM of biological specimens due to the need to dehydrate tissue prior to analysis. For example, the angular object in the centre of Figure 16 is not readily identifiable; the folding of the leaf tissue to surround it is almost certainly a product of the drying process and therefore the true localisation and positioning of the unknown object is not clear. In the lower left region of the image, rod-shaped structures are visible. As with EDIC micrographs, size and morphology are key indicators that these are likely to be bacterial in origin, but neither microscopy technique is sufficient to for absolute confirmation.

One of the greatest strengths of using EDIC microscopy techniques for phyllosphere study is rapidity. If samples are to be analysed without fixation or dehydration then their usable lifetime is typically less than 3 hours before wilting and tissue collapse occur. EDIC microscopy allows both single images and *z*-plane scans of the leaf surface to be obtained rapidly at high resolution; the fragility of fresh, unfixed leaf material prioritises rapidity as a major factor in successful analysis. Both EDIC microscopy and SEM present the limitation that images obtained make no distinction between bacterial species other than cell morphology; to study the colonisation of one species of interest using these techniques alone it would necessary to produce a gnotobiotic plant for study (Poonguzhali *et al.* 2008). In doing this however, a system unseen in nature is produced, and data must therefore be interpreted with extreme caution.

The higher magnification capabilities provided by SEM allow imaging of the leaf surface wax crystal structure. The leaf cuticle is formed by epicuticular wax secretion from leaf epidermal cells; this crystallises to form a three-dimensional matrix. SEM has been instrumental in characterising this matrix, which has been shown to vary with leaf surface (Gniwotta *et al.* 2005) and air humidity (Koch *et al.* 2006). The crystalline structure of the plant cuticle is almost certainly key to bacterial movement and attachment processes on a

leaf surface; SEM is one of the only techniques to produce the magnification necessary to examine leaf topography on such a small scale. Atomic Force Micrscopy (AFM) is also capable of high magnificxtion; however, it requires probe contact with the surface under study and so cannot be considered to be a non-destructive technique (Humphris *et al.* 2005). However, with such small scale comes a decline in ability to readily identify key topographical reference points, such as veins and stomata (Perkins *et al.* 2005). Although EDIC microscopy is performed at lower magnification, a detailed view of spatial patterning at an ecologically relevant scale can be rapidly achieved.

It should be noted that there is a considerable cost difference between the two types of microscopy. SEM is considerably more expensive due to both the capital purchase running costs of the electron microscope, as well has higher consumable costs due to the necessity of specialised mounting and coating. By contrast, EDIC microscopes are less costly to purchase, relatively inexpensive to run and can be used with very few consumables needed.

EDIC/EF microscopy has the advantage of being particularly rapid, reducing loss of quality through fading of the fluorophores used to stain samples. By contrast, CLSM, although high resolution, requires fixation and is markedly slower to scan in the *z*-plane (Keevil 2003). Laser excitation is unable to provide an image of the surface itself without negative staining; consequently, detailed data on spatial positioning of bacteria is more difficult to obtain. Red autofluorescence emitted by chlorophyll can be utilised to visualise leaf cells using CLSM; this technique has previously been used to demonstrate that GFP-expressing *Salmonella thompson* penetrate the interior of leaves (Brandl and Mandrell 2002). Chlorophyll autofluorescence alone however, does not always provide accurate definition of individual leaf cell shape as the intracellular distribution of chloroplasts determines the patterning of the fluorescence signal. By contrast, bright-field EDIC microscopy can be used to directly visualise leaf epidermal cells; epifluorescence micrographs can then be overlaid directly onto EDIC micrographs to create high resolution, spatial visualisation of bacterial distribution.

3.4.3 Evaluation of nucleic acid stains for quantitative *in situ* localisation of phylloplane bacteria

Plants maximise their photosynthetic capacity through the production of various photopigments that together harvest light of many different wavelengths. Chlorophylls fluoresce in the red region of the spectrum; chlorophyll A has maximum fluorescence emission at around 680 nm (Lang *et al.* 1991). Consequently, when leaves are stained with red fluorophores, high background fluorescence interferes extensively with fluorescence microscopy.

Acridine orange has previously been used to stain bacterial biofilms on intact leaf surfaces, on cuticle peels and on filters (Morris *et al.* 1997). Cuticle peels and filters were reported to give higher quality images than intact leaf pieces as they eliminated chlorophyll autofluorescence from the leaf tissue. The preparation of cuticle peels causes some loss of spatial data, but can remove most, if not all, autofluorescence associated with leaf tissue without dislodging or breaking up any biofilm which may be present. Here, bacteria on leaf samples stained with Acridine Orange produced only faint green fluorescence, whilst orange emission was largely obscured by high background chlorophyll autofluorescence levels. Samples stained with DAPI had a greater degree of clarity and showed many more bacteria per field of view, suggesting that Acridine Orange staining may be under-reporting the number of bacteria present.

When used as part of the BacLight assay, Propidium Iodide caused inorganic debris, such as soil particles, to fluoresce more intensely than stained bacteria; inorganic crystals appeared extremely bright, in some cases forming haloes of fluorescence which obscured the view of the immediate vicinity. As soil debris and bacterial contamination are often found at similar sites on the leaf surface, such as the veins, this makes it extremely difficult to reliably distinguish bacterial cells from the surrounding matter. The other component of the BacLight assay, SYTO-9, was readily able to penetrate the leaf cuticle, allowing it to associate strongly with plant cell walls thus producing intense green background fluorescence which almost completely obscured any stained bacterial epiphytes.

The BacLight bacterial viability assay was designed as a rapid way to directly enumerate live and dead bacterial cells using microscopy. Its use has been validated in conjunction with membrane filtration techniques for bacteria in water (Boulos *et al.* 1999) and food samples (Duffy and Sheridan 1998; Corich *et al.* 2004). These studies used epifluorescence microscopy for direct enumeration of bacterial cells. In phyllosphere microbiology, the

BacLight assay has been successfully used in conjunction with CLSM to visualise bacterial epiphytes (Boureau *et al.* 2002; Fett and Cooke 2005); successful epifluorescence techniques have not been reported. It should also be noted that these studies did not attempt quantitative analyses of leaf epiphytes using BacLight; the assay was used only for making qualitative observations. Whilst BacLight has been demonstrated as a quantitative determinant of bacterial viability in other systems (Boulos *et al.* 1999; Auty *et al.* 2001), the study presented here has shown that for light microscopy the tendency for SYTO-9 to penetrate and stain leaf tissue ensures it is too unreliable to allow quantitative bacterial viability assessment on intact leaves.

SYTOX Orange demonstrated more potential as a stain for use on leaf surfaces than the BacLight system; it was possible to produce images with a high degree of clarity of stained cells. However, the quality of staining was very inconsistent across the leaf surface and during the course of this study no suitable counterstain was found. SYTOX Orange has previously been combined with SYTO-13, a close analogue of SYTO-9 (Biggerstaff *et al.* 2006); however, given the high background staining produced by SYTO-9, this was not considered to be a suitable assay for use on leaves. Preliminary trials of combining DAPI or SYBR Green I with SYTOX Orange were unsuccessful as in both cases only the SYTOX Orange was visible, obscuring the counterstain. Therefore it can be concluded that whilst SYTOX Orange is potentially a useful indicator of bacterial viability on leaves, extensive optimisation of the staining conditions are needed as well as further tests to identify a suitable counterstain.

When spinach leaves were stained using DAPI, individual bacterial cells were readily distinguished from one another and from the background. Blue background fluorescence was present at a very low level, whereas DAPI-stained bacteria fluoresced bright cyan. The low level of background fluorescence meant that epifluorescence micrographs exhibited greater clarity than those obtained using either Acridine Orange or BacLight. Although the technique has been applied previously (Schreiber *et al.* 2005), actual images of DAPI-stained bacteria *in situ* on leaves had not been published until this study (Warner *et al.* 2008); the results presented here indicate that its use enables high resolution detection of both solitary and aggregated bacterial cells. DAPI staining combined with EDIC/EF microscopy demonstrates the potential for quantitative, *in situ* detection of bacterial epiphytes on leaf surfaces, providing insight into the spatial patterning of phyllosphere colonisation.

The use of DAPI staining on environmental samples has previously been criticised because of non-specific binding, particularly to silicates (Kepner and Pratt 1994), for this reason, quantitation of phylloplane bacteria on filters was validated against the newer, more DNA specific stain SYBR Green I. Contrary to previous work examining soil samples (Weinbauer *et al.* 1998), DAPI did not produce a significant quantity of non-specific binding; enumeration of phylloplane bacteria with the two stains was not significantly different. Since DAPI staining produces a consistently higher standard of imaging when used to view phylloplane bacteria, DAPI is the most suitable nucleic acid stain tested in this study for *in situ* enumeration of phylloplane bacterial populations.

3.4.4 Conclusions

Leaf surfaces provide a greater challenge for microscopy than abiotic surfaces such as stainless steel; they are both fragile and dynamic. One way to surmount these obstacles is to use fixation or dehydration to stabilise the surface and prolong the life of the sample. Unfortunately in doing so there is a high risk of generating artefacts, making it more difficult to produce a truly accurate representation of bacterial life on phylloplanes. EDIC overcomes this by being rapid and non-destructive; fixation and coverslips are not required, and images can be produced within minutes. This allows analysis of fresh leaf samples with minimal sample processing and no introduction of artefacts. EDIC/EF techniques are equally rapid; following screening of a number of differing fluorescent nucleic acid dyes, it was found that DAPI stained phylloplane bacteria with minimal staining of leaf tissue DNA and a consistently high quality of cell definition. Individual cells could be distinguished sufficiently well to potentially allow *in situ* quantitation; the suitability of DAPI for this was successfully validated for specificity and used in subsequent areas of experimentation (see Chapter 4). EDIC and EDIC/EF techniques also have the advantage of being much lower in cost than SEM and CLSM.

Salad leaf phylloplanes are clearly able to support large numbers of epiphytic bacteria; these exist as solitary cells, microcolonies and larger, three dimensional aggregates. The cells in larger aggregates are often surrounded by material that is slimy in appearance, suggesting extracellular matrix production, a key characteristic of biofilm formation. Leaf veins and epidermal cell margins appeared to be ‘hot spots’ of bacterial colonisation, often having a visually higher degree of surface contamination than the surrounding areas. The concentration of bacterial contamination in and around stomatal openings suggests that the

endophytic bacterial community is likely to be actively important in exchange of bacteria between the interior and exterior of the leaf. These data demonstrate that spatial patterning of bacterial colonisation on salad leaf phylloplanes in very closely linked to the topography created by the arrangement of epidermal cells forming the leaf surface.

CHAPTER 4

ENUMERATION OF TOTAL PHYLLOPLANE BACTERIAL POPULATIONS

4.1 Introduction

Leaf phylloplanes are capable of sustaining sizeable bacterial populations despite the apparent hostilities presented by environmental stress, limited water and low nutrient availability. However, complex three-dimensional bacterial aggregates have been reported on leaves (Monier and Lindow 2004; Warner *et al.* 2008) suggesting that phylloplanes can in fact sustain high numbers of epiphytes. To date, the quantitation of total phylloplane populations has, in the majority of studies, been achieved by recovery into suspension, culture on solid media and subsequent plate counts. The accuracy of plate counts on agar plates is limited primarily by the efficiency of detachment of bacteria from the sample matrix; suitability of the culture medium for growth of stressed and fastidious organisms can also reduce the number of bacteria enumerated (McDougald *et al.* 1998).

In Chapter 3, novel imaging techniques were used to view epiphytic bacteria *in situ* on salad leaves. *In situ* detection removes the inherent inaccuracies caused by both inefficiency of recovery and limited culturability, yet inherently samples a smaller volume of leaf material than culture techniques. The first aim of the work presented here was to stain phylloplane bacteria with the nucleic acid stain DAPI, thus creating a quantitative assay for *in situ* enumeration of total bacteria on salad leaves. The population sizes determined by DAPI direct count were compared with a traditional method of recovery and culture on agar media. The second aim was to apply both the culture-dependent and culture-independent techniques as described above to a selection of commonly eaten salad leaf varieties, thus providing ecological insight into the variation in ability of different leaf types to sustain bacterial life. With the exception of watercress, which is grown in specially designed water-filled beds, the leaf types tested were all field-grown under the same conditions; significant differences in bacterial population are therefore more likely to be due to the physiochemical environment presented by the particular leaf species rather than the wider environment.

All recovery of bacteria from leaves for culture based analysis that took place during this investigation was performed using the Pulsifier. Consequently, it was important to be able to understand the efficiency of the method, both in terms of the number of bacteria removed and the processing of aggregates and biofilm. Therefore the final two aims of this work were to quantify the efficiency of bacterial removal from salad leaf phylloplanes and to examine the effect of pulsification on aggregates of phylloplane bacteria. This

information provided an insight into the suitability of the Pulsifier for processing leaf samples during microbiological analyses.

4.2 Materials and methods

Details of the materials and methods used in this study are described in Chapter 2. *In situ* staining of salad leaf surfaces with DAPI was compared with recovery and R2A plate count methods of quantifying the total phylloplane bacterial population. The Pulsifier was also evaluated for both its efficiency of bacterial recovery from the spinach phylloplane and its ability to break up mixed-species biofilms in suspension.

4.3 Results

4.3.1 Enumeration of total bacterial phylloplane populations

The bacterial populations of baby salad leaves were quantified by both *in situ* enumeration using DAPI staining and R2A plate count methods. Figure 22 illustrates the numbers of bacteria per mm^2 of watercress, rocket and spinach leaf surfaces as determined using *in situ* detection and plate count methods.. Watercress leaves had markedly fewer bacteria than both rocket and watercress as determined by direct counting and plate count; there was less of a difference between rocket and spinach counts. A two way ANOVA showed significant differences in the data between both the leaf types ($p<0.001$; d.f. = 2,66) and the counting methods ($p<0.001$; d.f. = 1,66). All three leaf types recorded significantly different bacterial populations enumerated by the two different counting methods (paired *t*-tests; all at $p < 0.01$). It is also notable that spinach leaves had the most variation in their bacterial populations as determined by plate count, followed by rocket and then spinach. Direct counting using DAPI staining gave markedly smaller variation between samples, indicated by the size of the error bars in Figure 22; using this method the greatest variation was in rocket population numbers, then spinach, with watercress leaves again showing the least population variation.

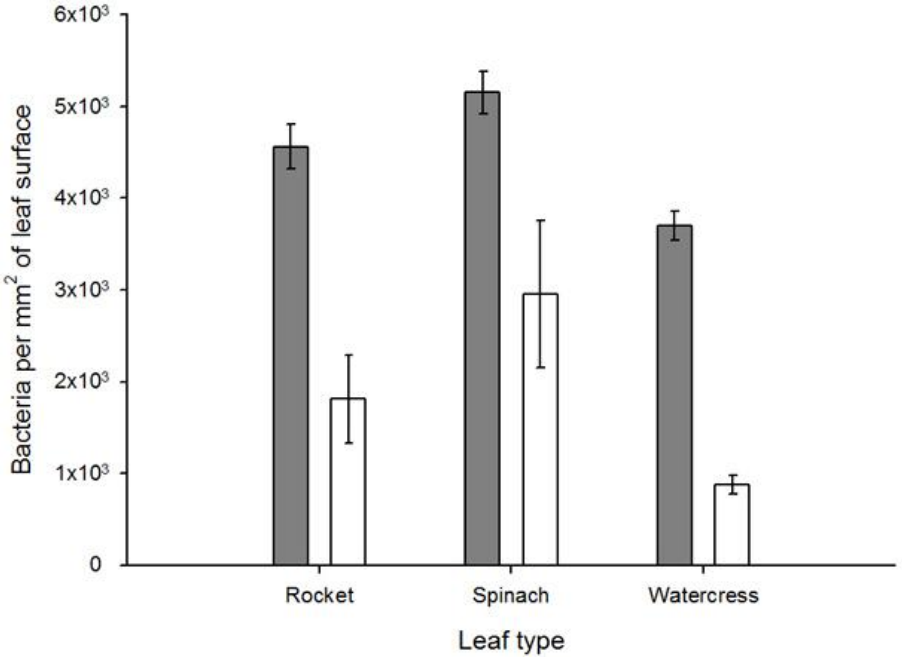


Figure 22: Total bacteria per mm^2 of leaf surface for individual baby spinach rocket and watercress leaves, obtained using either direct microscopic counting of DAPI stained bacteria on leaves (shaded bars) or recovery and culture of bacteria on R2A medium (blank bars. Error bars represent ±SEM of 18 leaves per method.

4.3.2 Comparison of red and green cos leaves as bacterial hosts

The bacterial populations of red and green cos leaves grown under the same conditions were compared as in Section 4.3.1. Figure 23 compares the leaf surfaces of the two cos varieties using EDIC microscopy. Whilst the colours present in EDIC micrographs cannot be said to be true colour, the differences in colour of the micrographs give a clear indication of the difference in leaf colouration. Aside from the colour difference, the leaf morphology itself is very similar; the sizes and arrangements of epidermal cells are very much alike in the two leaf surfaces. Bacterial colonisation of the leaves analysed features solitary cells and three-dimensional aggregates; the larger aggregates shown on both leaves in Figure 23 are very close in size to one another; there are two prominent aggregates in Figure 23A and one in Figure 23B which are all 8-9 µm in diameter and approximately circular in shape.

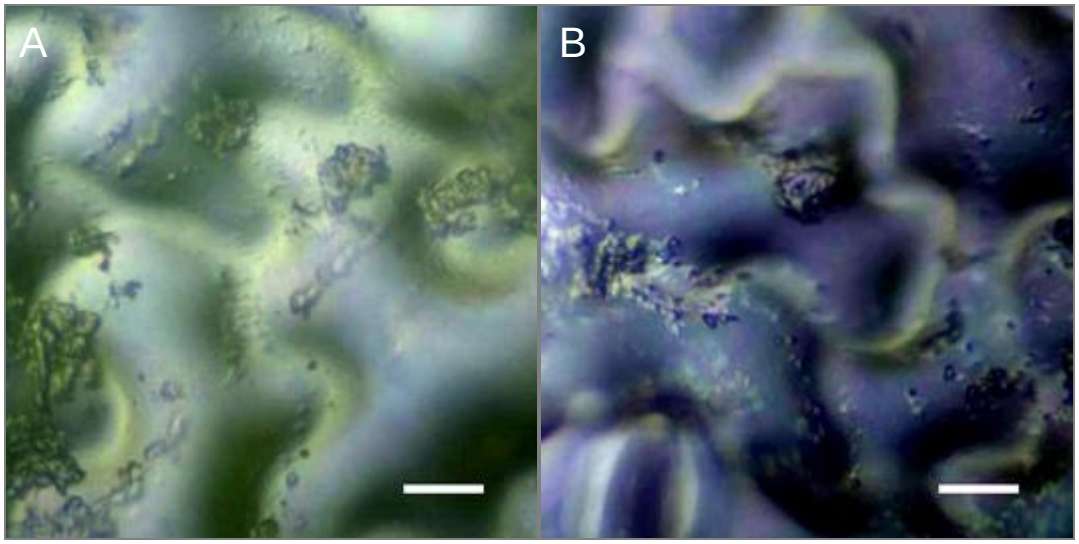


Figure 23: EDIC micrographs of the upper surface of green cos (A) and red cos (B) leaves showing surface contamination by bacterial epiphytes. Bar = 10 µm.

Figure 24 shows the DAPI and R2A counts for each leaf type, expressed as bacteria per mm^2 of leaf surface. When compared with the data in Figure 24, bacterial numbers on both cos varieties are three times higher than any of the other leaf species tested when enumerated by direct count. Comparing plate counts, however, suggests that the bacterial numbers were twice as high on cos varieties compared to other leaf types. Within the results for cos leaves, plate counts gave higher variation in the estimated bacterial population than direct DAPI counts, as observed with spinach, rocket and watercress. When the direct counts for the two cos varieties were compared with one another, there was no significant difference between the number of bacteria recorded on red cos and the number recorded on green cos (*t*-test, $p > 0.05$, d.f. = 34). This was also true of plate count data (*t*-test, $p > 0.05$, d.f. = 28).

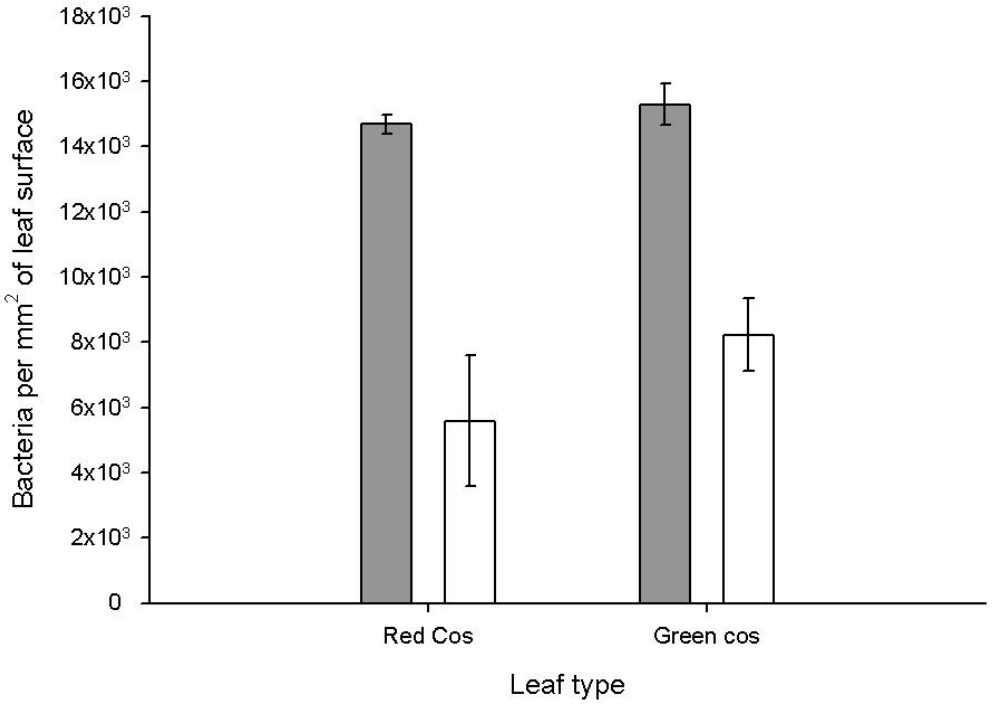


Figure 24: Total bacteria per mm^2 of leaf surface for individual baby spinach red and green cos leaves, obtained using either direct microscopic counting of DAPI stained bacteria on leaves (shaded bars) or recovery and culture of bacteria on R2A medium (blank bars). Error bars represent ±SEM of 18 leaves per method.

4.3.3 Efficiency of the Pulsifier for recovery of total phylloplane bacteria

Individual spinach leaves were subjected to pulsification for differing lengths of time in order to qualitatively examine the relationship between length of pulsification time and diversity of bacteria recovered. Figure 25 compares growth on R2A medium of bacteria recovered from spinach sample pulsified for 15, 30, 45 or 60 s. Various colony morphologies are apparent on all four plates; there is also a markedly high proportion of pigmented colonies. A high degree of similarity was observed in terms of the number and appearance of the colonies which grew following all four pulsification treatments. Time points above 60 s were also tested (data not shown); however, above 60 s leaf tissue suffered considerable damage, resulting in bacterial suspensions contaminated with leaf material; this appeared to have a negative effect on colony growth of all species present.

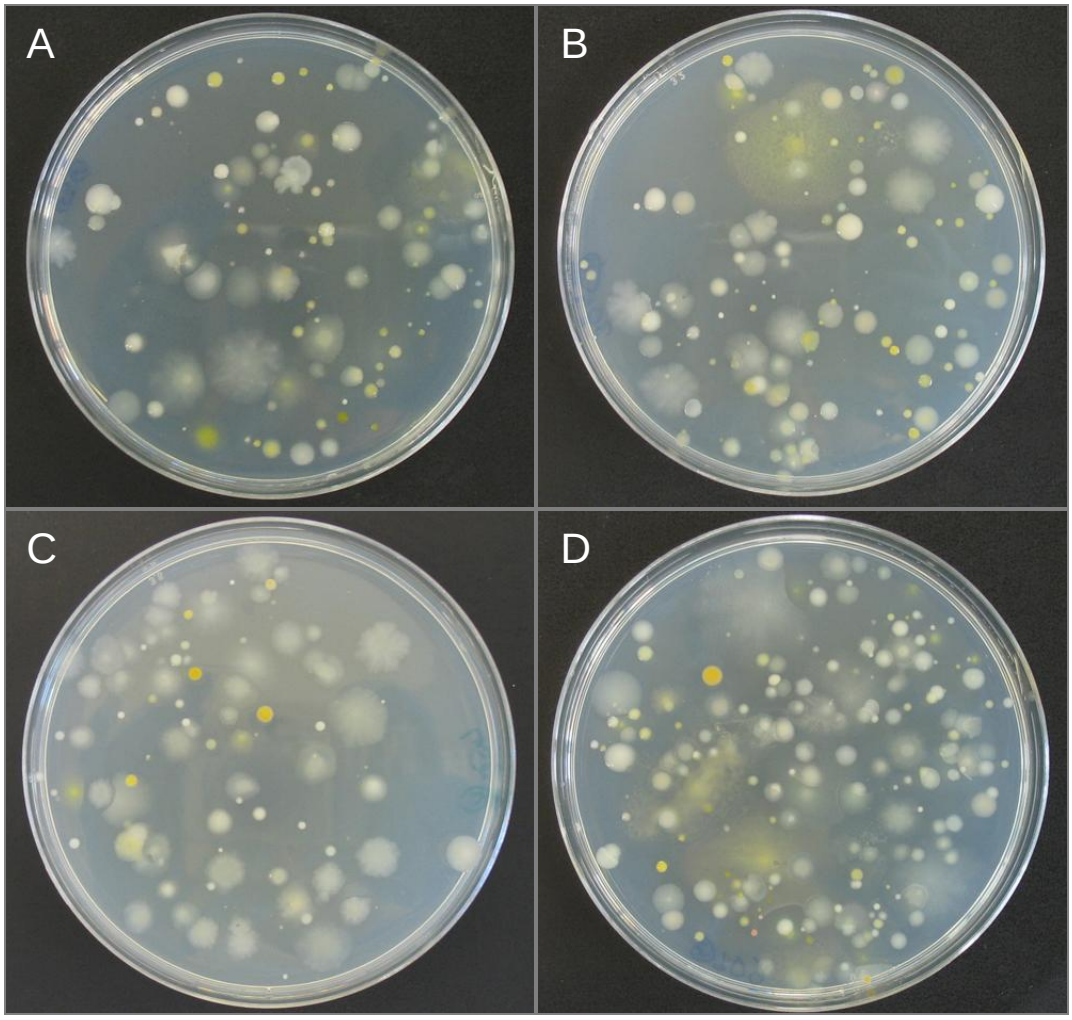


Figure 25: Growth on R2A medium of bacteria recovered from spinach leaves using pulsification treatment of 14 s (A), 30 s (B), 45 s (C) or 60 s (D).

Total phylloplane bacterial populations of unpulsified baby spinach leaves were compared with those remaining after pulsification or removal by manual shaking of the leaves in a diluent. Figure 26 presents EDIC micrographs of the surfaces of untreated and pulsified spinach leaves. There is little discernable difference between pulsified and unpulsified salad leaves; both micrographs reveal a mixture of solitary bacteria and aggregated material. The similarity between the samples is particularly noticeable when considering the material on the stomatal guard cells, which shows a high degree in morphology between the pulsified and unpulsified leaves. Pulsified leaves did, however, frequently appear to harbour fewer solitary bacterial cells than untreated leaves, although this was not quantified from EDIC micrographs alone; DAPI staining was instead used to differentiate between genuine bacterial cells and inorganic particles.

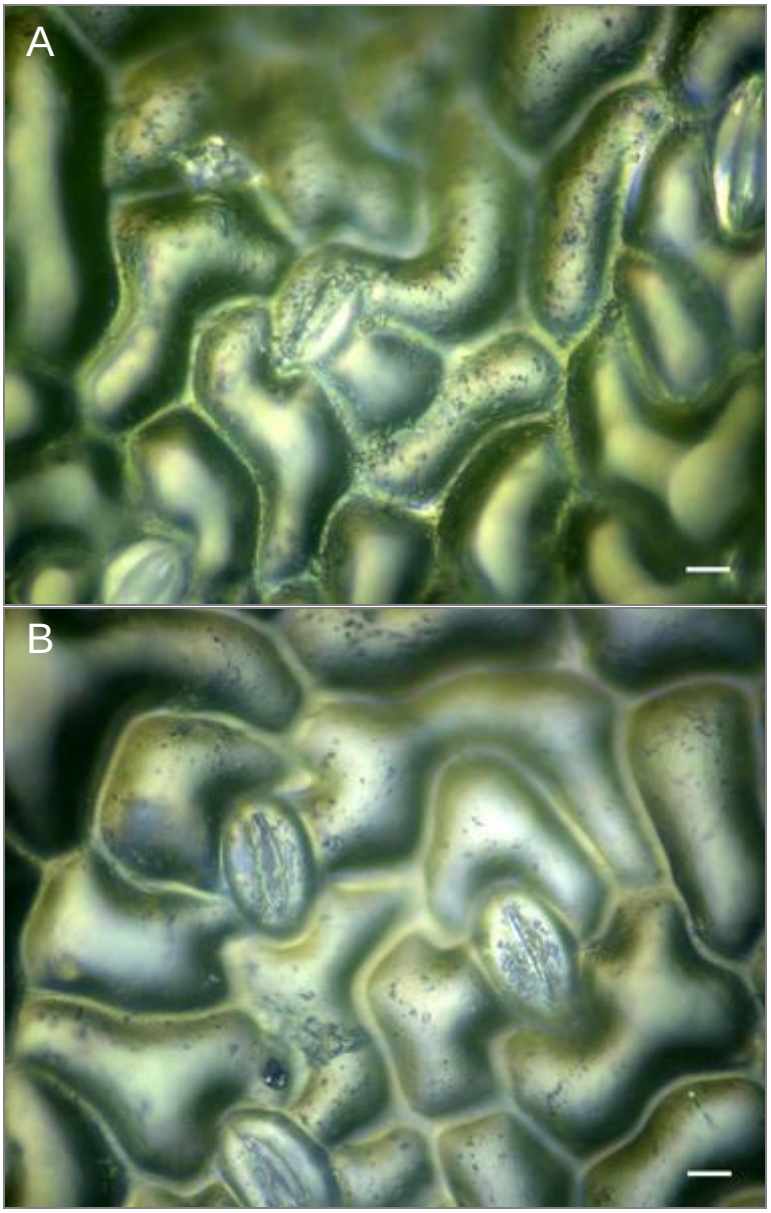


Figure 26: EDIC micrographs of the upper surface of an untreated spinach leaf (A) and one pulsified for 30 s (B). Bar = 10 µm.

Figure 27 shows the numbers of bacteria recorded *in situ* on untreated, manually shaken and pulsified spinach leaves. Single leaves did not lose a significant number of bacteria from their surfaces relative to control leaves during manual shaking (Holm-Sidak one-way ANOVA, $p > 0.05$, d.f. = 2, 24). The same was true of 25 g leaf samples. However, when leaves were pulsified, significantly more bacteria were removed relative to control and untreated samples in single leaves and 25 g samples (Holm-Sidak one-way ANOVA, $p < 0.001$, d.f. = 2, 24) and 25 g samples. It is important to note that despite the significance of

the pulsification process on bacterial removal, bacterial populations observed on pulsified leaf surfaces corresponded to almost half of the population observed on control leaves. Pulsification was less efficient when applied to 25 g samples than to individual leaves, removing an average of 46.2 % of total bacteria on leaf surfaces compared with 52.6 % removed from leaves pulsified individually.

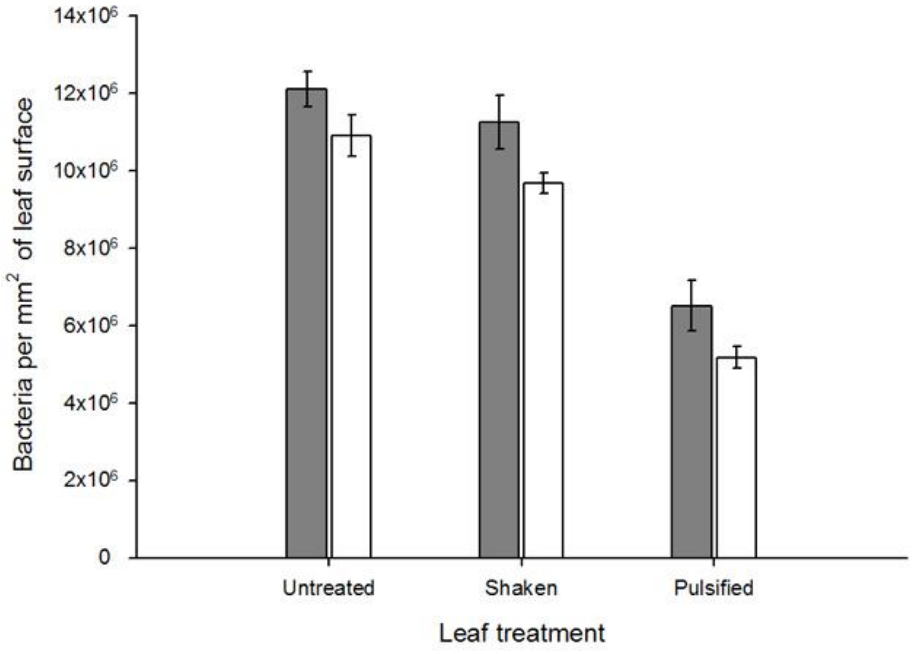


Figure 27: Bacterial numbers detected in situ *on spinach leaves from untreated, manually shaken and pulsified 25 g leaf samples in 225 ml PBS (shaded bars) or individual leaves in 50 ml PBS (blank bars). Error bars represent standard error of the mean of three samples per treatment.*

Mixed phylloplane bacteria were recovered from spinach leaves and cultured into biofilms in order to examine the ability of the Pulsifier to break up bacterial aggregations. Figure 28 represents a cultured biofilm grown from mixed spinach phylloplane isolates; similar biofilms were manually broken up and suspended in PBS in order to examine the effect of pulsification on mixed-species biofilm. In Figure 29A, it is possible to see biofilm fragments from the control suspension, before the application of any pulsification

treatment. There is a marked difference between this image and Figure 29B, which shows the biofilm suspension after pulsification in aliquots of 225 ml (Figure29D). Fewer biofilm aggregates can be seen, and they are generally smaller in size than the control sample. By contrast, when 50 ml samples were pulsified (Figure 29C), EDIC /EF imaging of the control and treated samples are much closer in appearance; the size and concentration of biofilm is visually similar.

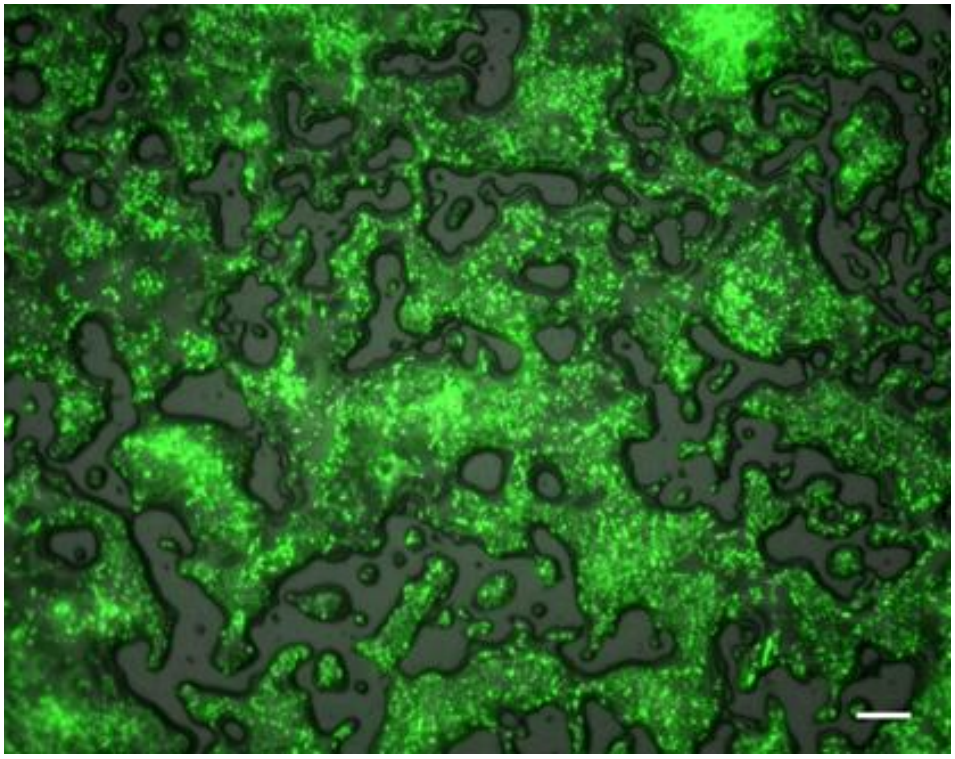


Figure 28: EDIC/EF micrograph of SYTO-9 stained bacterial biofilm grown for 7 days, overlaid onto the corresponding EDIC bright-field image. Bar = 10 µm.

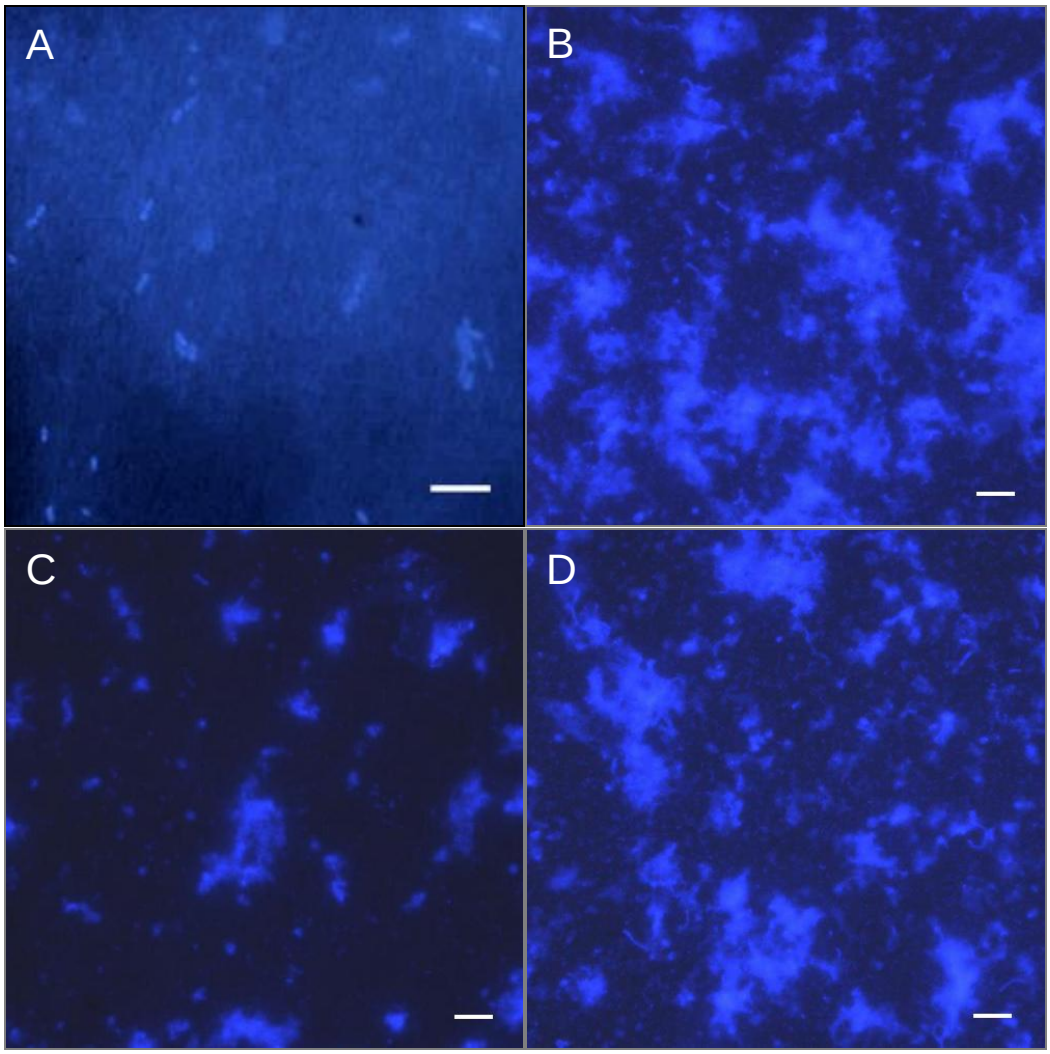


Figure 29: EDIC/EF micrographs of DAPI-stained mixed phylloplane bacteria. A represents typical recovery of bacteria from leaf materialusing a Pulsifier; B, C and D are fragments of cultured biofilm suspended in PBS and unpulsified (B) or pulsified in aliquots of 50 ml (C) or 225 ml (D). All pulsification steps were 30s in duration. Bar = 10 µm.

The differences between the samples were quantified by software-assisted measurement (ImagePro Plus v5.0.2.9; MediaCybernetics) of biofilm fragments recovered during the experiment. Table 6 presents the average length of biofilm fragments at their longest point for each treatment. Pulsification using either sample size reduced the average biofilm size by at least 48 %, a significant percentage (Holm-Sidak one-way ANOVA, $p < 0.001$, d.f. = 2,67); however this could be improved by a further 31 % of the control by selecting the more efficient method, which was the use of 225 ml aliquots; the improvement in relation

to the 50 ml samples was found to be statistically significant (*t*-test, $p < 0.001$, d.f. = 58). The larger of the two aliquot sizes also produced a slightly more consistent effect on biofilm fragment size as indicated by the lower standard error values.

Table 6: Average length of biofilm fragments in saline suspension, before and after pulsification treatment. Values in parentheses represent standard error of the mean of three samples per treatment.

Treatment	Average biofilm fragment length (µm)
Unpulsified	90.0 (±1.55)
Pulsified	
30s, 50 ml PBS	47.2 (±1.18)
30 s, 225 ml PBS	28.6 (±0.90)

4.4 Discussion

4.4.1 Comparison of bacterial population sizes observed on commonly eaten salad leaves

In this study, considerable variation has been observed between the epiphytic bacterial population sizes on different varieties of salad leaf. For example watercress leaves were host to significantly fewer bacterial epiphytes than any of the other leaves examined; the growing process for watercress, which uses raised water-filled beds, is unique to this leaf type and so a difference in bacterial colonisation is not unexpected. Watercress grows in semi-aquatic conditions, in which the rot system grows below the water whilst the stem and leaves exist above the water level (Howard and Lyon 1952); the growth conditions of watercress could therefore reasonably be expected to produce a higher bacterial population, as the risk of death by desiccation is likely to be reduced due to both a higher relative humidity level and splashing from the water. In this study, however, the reverse appeared

to be the case; it may therefore be the case that soil is the predominant source of bacterial immigrants to salad leaves during their growth, and in its absence either fewer immigrants are available or water-borne bacteria are less efficient colonists of the phylloplane.

When comparing the four types of soil-grown salad leaf examined during this study, further significant differences in epiphytic population size were observed. Red and green cos leaves had significantly higher bacterial populations than all other leaf varieties examined, around three times the number of bacteria recorded on spinach and four times that on rocket. Morphologically there are differences between cos, spinach and rocket, both at the leaf and whole plant level, which could account for this variation. Cos leaves are physically larger than spinach and rocket, and have a markedly more uneven surface; to the naked eye, a cos leaf has a much more crinkled appearance that a rocket or spinach leaf. Complex surface morphology is likely to have a strong effect on the movement of water across the leaf, and may provide greater heterogeneity in UV exposure and temperature fluctuation through shading.

The coloration in red leaves is in general due to localised vacuolar accumulation of anthocyanins; pigmented phenolic compounds which have previously been linked with increased antioxidant activity in red lettuce varieties (Gould *et al.* 2002; Llorach *et al.* 2008) and antimicrobial activity in *vitro* (Kahkonen *et al.* 1999; Lee *et al.* 2003). However the results of this study demonstrate that accumulation of anthocyanins in red cos had no effect upon conditions in the phylloplane, as neither the patterning of colonisation nor the total bacterial population were significantly different between red and green cos leaves. This homogeneity of bacterial populations between red and green cos leaves suggests that although anthocyanins have been shown to have antibiotic properties, they are not part of the constitutive defences of the plant, although they may still be involved in inducible responses to pathogen attack (Creelman and Mullet 1997). *In vivo* studies of the role of anthocyanins in red leaves suggest that the antibiotic properties of the compounds may not be utilised at all; scavenging of reactive oxygen species in response to UV radiation has instead been proposed as their primary role (Steyn *et al.* 2002; Park *et al.* 2007). It is a testament to the complexity of plant tissues that such a visually abundant chemical is compartmentalised within cells in such a way that its antimicrobial properties have no discernible effect on phylloplane epiphytes.

4.4.2 Comparison of methods for enumeration of phylloplane populations

Direct i*n situ* enumeration of phylloplane bacterial populations has both key advantages and limitations when considered in relation to using traditional recovery and culture techniques as a means of quantitation. As demonstrated in Section 4.3.3, techniques involving recovery of bacteria prior to culture are severely limited by the efficiency of detachment from the sample matrix. Direct counting using DAPI staining circumvented this issue; the difference in efficiency is reflected in the higher populations indicated by direct counting as opposed to plate counts. Nucleic acid staining also avoids the potential pitfalls associated with attempting to culture nonculturable species of sublethally stressed cells; these bacteria are capable of being stained on the leaf surface despite being unable to produce colonies on agar media. Direct counting allows for more rapid screening of leaf samples; counts can be obtained within one day instead of requiring lengthy incubations necessary for optimum R2A plate counts. Spatial patterning of bacterial colonisation is also apparent when using *in situ* detection, yet is lost during recovery of bacteria for plate counting; patterns of epiphytic colonisation may provide key information as to factors affecting bacterial growth and survival, which are important when analysing the colonisation of a specific immigrant species. This will be explored in more detail in Chapter 5.

Despite the key advantages already mentioned, *in situ* enumeration of phylloplane bacteria is not yet a definitive replacement for traditional culture techniques. A key strength of the use of culture media is the determination of viable bacteria, as only live cells are able to form colonies; DAPI staining alone does not provide a means of viability assessment. Evaluation of viability stains for *in situ* use on salad leaves in this study did not yield a successful assay (see Section 3.3.3). Plate counts, though slower than staining techniques, can sample a much higher volume of leaf material as plate counts can typically be obtained from 25 g samples of material as opposed to randomly selected fields of view on a sample measuring just a few millimetres. When used together to compare bacterial populations in leaves grown under differing environmental conditions of interest, both techniques can therefore complement one another in providing an insight into the number and spatial arrangement of phylloplane epiphytes.

4.4.3 Efficiency of the Pulsifier for recovery of bacteria from salad leaf phylloplanes

Following its introduction by Sharpe and Jackson (1972), the Stomacher rapidly became established as the laboratory standard for the processing of environmental samples for microbiological analysis. However, whilst it is a well-suited system for culture-based analyses of bacteria, the increasing focus on culture-independent techniques including molecular and microscopic methods demand a higher degree of sample purity and processability than the Stomacher can provide. Consequently the Pulsifier was developed to meet this demand (Fung *et al.* 1998). Its superiority over the Stomacher in terms of both number of bacteria recovered and quality of bacterial suspensions produced has been demonstrated for a variety of foodstuffs including a number of fruits and vegetables (Fung *et al.* 1998; Wu *et al.* 2003).

Bacterial loads of pulsified and unpulsified spinach leaves did not appear visibly different when viewed by EDIC microscopy alone, despite the fact that DAPI counts demonstrated the removal of approximately 50 % of epiphytic bacteria .One explanation for this may be the attachment of bacteria to leaf surfaces using secreted exopolymers; this could potentially leave an impression of a cell or aggregate even after actual bacteria are removed. Material present on stomatal guard cells seemed particularly predisposed to remain after pulsification; given the need for guard cells to alter their morphology for stomatal closure, the physiochemistry of the cuticle may differ from that of the surrounding cells. This or the change in topography arising from the stomata frequently being sunken relative to the plane of the surrounding leaf epidermis may lead to reduced efficiency of bacterial detachment from guard cells.

There were no palpable differences between the diversity of bacteria recovered from spinach phylloplanes when the length of pulsification time was altered. A difference might be expected if the phylloplane community employed a variety of attachment strategies with different levels of effectiveness. It is possible that all methods of bacterial attachment to leaves are equally susceptible to the Pulsifier, or indeed that all bacterial species use the same mechanism; however, given the sheer diversity of bacterial genera previously reported on leaf surfaces (Ercolani 1991; Yang *et al.* 2001) this seems unlikely. Instead, given the frequency with which aggregates and biofilm have been observed on leaves during this study and others (Morris *et al.* 1997; Monier and Lindow 2004; Warner *et al.* 2008), it is likely that instead the Pulsifier simply detaches whole or fragmented mixed-species aggregates of bacteria; this would negate the individual attachment mechanisms

employed by each species in isolation. To further investigate whether the Pulsifier has any species bias when detaching phylloplane epiphytes, it would be necessary to identify the isolates recovered onto R2A medium and observe their relative abundance in samples pulsified for differing lengths of time. However, accurate and comprehensive analysis of complex bacterial communities such as those on phylloplanes is a major undertaking deemed beyond the scope of this investigation.

In common with the Pulsifier, shaking by hand produces non-destructive agitating forces within the bag, yet, unlike pulsification, is difficult to replicate with any degree of consistency; the speed of agitation is also limited by the ability of laboratory worker. As a tool for the removal of phylloplane bacteria it is of little use, but serves to highlight the benefits of the Pulsifier technology. Though the improvement is obvious, the Pulsifier is still only able to remove only around half of all phylloplane bacteria; those remaining are either resistant to the high frequency waves generated during pulsification, or else are physically shielded from their effects by surface topography or dynamics of the leaves in the sample bag. When single leaves were pulsified the improvement in efficiency was only slight despite differing dynamics arising from a difference in bag size and diluent volume; resistance and topographical shielding are therefore the more likely causes. Direct comparison of the comparative efficiency of the Stomacher and Pulsifier has previously been obtained using culture-based enumeration (Fung *et al.* 1998), unfortunately salad leaves processed using the Stomacher become too damaged to be suitable for *in situ* direct microscopic counting (Sihota 2008).

4.4.4 Effect of the Pulsifier on aggregated phylloplane bacteria

Following recovery from leaf phylloplanes, bacterial growth on agar media and staining of suspensions inoculated onto membrane filters showed few signs of cell clumping; EDIC/EF microscopy showed that aggregates in pulsified suspensions rarely exceeded ten cells. However, this could be due to forces within the diluent breaking up cell clumps during pulsification, immediately after detachment from the sample matrix has occurred. Previous studies evaluating the use of the Pulsifier in recovery of bacteria from food matrices have focussed almost exclusively on bacterial numbers recovered and clarity of suspensions produced (Kang *et al.* 2001; Wu *et al.* 2003). Differential recovery efficiency by the Pulsifier against solitary and aggregated bacteria from sample matrices may introduce a sampling bias which could affect the accuracy of downstream analysis;

individual species within complex communities such as those found on phylloplanes could be at risk of misrepresentation due to a preference for either solitary or aggregated survival strategies. Therefore it was necessary to further explore the effect of the Pulsifier on mixed-species aggregates such as those found on a leaf, in order to evaluate the possibility of the Pulsifier being able to detach bacterial aggregates and/or biofilm fragments from leaf surfaces.

Mixed phylloplane isolates from spinach readily formed biofilms in static liquid culture, in which coccoid and bacillar cells could be seen interspersed with one another; low nutrient availability and ambient temperature were used to partially emulate conditions on a leaf. Although not all conditions could be controlled, the model biofilms offered a means to challenge the action of the Pulsifier and reveal whether or not it could significantly break up aggregated phylloplane epiphytes. Pulsification did lead to a significant reduction in the size of biofilm fragments, although biofilm fragments that remained were markedly larger than anything seen in the bacterial suspensions produced from actual leaves. Size and morphology of fragments were, however, comparable with structures observed *in situ* on leaf phylloplanes. The marked increase in surface coverage and biofilm depth observed in cultured biofilms of leaf-associated bacteria compared to colonisation patterns observed on leaves highlights the extent of growth limitations placed on epiphytes by the hostility of conditions on the phylloplane.

In phylloplane microbiology, aggregation and biofilm formation have been demonstrated to be central to bacterial community dynamics (Monier and Lindow 2003; Monier and Lindow 2005a). Mechanical removal risks being preferentially more effective at recovering either solitary or aggregated cells; subsequent analyses in which bacterial diversity or physiology are important could therefore be skewed by sampling bias. The fact that aggregates were observed on leaves that had been pulsified in similar numbers to those recorded on unpulsified leaves suggests that the Pulsifier does not have a bias for removing aggregations of bacteria from phylloplanes. Bacterial suspensions from pulsified leaves typically took the form of single cells or clusters of fewer than ten cells; thus study has established that the Pulsifier appears to recover cells in such configurations, rather than detaching larger biofilms then fragmenting them in suspension. This could lead to a bias towards recovery of solitary cells, or those in the periphery of aggregates and biofilm; during analysis of community diversity this may lead to incorrect determination of the dominant bacterial species present. The better the mechanisms of bacterial recovery from

complex matrices such as food is understood, the greater the ability to interpret the biological significance of the results of downstream analyses of such samples.

4.4.5 Conclusions

Bacterial community sizes have been shown to vary between species of commonly eaten salad leaf; differences were observed that could not be attributed to differences in growth environment. Differences in leaf morphology, surface topography and cuticular composition could all be responsible for creating refuges from potential lethal stresses such as UV radiation and desiccation. Total phylloplane populations determined on red and green cos leaves suggested that the chemistry of the leaf interior did not affect conditions on the surface; the leaf cuticle is likely to form an effective barrier to constitutive solute loss onto the leaf surface.

The tendency for phylloplane epiphytes to colonise epidermal cell margins may not only confer resistance from environmental stress, it may also be a major factor in the resistance of bacterial cells to detachment in the laboratory. The mechanism of action of the Pulsifier is yet to be fully understood, though its effectiveness in processing fragile sample matrices such as leaves demonstrates great potential for a wider application. The Pulsifier may only be able to remove around half of the bacterial epiphytes on the leaf, although until superior techniques are developed this can be viewed as more of a testament to the complexity of the interaction between bacteria and leaf than a criticism of the technology itself. During this study the Pulsifier appeared to demonstrate a bias towards the removal of solitary cells rather than aggregates or biofilm from spinach phylloplanes; this must be taken into account when interpreting any results generated following its use.

It is interesting to note that despite the advantages and disadvantages of both DAPI staining and R2A plate counts for quantitation of leaf epiphytes, neither assay is optimally suited to processing samples containing aggregates and biofilm. Such structures have frequently been observed throughout this investigation; it must therefore be concluded that the search for the optimum assay for quantifying total phylloplane bacteria is not yet at its end.

CHAPTER 5

ROLE OF CURLI FIMBRIAE IN ATTACHMENT OF *SALMONELLA* TO SPINACH LEAVES

5.1 Introduction

Human gastrointestinal pathogens are most commonly found in environments which are consistently warm, moist and nutrient rich (Winfield and Groisman 2003). If optimum population growth occurs under such conditions what happens when they arrive on a leaf surface? The phyllosphere is hostile to bacteria; nutrients are scarce and epiphytes must avoid or tolerate fluctuations in temperature, solar UV radiation and desiccation stress (Hirano and Upper 2000).

The initial aim of this investigation was to quantitatively and spatially track the colonisation of spinach leaves by *Salmonella thompson* using GFP reporter technology. The green fluorescence reporter allowed spatial tracking of the pathogen following inoculation using EDIC/EF microscopy. Direct microscopic observation of *Salmonella* on leaves was used in order to provide information on the spatial patterning of colonisation. Inoculation of single leaves by immersion in a bacterial suspension was chosen because it allowed bacteria equal access to both the upper and underside of leaf surfaces, allowing quantitative comparison of surface preference for bacterial attachment. Leaves were washed manually in tap water to represent a simulation of produce washing by consumers in the home prior to consumption. Remaining bacterial contamination was enumerated in order to comparatively assess the strength of bacterial attachment to spinach leaf phylloplanes

Previous work by Sihota (2008) demonstrated that treatment with nitric oxide improved the recovery of *E. coli* from leaves but not the recovery of *Salmonella*. One possible explanation for this was that *Salmonella* may utilise different mechanisms of attachment from those used by *E .coli*. Both *E. coli* and *Salmonella* possess curli fimbriae, short pili on the cell surface which have been demonstrated to be involved during *in vitro* attachment assays (Cookson *et al.* 2002; Jonas *et al.* 2007). Interestingly though, in pathogenic *E. coli*, curli were not found to be necessary for leaf surface attachment (Jeter and Matthysse 2005). Therefore the second aim of this study was to examine the role of curli fimbriae in the attachment of *Salmonella* to spinach leaves. In order to test for a correlation between the amount of curli produced and the level of attachment conferred, a selection of different *S. typhimurium* mutants each lacking one or more genes involved in the curli biosynthesis pathway were employed. *Salmonella* biofilms were grown in polystyrene 6-well plates, allowing comparison of surface coverage and biofilm architecture using direct microscopy. In addition, spinach leaves were inoculated with the same *Salmonella* strains as those used

in the 6-well plate assay, allowing comparison of the ability of curli-defective mutants to colonise the two environments.

5.2 Materials and methods

Details of the materials and methods used in this study are described in Chapter 2. *Salmonella thompson* GFP RM2311 was used for *in situ* detection of colonisation of spinach leaves due to its constitutive green fluorescence using epifluorescence microscopy. *Salmonella typhimurium* ATCC 14028 was used for studies involving expression of curli fimbriae as a variety of different gene mutations in the curli biosynthesis pathway were available. Analysis of *S. typhimurium* strains on leaves was performed using recovery and culture on selective agar media only.

5.3 Results

5.3.1 Patterns of *S. thompson* colonisation of the spinach phylloplane

S. thompson GFP was inoculated onto spinach leaves in order to observe its spatial patterns of colonisation on the spinach phylloplane. In Figure 30, numerous green-fluorescing cells are apparent on the leaf surface. The cells present are largely solitary; there is little evidence of aggregation or microcolony formation. Other samples, including those left for up to four hours post-inoculation (not shown), displayed a very similar lack of aggregation. However, as early as two hours after inoculation the numbers of green-fluorescent cells present inside stomatal openings (Figure 31) increased markedly; incubation times extending beyond four hours did not appear to produce any further increase in stomatal penetration.

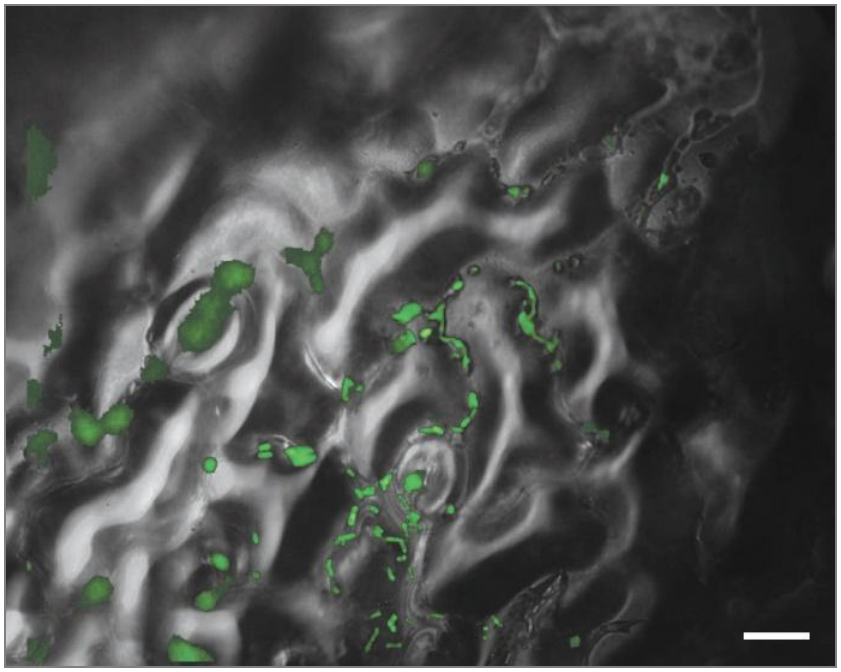


Figure 30: EDIC/EF micrograph of S. thompson *GFP inoculated directly onto the upper surface of a spinach leaf (shown as EDIC background image). Bar = 10 µm.*

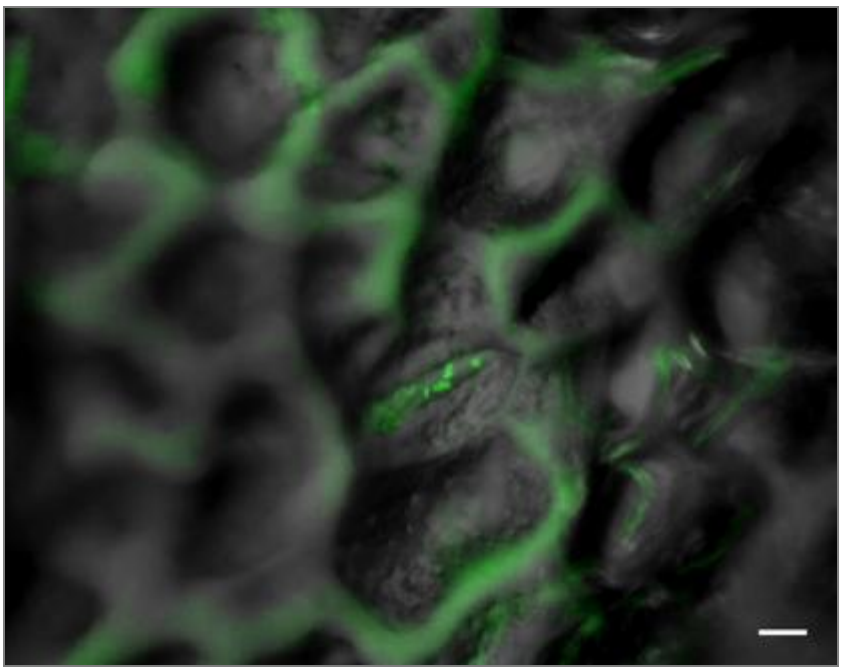


Figure 31: EDIC/EF micrograph of S. thompson *GFP within a stomatal opening of a spinach leaf, observed two hours after direct inoculation onto the leaf surface (image overlaid onto EDIC micrograph of leaf). Bar = 10 µm.*

Individual leaves inoculated by immersion in an *S. thompson* GFP suspension were analysed to determine whether cells exhibited a leaf surface preference when given equal access to both surfaces. The data presented in Figure 32 confirms that cells suspended in water did not show a significant preference in the surface they attached to; the proportion of attached cells enumerated on the upper and lower leaf surfaces was not significantly different (paired *t*-test; $p > 0.05$, d.f. = 8). By contrast, when cells suspended in PBS were used, on average 18 % more cells attached to the lower surface than the upper surface, though this was not a significant preference (paired *t*-test; $p > 0.05$, d.f. = 7). Low standard error values indicate a high degree of reproducibility in the patterns observed.

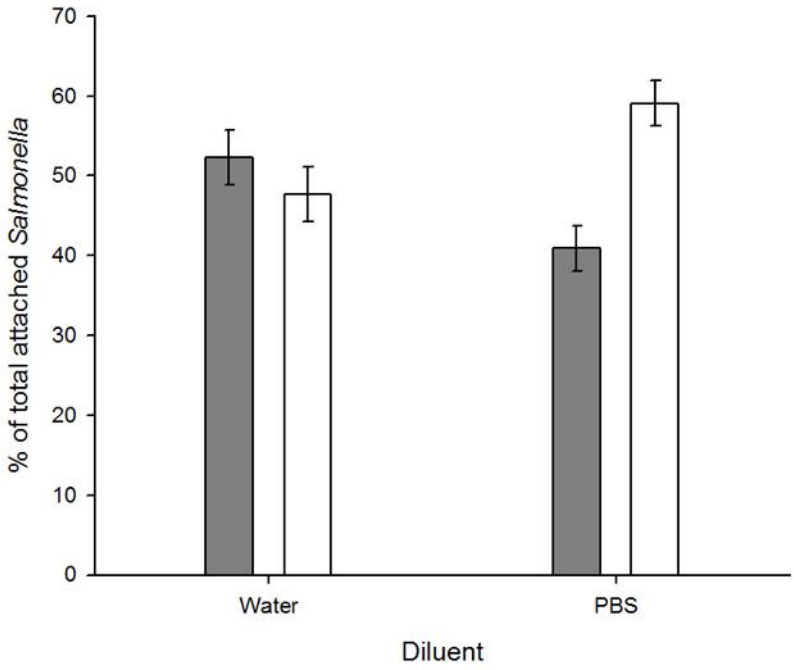


Figure 32: Percentage of attached S. thompson *GFP recorded on the upper (shaded bars) and lower (blank bars) of individual spinach leaves following a 24 h inoculation period in either a water or PBS suspension of 5 × 10^6 CFU/ml* Salmonella *cells. Error bars represent standard error of the mean of eight leaves per treatment.*

Some leaves were hand-washed in tap water following inoculation in order to assess whether the strength of attachment was sufficient to resist manual detachment from the spinach phylloplane, such as might occur in the home prior to consumption. *S. thompson* inoculated onto spinach leaves in PBS suspensions were not significantly reduced in number by washing (Figure 33). Interestingly, cells inoculated in a water suspension were significantly reduced in number by washing (*t*-test; $p<0.05$, d.f. = 4). Using either type of cell suspension, washed leaves showed considerably less variation between samples than unwashed leaves. PBS-suspended cells and water-suspended cells did not show a significant difference in the overall numbers of cells attached per mm^2 of leaf surface (*t*-test, $p>0.05$, d.f. = 9).

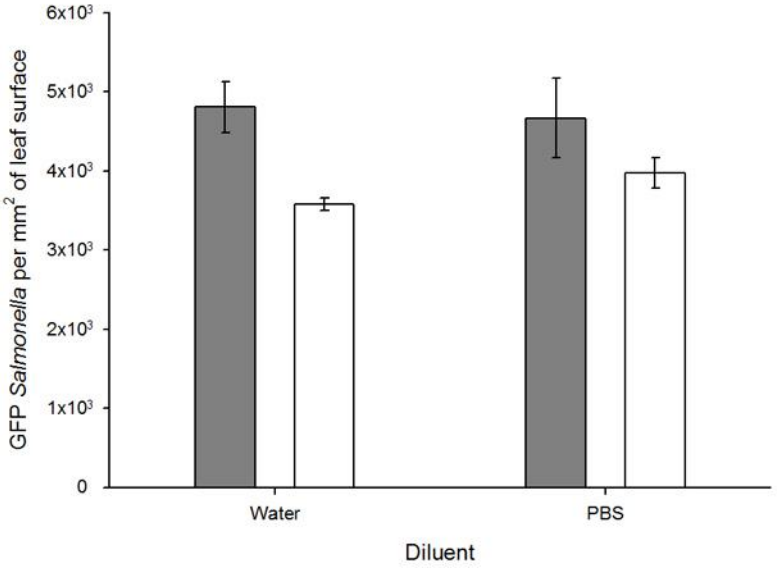


Figure 33: Numbers of S. thompson *GFP recorded following a 24 h inoculation period in either a water or PBS based suspension of 5 × 10^6 CFU/ml* Salmonella *cells. Shaded bars indicated leaves examined directly after inoculation; blank bars indicated leaves washed manually for 2 min before examination. Error bars represent standard error of the mean of three leaves per treatment.*

5.3.2 Determination of curli production in *S. typhimurium* mutants

The level of curli production in *S. thompson* GFP, *S. typhimurium* ATCC 14028 and four derived curli mutants was compared using growth on CRI agar plates and by determination of Congo Red binding in suspension. Figure 34 presents photographs of growth on CRI plates; red coloration of colonies is indicative of Congo Red binding to curli fibres. *S thompson* and wild type *S. typhimurium* produces colonies with the most intense red coloration; less intense but similar to one another were the colonies of the *Δcrl* and *ΔcsgB* mutants. The remaining two *S. typhimurium* mutants, *ΔrpoS* and *ΔcrlΔcsgB*, showed very little red coloration.

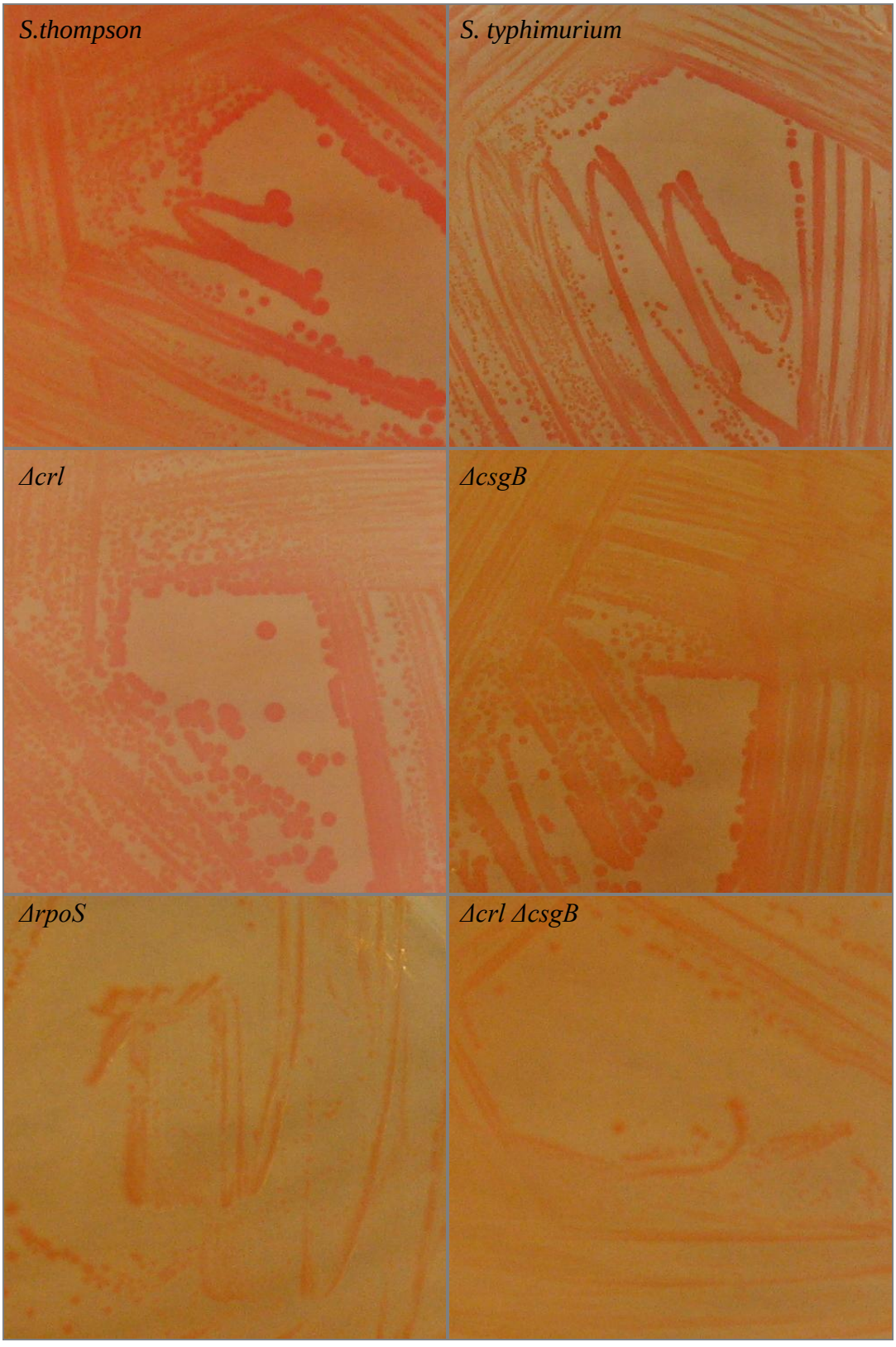


Figure 34: Colony pigmentation of Salmonella *strains grown for on CRI plates for 72 h at 28 °C; pigmentation results from binding of Congo Red contained within the medium. Mutant genotypes are derivatives of the parent strain* S. typhimurium *ATCC14028.*

When Congo Red binding activity was assessed quantitatively (Figure 35) a similar pattern of relative curli expression was observed amongst the six *Salmonella* strains analysed. *S. thompson* bound the greatest amount of Congo Red, more than wild type *S. typhimurium*. As suggested by analysis of CRI plates, *ΔrpoS* and *ΔcrlΔcsgB* respectively had the two lowest levels of binding activity. The binding activity of all six strains was significantly different from one another (one-way ANOVA, $p<0.05$ d.f. = 35). Unexpectedly, the *Δcrl* mutant bound more Congo Red than its wild-type parent strain, while the greatest difference in binding activity amongst the *S. typhimurium* strains under scrutiny was between *Δcrl* and the *ΔcrlΔcsgB* double mutant.

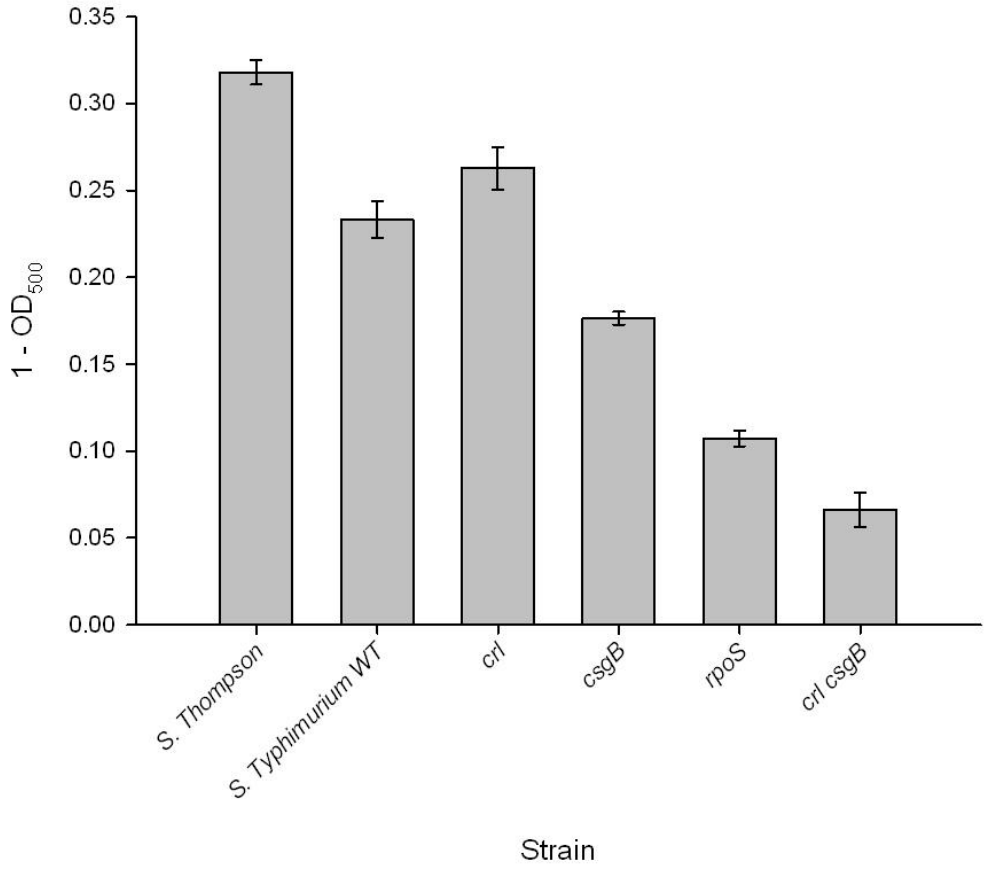


Figure 35: Congo Red binding activity of S. thompson *GFP,* S. typhimurium *and its mutant derivatives as determined by the change in the light absorbance at 500nm of a*

Congo Red solution following a ten minute incubation with Salmonella *cells. Error bars represent standard error of the mean of six replicates per treatment.*

5.3.3 Attachment of *Salmonella* curli mutants to polystyrene surfaces and spinach phylloplanes

Attachment of *S.* t*yphimurium* mutants to abiotic surfaces was evaluated using polystyrene 6-well plates. As in Section 5.3.2, *S. thompson* GFP and wild-type *S. typhimurium* were included for comparative purposes. Photomicrographs of 48 h biofilms of each of the six strains under examination are shown in Figure 36. It is immediately apparent that only wild-type *S. typhimurium* and *S. thompson* GFP were able to form three dimensional structures; all four mutant strains formed only a single layer of cells. Although they were restricted to the production of monolayers, *Δcrl* and *ΔcsgB* mutants exhibited a markedly higher degree of aggregation, than the remaining two mutant strains; *ΔrpoS* and *ΔcrlΔcsgB* mutants attached in markedly lower numbers than any of the other four strains and showed no evidence of aggregation or microcolony formation. As predicted from initial visual examination of photomicrographs, *ΔrpoS* and *ΔcrlΔcsgB* mutants covered the lowest percentage of the surface, whilst *S. thompson* and the *S. typhimurium* parent strain covered the greatest percentage (Figure 37). The *Δcrl* mutant of *S. typhimurium* was able to cover as high a proportion of the surface as both its wild type parent and *S. thompson* (one-way ANOVA; $p > 0.05$, d.f. = 35), though its surface coverage was also the most variable. Despite the overall similarity in coverage, biofilm architecture differed markedly between mutant and parent; loss of *crl* function greatly reduced the size and frequency of three-dimensional structures compared with wild-type biofilms, instead predominantly producing only monolayers.

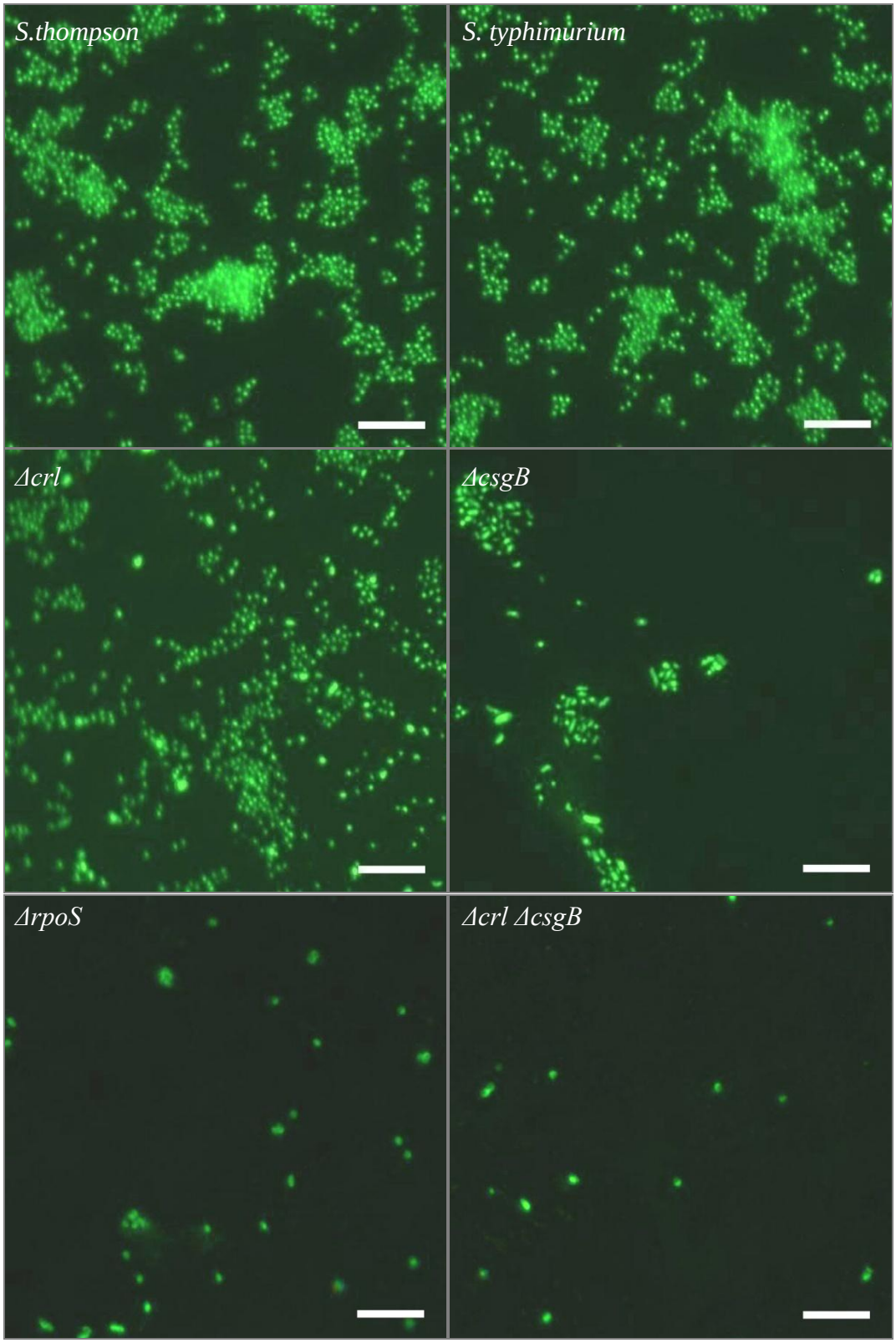


Figure 36: Salmonella *biofilms grown in polystyrene 6-well plates for 48 h at 28 ^{o}C and stained using SYBR Gold. Bar = 10 µm.*

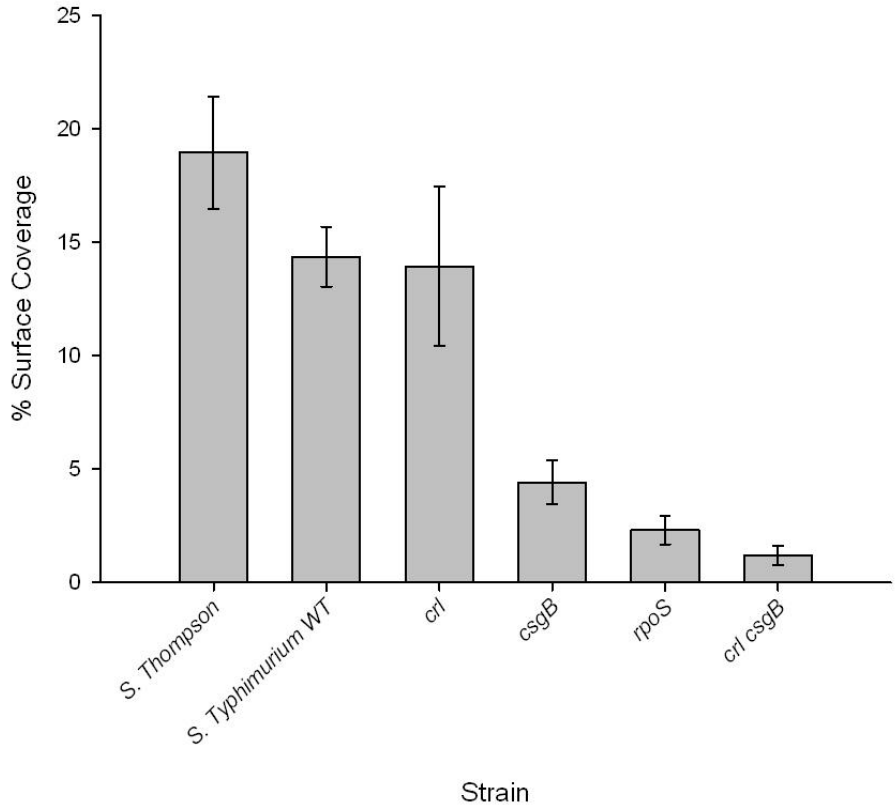


Figure 37: Percentage coverage of 48 h Salmonella *biofilms grown in polystyrene 6-well plates. Error bars represent ± standard error of the mean of six wells per strain.*

All six *Salmonella* strains evaluated during this study were inoculated onto individual spinach leaves in order to see whether curli synthesis mutants were altered in their ability to colonise the spinach phylloplane. Attachment efficiencies determined by recovery and enumeration on selective agar media did not follow the trends observed in 6-well plates, nor did they correlate with Congo Red binding activity as presented in Figure 35. Two determinants of attachment were recorded; the number of cells remaining in inoculum suspensions after incubation and the number of *Salmonella* cells recovered from each leaf using the Pulsfier. The counts obtained for each strain are presented in Table 7. Cell counts following pulsification of leaves did not reveal any significant difference in the level of

attachment displayed by the six strains (one-way ANOVA; $p > 0.05$, d.f. = 17). A observed previously in 6-well plate assays, on leaves *Δcrl* samples produced the most variable rates of attachment. Overall variation in leaf surface attachment within replicate samples of each strain was markedly higher than replicate samples on polystyrene.

The *Δcrl* mutant produced a marked decline in inoculum numbers compared to the other strains, however, this was not reflected in the number of cells recovered from the leaf surface. *ΔrpoS* and *ΔcrlΔcsgB* mutants, which had demonstrated the lowest surface coverage of 6-well plates, showed markedly less of a decrease in inoculum numbers than the other four strains tested, though not statistically significantly so (one-way ANOVA; $p < 0.05$, d.f. = 17). Again, this difference was not reflected in the cell numbers recovered from leaves following inoculation.

Table 7: Numbers of Salmonella *cells detected by Rambach agar plate count when individual spinach leaves were inoculated with different* Salmonella *strains. Counts were determined for* Salmonella *cells recovered from leaf surfaces using a Pulsifier, as well as cells remaining in the inoculum suspension after a 24 h period of inoculation. Values in parenthesis indicate ± standard error of the mean of nine leaves per treatment.*

Strain	CFU/ml recovered from leaf	CFU/ml remaining in inoculum
S. thompson GFP	$1.19\ (\pm 0.57) \times 10^6$	$1.62\ (\pm 0.35) \times 10^7$
S. typhimurium		
wild-type	$2.92\ (\pm 1.56) \times 10^6$	$9.94\ (\pm 1.89) \times 10^5$
Δcrl	$6.03\ (\pm 5.03) \times 10^6$	$2.16\ (\pm 0.32) \times 10^5$
ΔcsgB	$2.92\ (\pm 1.83) \times 10^6$	$5.89\ (\pm 1.98) \times 10^7$
ΔrpoS	$8.67\ (\pm 0.33) \times 10^6$	$1.97\ (\pm 0.65) \times 10^8$
Δcrl ΔcsgB	$2.10\ (\pm 1.02) \times 10^6$	$2.98\ (\pm 1.06) \times 10^8$

5.4 Discussion

5.4.1 Patterns of leaf colonisation by *S. thompson* GFP

S. thompson followed a similar pattern of leaf surface colonisation on spinach to that observed in indigenous bacterial communities (see Chapter 3). Cells preferentially attached at epidermal cell margins and around stomata; comparatively few cells were located more centrally on epidermal cells. There are several potential survival benefits to immigrant cells of co-localisation with native leaf microflora; Monier and Lindow (2005a) demonstrated that contact with resident bacterial aggregates increased the survival of immigrant Pseudomonads. Aggregation and biofilm are thought to be key survival strategies in the phylloplane environment (Molina *et al.* 2003); immigrant *Salmonella* cells may become either passively or actively incorporated into mixed species biofilms on the leaf surface. Once established, the mature biofilm could provide protection from environmental stresses (Monds and O'Toole 2009) and chemical challenge (Xu *et al.* 2000). The co-localisation of immigrant cells with native bacterial aggregates observed during this study suggests that native leaf microflora may be critical in determining the fate of non-native colonists.

Immigrant bacterial cells, especially from non-native species such as enteric pathogens, face immediate and severe environmental stresses upon arrival at the phylloplane surface. The detrimental effects of desiccation and DNA damage from UV radiation have the potential to inactivate cells within hours of colonisation; sub-lethal stress responses, such as ice nucleation (Hirano and Upper 2000) or endospore formation (Maduell *et al.* 2008) may be initiated, enabling survival but halting population growth. In this study, two potential coping strategies were observed to occur in *S. thompson* cells: co-localisation with native bacterial aggregations and penetration of leaf stomata. Localisation activity was not identified as a passive or active process using he techniques described here, however, observation of typical movements of free water on the leaf surface suggests that deposition of bacteria in epidermal cell margins is a largely passive process. Integration into a biofilm is likely to provide protection from desiccation, some UV resistance and physical adhesion protecting against detachment thorough shear stress (Cao and Alaerts 1995). In addition, if virulent plant pathogens are present nearby, they may be able to

induce nutrient leakage from host plant cells, improving food availability to nearby opportunists (Beattie and Lindow 1995).

Little evidence of aggregation of *S. thompson* GFP cells with itself was observed in the hours following inoculation onto the spinach phylloplane. This may have been due to the process of aggregation occurring too slowly to have been recorded in the duration of the study; alternatively such processes may have been absent altogether. Aggregation and biofilm formation are key growth phases in both pure culture systems (Prouty and Gunn 2003) and during infection of animal hosts (Esteves *et al.* 2005). Such activity in the phylloplane environment would be dependent on successful transmission of quorum sensing signals; water scarcity, plant responses and high numbers of competing bacterial species could both act to disrupt the transmission of chemical signals necessary for multicellular behavioural responses (Bauer and Robinson 2002; Bauer and Mathesius 2004).

In this study *S. thompson* GFP cells demonstrated an ability to penetrate the leaf interior thought stomatal openings. The leaf interior, or endosphere, is regarded as a much less stressful environment for bacterial survival, and its colonisation is key in the pathogenesis of plants by many bacterial and fungal pathogens (Beattie and Lindow 1995; Beattie and Lindow 1999). The relative humidity of the endosphere is higher than the phylloplane because of transpiration processes in the plant, whilst the leaf epidermis provides physical shielding from UV radiation. Temperature fluctuations may also be lessened slightly by the thermal insulation of surrounding leaf tissue (Rasche *et al.* 2006). *Salmonella* has previously been observed in sub-stomatal cavities using CLSM techniques (Brandl and Mandrell 2002). Where the plant tissue involved is destined for human consumption, especially salad crops and other foods eaten raw, this potentially poses a serious risk to human health. Enteric pathogens resident in the endosphere are protected from both chemical and mechanical challenge, making them strongly resistant to sanitisation processes. In this study, stomatal penetration was observed just 2-4 hours after leaf inoculation of postharvest spinach; a phenomenon previously observed on whole plants (Brandl and Mandrell 2002). Produce affected in this way is effectively unsafe for human consumption as there currently appears to be no suitable way to remove colonising pathogens from the endosphere.

Field-grown leafy salad crops are heterogeneous habitats for bacteria at the whole-plant level; spatial arrangement of the uppermost leaves can create microclimates of sunlight and

shade on lower leaves, which exacerbate fluctuations in temperature, relative humidity, water availability and UV radiation levels. In addition, the conditions on the upper and lower surfaces of a single leaf offer differing levels of these same stresses. Despite this, in Chapter 4 it was observed that the two surfaces of several types of salad leaf did not carry a significantly different total epiphytic bacterial load from one another. When temperature, light and water availability were equalised across the two surfaces of spinach leaves during this study, waterborne *S. thompson* GFP attached in equal numbers to each surface. Therefore the physiochemistry of the leaf surfaces, which differ from one another in many higher plant species (Gniwotta *et al.* 2005; Nawrath 2006), did not affect colonisation by *S. thompson* suspended in water. Both sterile water and PBS were used as diluents during inoculation experiments; PBS was used as an isotonic diluent to reduce the likelihood of bacterial osmotic stress responses becoming induced prior to inoculation , such as may occur in waterborne cells. Interestingly, whilst the overall number of *S. thompson* cells attaching to spinach leaf surfaces was not affected by the choice of diluent, the use of PBS caused a markedly higher rate of attachment to the lower leaf surface than the upper. Regarding the upper and lower leaf surfaces of spinach leaves, examination using the naked eye indicates that the thickness of the waxy cuticle is noticeably different; in addition further investigation may identify differences in the chemical compositions of each surface. What was apparent in this study was that cells not subjected to osmotic stress exhibited a significant preference for the lower surface, whilst cells suspended in water and therefore subjected to osmotic pressure colonised both leaf surfaces equally. Enumeration of attached cells gave no assessment of the strength of attachment, which may be affected by stress-induced phenotypic responses; manual wash treatments did not reveal a significant difference in the strength of attachment in relation to the choicer of diluent. A variety of environmental stresses are known to promote surface attachment relative to a planktonic mode of life (Donlan 2002); it is possible that the lower leaf surface is physiochemically more suitable for attachment, yet osmotically challenged cells induce alternative mechanisms of attachment in order to promote surface attachment, which would account for the loss of surface preference. It is clear that the plant-microbe interface is a complex system and there are likely to be several mechanisms involved in the attachment process, as has previously been suggested (Barak *et al.* 2002; Barak *et al.* 2005).

5.4.2 Role of curli fimbriae in colonisation of polystyrene and the spinach phylloplane by *Salmonella*

Curli fimbriae on bacterial cell surfaces are able to bind the Congo Red when the dye is added to growth media or applied directly to the cell, making it extremely useful for assessing curli activity *in vitro* (Collinson *et al.* 1993). The gene expression pathway leading to curli synthesis is already well characterised (Robbe-Saule *et al.* 2006); the strains used in this study were chosen from a larger collection of curli expression mutant genotypes in order to give a range of curli expression levels, thus allowing a search for correlative attachment effects linked to curli production.

Wild type *S. typhimurium* and *S. thompson* GFP appeared to have the highest Congo Red binding activity and therefore, as expected, higher number of curli fibres than the mutants. Unexpectedly, loss of *crl* gene function appeared to increase curli production; the role of *crl* is thought to be involved in the regulation of *rpoS* via an unknown mechanism, and in addition associates directly with the csgBAC promoter, which governs synthesis of curli subunits, however, in some *E. coli* strains curli can be produced independently of *crl* (Provence and Curtiss 1992). By contrast, the absence of *csgB* function caused significant reduction in curli, though as the gene governs only one type of curli subunit, the curli phenotype was not lost altogether.

The remaining two strains displayed a significantly lower level of curli expression than the *Δcrl* and *ΔcsgB* mutants. The *ΔcrlΔcsgB* double-mutant had previously been reported to eliminate curli production entirely (Robbe-Saule *et al.* 2006); however, during this study both CRI plates and the quantitative assay demonstrated a very low but detectable level of Congo Red binding. This could be due to partial restoration of the phenotype via an unknown genetic mechanism, or simply to non-specific binding of Congo Red to other cell structures, although a similar assay suggested the latter to be unlikely (Gophna *et al.* 2001). Interestingly, the *rpoS*-defective mutant produced only slightly more Congo-red binding activity than the minimal amount observed in the *ΔcrlΔcsgB* double-mutant strain. The *rpoS* gene is a global regulator of stress response as it encodes a sigma subunit of RNA polymerase (O'Neal *et al.* 1994); whilst it is downstream of *crl* in the curli biosynthesis pathway, the loss of *rpoS* function resulted in a more pronounced reduction in curli production than the loss of *crl*. This demonstrates the complexity of the regulatory elements of the curli biosynthesis pathway; an important consideration when examining bacterial responses to differing environments.

S. thompson and *S. typhimurium* in pure culture are both effective colonisers of polystyrene; in addition, they both show the formation of complex 3D aggregations which are likely to be biofilm or its precursors. *Salmonella* is known to form biofilm on polystyrene and other plastics (Joseph *et al.* 2001; Stepanovic *et al.* 2004); having two clinically relevant *Salmonella* strains behave in a similar matter supports the view that more *Salmonella* strains may exhibit this behaviour, suggesting that the findings of this study relating to curli and surface attachment to could be applied more widely amongst the *Salmonellae*. *ΔrpoS* and *ΔcrlΔcsgB* mutant strains of *S. typhimurium* produced almost no curli fimbriae; in addition, attachment to polystyrene surfaces by these strains was only observed in very low numbers. The strains did not display aggregative or biofilm-forming activity, indicating that curli production is a fundamental requirement for effective colonisation of polystyrene. In a previous study, the same two strains had demonstrated altered multicellular behaviour during colony formation on agar media, resulting in distinctive colony morphotypes relative to the wild-type parent strain (Robbe-Saule *et al.* 2006).

Both the *Δcrl* mutant and to a lesser degree the *ΔcsgB* mutant showed aggregative cell behaviour, although it should be noted that these strains cells failed to produce 3D structures, remaining instead as monolayers. Previous work using these strains indicated altered colony morphology including a unique morphotype in the *Δcrl* mutant, which was suggestive of a change in multicellular, aggregative responses. *Δcrl* and *ΔcsgB* mutants behaved more similarly to one another in the 6-well plate activity than their Congo Red binding activities would suggest; this gives support to the view that more than one attachment mechanism is involved. Indeed, previous work has implicated flagella as playing a prominent role in surface attachment in conjunction with fimbriae and other unknown mechanisms (Lillard 1986). In addition, non-specific surface interaction facilitated by hydrophobicity and cell surface charge are likely to play an important role (Ukuku and Fett 2002; Dykes *et al.* 2003). Curli fibres clearly play a major role in binding to polystyrene as all curli gene mutants examined in this study produced a significant reduction in effective colonisation of the surface, both in terms of coverage and 3D structure. In addition, surface coverage of 6-well plates correlated with curli production as determined by Congo Red binding, suggesting that curli may even be the primary cell surface structure involved in attachment to polystyrene. Further characterisation of the mutant strains used in this study, in particular attachment responses under different

environmental conditions, could be key to establishing the extent of the influence of curli fibres on attachment to polystyrene and other synthetic surfaces.

Unlike during culture in 6-well plates, attachment to plant leaves by the six strains tested during this study does not correlate well with OD_{500} values for Congo Red binding. The six strains were not observed to attach in significantly different numbers to the spinach phylloplane; this suggests that curli fibres do not play a prominent role in colonisation of the leaf surface by *Salmonella*. The behaviour recorded in phylloplane experiments presented a stark contrast to the patterns observed during colonisation of man-made polystyrene, where attachment correlated positively with curli expression, indicating that very different mechanisms are involved in attachment to the two surfaces. Curli produced by *E. coli* has previously been implicated in attachment to alfalfa, (Jeter and Matthysse 2005; Torres *et al.* 2005), although it was noted that in pathogenic strains curli production was not necessary for effective colonisation and that such strains appear to employ multiple mechanisms of attachment.

There was a markedly higher degree of variation between samples than had been observed during attachment to polystyrene. The variation may account for a spectrum of responses to the surface; the leaf has a greater degree of spatial heterogeneity than many abiotic surfaces and is able to induce responses to bacterial signalling (Bauer and Mathesius 2004; Melotto *et al.* 2006). It is impossible to eliminate these sources of variation, indeed, to do so would be an inaccurate presentation of the spectrum of challenges and stresses which may affect immigrant cells. Loss of the *crl* gene produced the most variation in phylloplane colonisation efficiency; this is not unexpected as Crl is a regulatory protein thought to be involved in multiple biosynthesis pathways including that of cellulose (Robbe-Saule *et al.* 2006). Further studies employing genes associated with cellulose biosynthesis, such as *bcsA* (Römling 2002), would help to further elucidate the individual roles of curli and cellulose production in phylloplane colonisation. A role for cellulose in plant colonisation has previously been implicated (Barak *et al.* 2007); expression of *BcsA*, a cellulose-regulating gene downstream of *crl*, was shown by RT-PCR to be upregulated during the colonisation of alfalfa sprouts. Given the cross-linkage between regulatory elements of the curli and cellulose biosynthesis pathways, it is interesting that cellulose appears to play a role in colonisation and curli does not; RT-PCR analysis of *crl* activity during colonisation may provide a valuable insight into how these cell surface structures are induced in response to plant surfaces.

In some samples, most notably wild-type and *Δcrl* mutant *S. typhimurium*, there was a disparity between the drop in inoculum concentration and the number of attached cells. Cells unaccounted for may have been the result of the limitations of culture-based quantitation of bacteria from environmental samples. As explored in detail in Chapter 4, recovery of bacterial cells from a sample matrix is typically well below 100 % due to strong adherence to the matrix surface. In addition, cells stressed by their environment or by removal from it can enter a VNC state, causing them to be undetectable in culture media despite being viable. During this assay, weakly or transiently attached cells may have been lost during leaf rinsing. Rinse solutions would have contained cells in various stages of association with the leaf surface, along with planktonic cells contained within surface water as the leaves were removed from their initial inoculum. The population of cells in the rinse solution was therefore considered to be a mixture of planktonic and attached cells and could not be attributed to either the inoculum count or the cells recovered from the leaf; it was for this reason that rinse solutions were not enumerated during this study.

5.4.3 Conclusions

Immigrant *Salmonella* cells deposited onto a leaf surface encounter well established bacterial communities. In this study it has been demonstrated that colonisation patterns of *Salmonella* cells closely associate with the regions containing aggregates or biofilms indigenous microflora: predominantly epidermal cell margins, stomatal guard cell surfaces and leaf veins. Co-location may promote survival through opportunistic access to nutrients and incorporation into established biofilms, mitigating in part the hostility of the phylloplane environment to enteric bacteria. Through comparison of colonisation patterns across the two surfaces of a leaf, this study found that leaf surface physiochemistry did not appear to play a significant role in colonisation patterns; however, at the whole plant level there is potential for considerable variation in environmental conditions between leaf surfaces; this is likely to be due to leaf arrangement and shading potentially affecting the survival of all epiphytic bacteria.

Curli fimbriae are a major player in attachment of *Salmonella* to polystyrene surfaces according to the findings of this study and others (Römling *et al.* 2000; Brombacher *et al.*

2006); however they have little or no involvement in attachment to the spinach phylloplane. *Salmonella* must therefore be able to utilise multiple mechanisms to facilitate survival in a variety of non-host environments, whether abiotic or biotic. Extensive research has demonstrated physiological differences between planktonic and surface modes of life in bacteria (Allison and Gilbert 1995; Hall-Stoodley and Stoodley 2002); however, differing surfaces clearly require differing colonisation strategies and phenotypic responses to surface life should not be generalised across environments. The data presented in this chapter highlights the fact that synthetic models are never an effective solution for studying the interaction of bacteria with a biotic surface such as the phylloplane. Pure culture systems remove the influence of neighbouring bacterial species, which may be beneficial or detrimental to the species of interest but will certainly exist, regardless of the environment under study. Bacterial species simply do not exist in isolation from one another, and this study demonstrates that the behaviour of *S. typhimurium* in pure culture on an abiotic surface bears almost no resemblance to its colonisation a natural system, in this case the phylloplane. In addition to playing host to complex epiphytic communities, it must be noted that the phylloplane is a biotic surface and elicits the capacity to react dynamically to the presence of bacterial colonists (Anderson *et al.* 2004). Biotic surfaces, particularly the surfaces of fresh foodstuffs, continue to be commercially important in the fields of plant pathology and food safety; there is also a great deal of fundamental ecological knowledge still to be gained from studying genuine environmental plant-microbe interactions.

CHAPTER 6

EFFECT OF CHEMICAL BIOCIDES ON SPINACH-ASSOCIATED *SALMONELLA* THOMPSON

6.1 Introduction

Currently, pathogen monitoring in the food industry relies almost exclusively on detection and enumeration using growth in culture media. However the existence of a viable but nonculturable (VNC) state has been reported for a number of bacterial pathogens (Desmonts *et al.* 1990; Byrd *et al.* 1991; Oliver *et al.* 1991; Juhna *et al.* 2007a); having entered this state, bacteria will no longer grow under routine culture conditions (Oliver 2005). Furthermore, bacteria in this state have been observed to maintain their virulence (Caro *et al.*, 1999). The VNC state is believed to be a stress response; it can be induced in response to a number of experimental stresses such as oxidative stress, starvation and osmotic pressure (Kogure *et al.* 1979; Leriche and Carpentier 1995; Caro *et al.* 1999; Gupte *et al.* 2003). The presence of VNC pathogens in food poses a risk to consumers because inadequate quality control procedures may fail to detect microbial contamination on food at levels high enough to constitute an infective dose if eaten. Processing practices used to sanitise pre-packaged, ready to eat foods such as bagged salads may cause stress to adherent pathogens and induce the VNC state. For example, chlorine treatment is known to cause cultured biofilms of *Salmonella* to become VNC (Leriche and Carpentier 1995). similarly for *Legionella pneumophila* in aquatic biofilms, this pathogen can be resuscitated from the VNC state and shown to remain infective following passage of chlorine-stressed cells to amoebae (Giao *et al.* 2009).

The primary aims of this study were to evaluate the effect of hypochlorite and Citrox (Citrox Ltd, UK) as biocidal agents against spinach-associated *Salmonella*; biocidal activity was assessed in terms of its effect on both viability and culturability of cells recovered from the phylloplane following sanitisation of leaf samples. Hypochlorite solutions display biocidal activity primarily as a result of strong oxidising action of free chlorine in the solution causing structural damage to bacterial cells (Camper and McFeters 1979). By contrast, Citrox is a proprietary mixture of plant-derived organic acids; producing low pH which causes cell damage (Allende *et al.* 2008). Biocidal activity against leaf-associated *S. thompson* was determined by recovery from leaf samples and enumeration in culture and using microscopy. Induction of the VNC state could be inferred by comparison of culture-independent Direct Viable Count (DVC) values with standard plate counts.

A secondary aim of this study was to investigate the effect of hypochlorite solutions of the degree of stomatal opening found on spinach phylloplanes. Stomata are known to be key

points for leaf entry during bacterial and fungal plant pathogenesis; in Chapter 5, it was observed that *S. thompson* GFP frequently entered stomatal openings, which may allow cells protection from biocidal agents. A change in stomatal opening pattern during sanitisation could therefore be an important factor in the effectiveness of sanitisation treatment, particularly in cases of postharvest contamination where immigrant human pathogens are less well established on the phylloplane.

6.2. Materials and methods

Details of the materials and methods used in this study are described in Chapter 2. Spinach leaves were inoculated with *S. thompson* GFP and subjected to 2 minute washes in water or differing concentrations of chemical sanitising agents. *Salmonella* cells were recovered and enumerated by DVC using epifluorescence microscopy and also by standard plate count on selective agar. Citrox was prepared in solutions containing the organic agent Antifoam (Basildon Chemicals, UK), added at industrial scale to prevent foaming of the wash water.

6.3 Results

6.3.1 Assessment of biocidal activity of sanitising agents against spinach-associated *S. thompson*

6.3.1.1 Hypochlorite

EDIC microscopy was used to make a qualitative comparison between the surfaces of unwashed and chlorine-washed baby spinach leaves. Unwashed leaf surfaces were frequently observed to have contamination of a size and morphology consistent with solitary bacterial cells; putative bacterial biofilms were also observed (Figure 38A). Chlorine washed leaves had a very similar appearance to unwashed leaves; unlike in unwashed samples soil particles were largely absent from washed leaves, though bacterial contamination appeared unchanged (Figure 38B). Crystalline soil particles were readily distinguished by their bright reflection of both episcopic light and epifluorescence in all fluorescence channels of the microscope. Leaf cells themselves did not appear to differ morphologically as a result of exposure to chlorine.

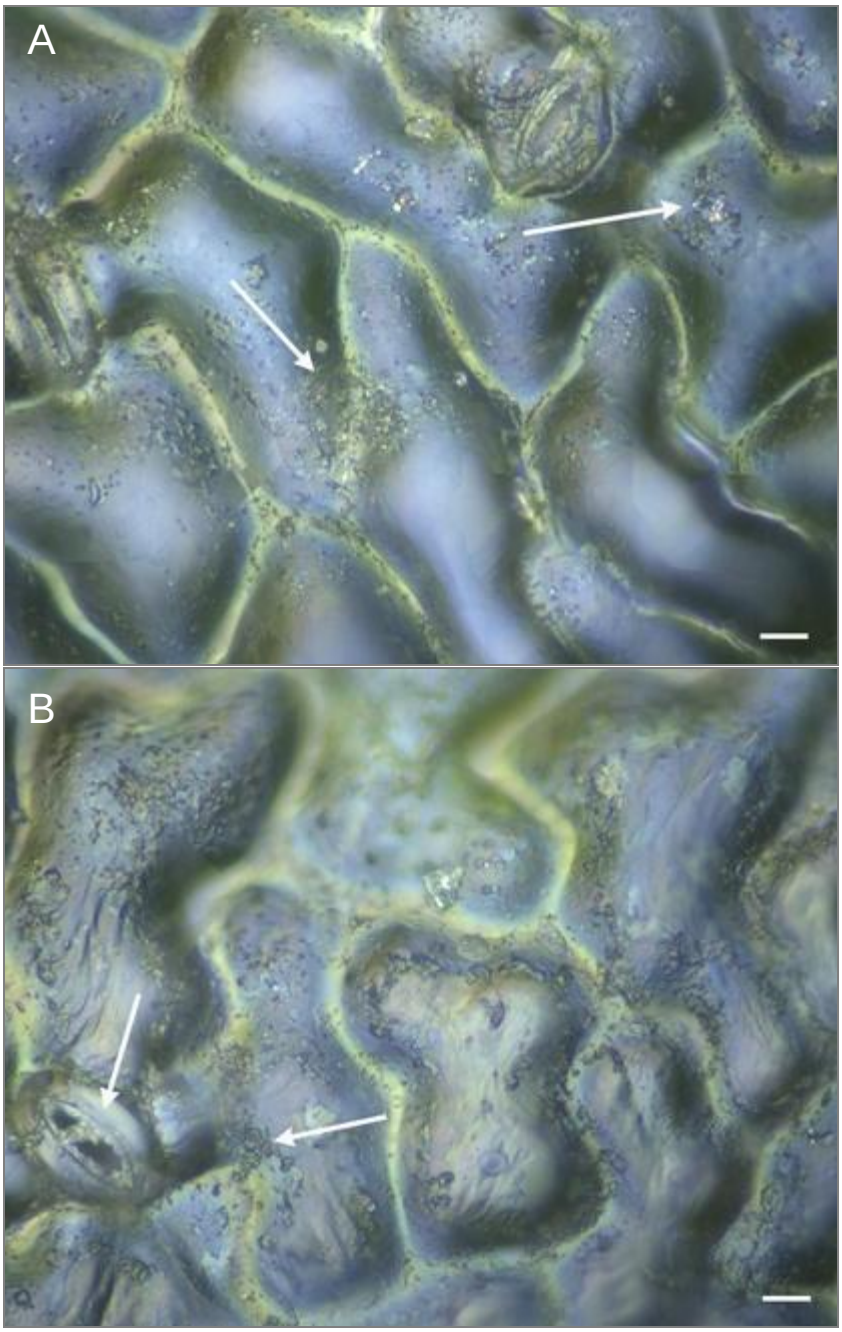


Figure 38: EDIC micrographs of spinach leaves unwashed (A) or washed in 100 ppm chlorine (B), obtained using z-plane scanning of the leaf surface. The chlorine-washed leaf still retains a similar degree of surface contamination to the unwashed leaf, both samples had numerous regions of bacterial aggregation and putative biofilm (indicated by arrows). Bar = 10 μm.

Pure cultures were used initially to validate the DVC procedure for the *S. thompson* GFP strain used in this study. Cells elongated in the presence of pipemidic acid and were fewer in number than cells from the same dilution of the starter culture incubated in $R2B_{50\%}$ medium without antibiotic (Figure 39B). The degree of elongation was consistently no less than 3-5 times that of control cells (Figure 39A), with a small proportion of the cells showing no elongation relative to the control group. These and all non-elongated cells in the study were presumed to be dead as they had shown no growth response to the treatment. The consistent degree of elongation allowed visual distinction of responsive versus unresponsive cells. A slight loss of fluorescence was observed in the elongated samples relative to the control cells, although the decline did not affect the ability to clearly visualise the cells and distinguish cell morphology. Slight indentations were sometimes observed along the length of elongated cells, indicating that the cell division process had begun and subsequently been halted by the antibiotic over several growth cycles.

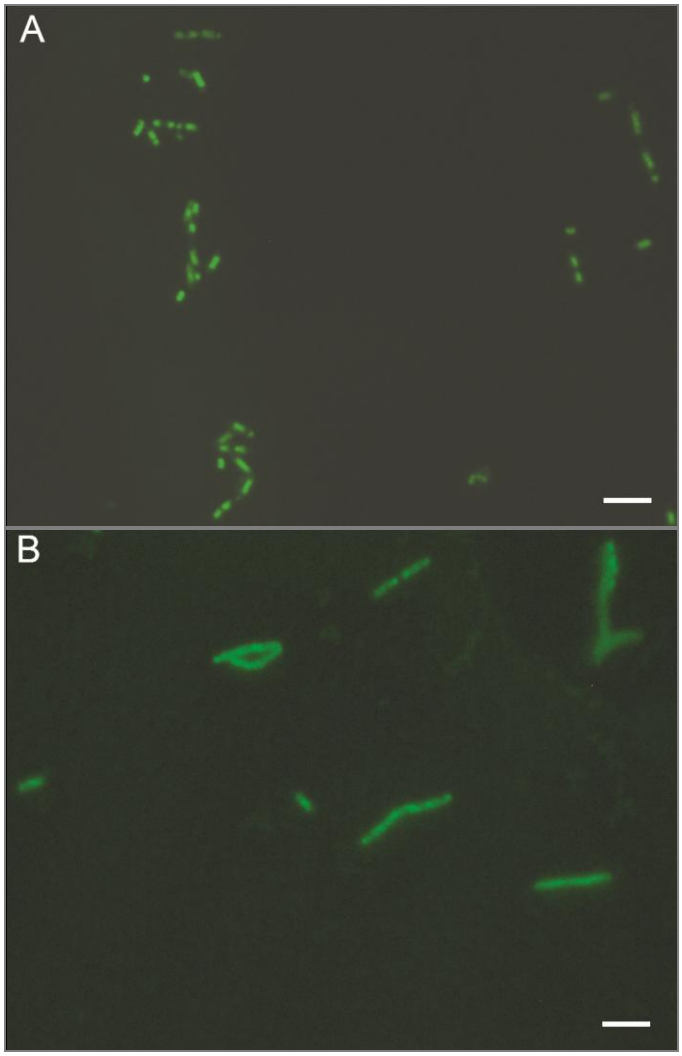


Figure 39: Epifluorescence micrographs of green fluorescent Salmonella thompson *GFP grown for 16 h in 50 % R2 broth without supplements (A) or with the addition of 10 µg/ml pipemidic acid (D), which inhibits cell division and causes elongation of metabolically active cells. Dead cells remain unchanged by the antibiotic. Bar = 10 µm.*

DVC counts obtained from hypochlorite-washed spinach samples showed that for all concentrations tested, the number of viable *Salmonella* recovered from the salad leaf surfaces following washing were not significantly different from the samples washed in water, as shown in Figure 40 (one-way ANOVA, $p > 0.05$, d.f. = 3,16). In contrast to the DVC counts, increasing the hypochlorite concentration of the wash water led to a sharp fall in the number of *Salmonella* colonies observed on agar plates. Plate counts were significantly lower than DVC values for samples washed at 50 and 100 ppm chlorine (two-way ANOVA, $p<0.01$, d.f. = 1,32); at 50 parts per million (ppm) chlorine the number of

colonies counted on agar plates represented less than half of what was observed using DVC. At 100 ppm no colonies grew on agar despite DVC clearly showing high numbers of metabolically active cells recovered onto filters. The decline in colonies counted on agar plates correlates with increasing chlorine concentration with the greatest decline occurring between 50 and 100 ppm free chlorine; washing in water alone produced plate counts which no lower than corresponding DVC values, indicating that all of the *Salmonella* cells recovered from these samples were capable of growing in culture.

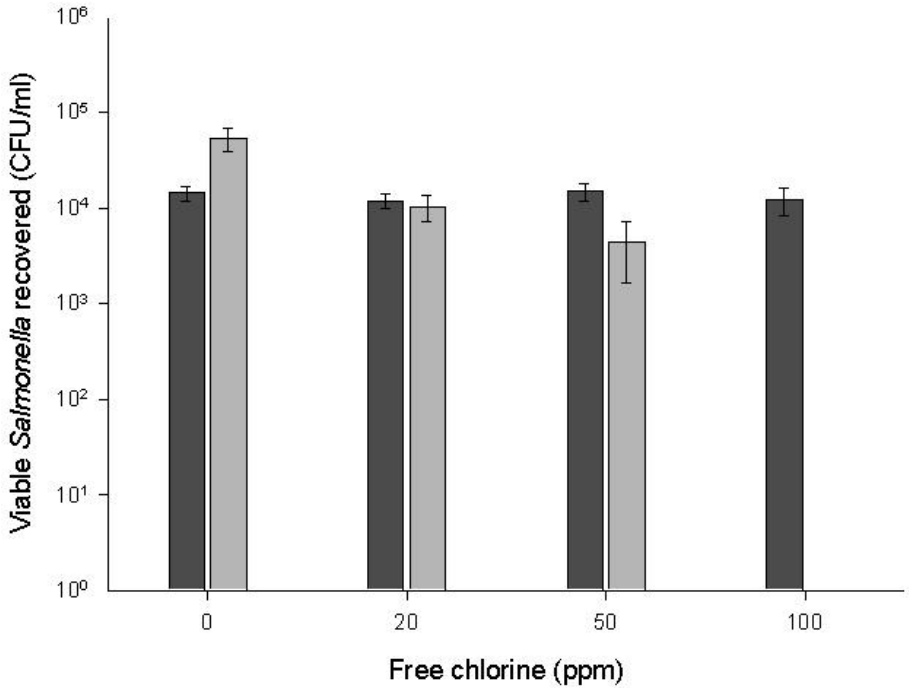


Figure 40: Numbers of viable Salmonella *recovered from spinach leaves following wash treatments and enumerated by DVC (dark grey bars) or plate count (pale grey bars). Error bars represent ± standard error of the mean of six experiments.*

For some samples, a resuscitation step was included in the culture procedure, involving pre-culture of the membrane filters on Tetrathionate medium-soaked glass wool pads for 24 hours at 37 °C before transfer to the agar medium (Horan *et al*., 2004) This additional

step did not recover significantly more *Salmonella* than plate-count enumeration alone in any of the samples tested (data not shown).

6.3.1.2 Citrox and antifoam

Citrox was assessed for its biocidal activity against spinach-associated *S. thompson* at relative concentrations up to twice the dosage used at industrial scale. Figure 41 presents DVC values obtained for samples washed in each solution alongside their corresponding plate counts. As observed when hypochlorite solutions were tested, there was no significant difference between the DVC values for any of the concentrations tested (one-way ANOVA, $p>0.05$, d.f. = 4,24).

Plate count data showed a significant drop in culturable *Salmonella* with each incremental chlorine concentration (one-way ANOVA, $p<0.01$, d.f. = 4,24) except for 1 to 1.5, where the change was not significant. The change from 0 (water) and 0.5 relative concentration was the largest incremental decrease in culturable *Salmonella* as the Citrox concentration was increased. Overall, even when double the Citrox concentration used at industrial scale was tested, plate counts showed a drop in culturable *Salmonella* of less than 2-log.

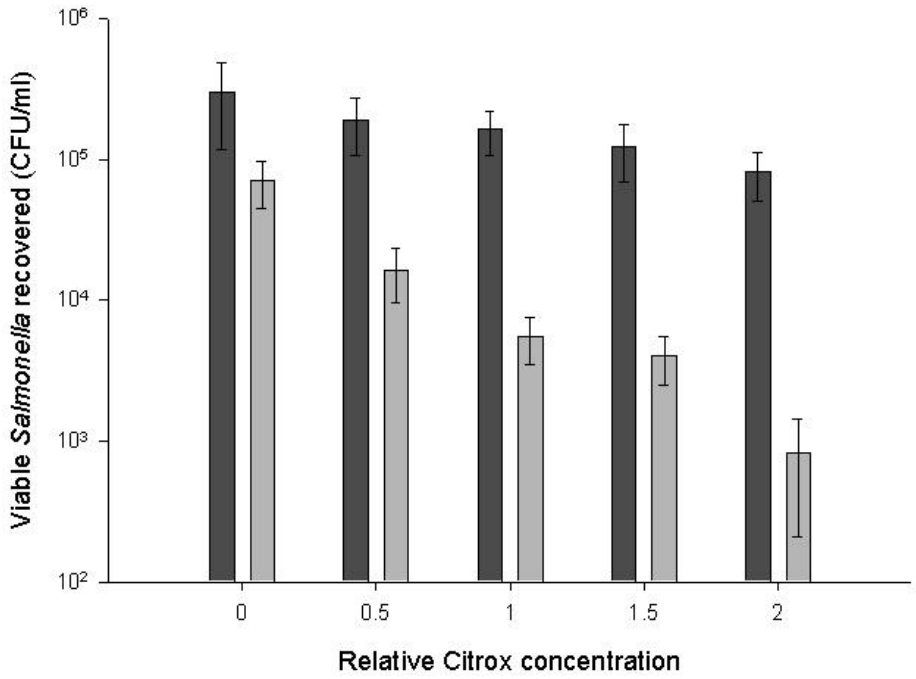


Figure 41: Salmonella recovered and enumerated on filters by DVC counts (shaded bars) and by counts on Rambach agar (blank bars) following 2 minute washes in Citrox 14W solutions plus Antifoam, of differing concentrations relative to that used on spinach at industrial scale, or in sterile distilled water (relative concentration of 0). Error bars represent ± standard error of the mean of six experiments.

Antifoam was a necessary addition to the wash process, firstly in order to replicate the wash conditions used at Vitacress Salads limited which formed a reference point for the trial of Citrox, and secondly as preliminary trials indicated that even small amounts of Citrox residue on leaf samples caused foaming during processing with the Pulsifier. In order to verify that Antifoam did not have any biocidal or VNC-inducing effects itself, samples were washed in Antifoam alone at the same doses used in conjunction with Citrox. Figure 42 shows DVC and plate count data following Antifoam wash treatments; neither the DVC data nor the plate counts showed a change with increasing Antifoam concentration (one-way ANOVA; $p>0.05$, d.f. = 4,24). In addition, plate counts and DVC values did not differ significantly from one another at any of the Antifoam concentrations tested (two-way ANOVA, $p>0.05$, d.f. =1,32). Thus the differences seen in the data in Figure 42 can be attributed to Citrox alone.

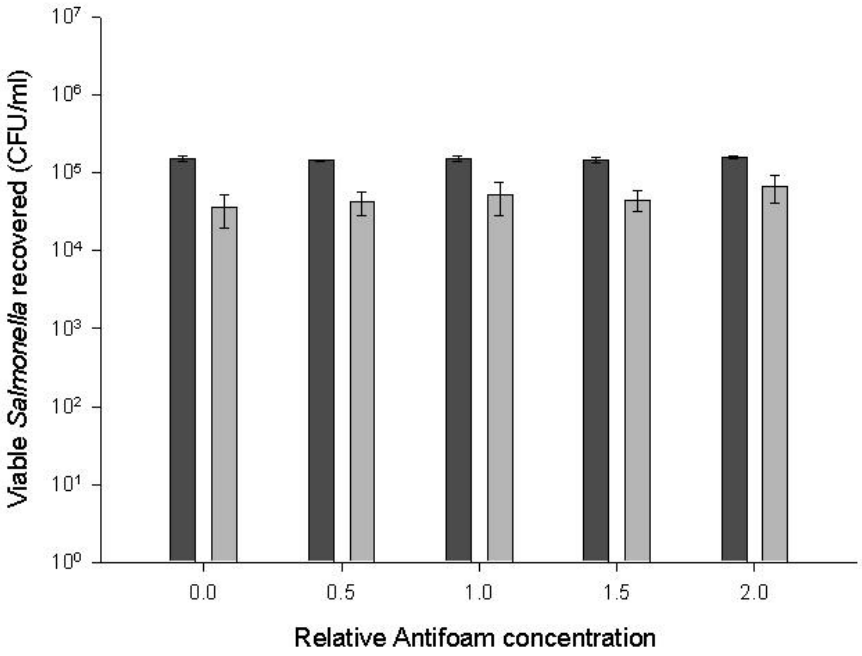


Figure 42: Salmonella recovered and enumerated on filters by DVC counts (shaded bars) and by counts on Rambach agar (blank bars) following 2 minute washes in differing Antifoam concentrations relative to that used on spinach at industrial scale, or in sterile distilled water (relative concentration of 0). Error bars represent ± standard error of the mean of three experiments.

6.3.2 Determination of VNC populations following sanitisation

The proportion of *Salmonella* cells recovered from leaf samples in the VNC state following chemical sanitisation was estimated by subtracting plate counts from DVC data and expressing the result as a percentage of the DVC value. Table 8 presents VNC estimates for all chlorine and Citrox concentrations tested in this study, alongside the percentage of viable *Salmonella* cells recovered as determined by DVC count. In both sets of experiments it is interesting to note the low viability of *Salmonella* cells recovered from control samples, which were washed in water with no added biocides, which was 57.3 % in the Citrox experiments and just 31.7 % in the hypochlorite experiments. Water-washed samples examined during hypochlorite experiments showed markedly higher variation between replicate samples than any of the chlorine concentrations tested.

Table 8: Percentage viability of Salmonella recovered following wash treatments of 25 g spinach samples as determined by Direct Viable Count. Values in parentheses are ± standard error of the mean of six experiments. Also included is the percentage of the recovered population estimated to be in the VNC state, calculated by comparing DVC counts with plate counts.

Wash treatment	% viability of recovered *Salmonella* cells detected using DVC	% estimated to be VNC
Chorine (ppm)		
0	31.7 (±9.0)	0
20	18.2 (±4.7)	30.5
50	14.8 (±3.7)	70.6
100	9.9 (±2.0)	100.0
Citrox and Antifoam (relative)		
0	57.3 (±15.0)	50.3
0.5	53.0 (±15.0)	77.7
1	53.3 (±15.9)	88.7
1.5	37.6 (±16.6)	92.8
2	42.3 (±26.3)	99.5

The percentage survival declined with increasing chlorine concentration, despite the viable count remaining unchanged (see Figure 40) due to the fact that at higher chlorine concentrations, a greater number of dead cells were observed on the filters. A similar phenomenon occurred at the two highest Citrox concentrations tested. The same two Citrox concentrations also showed a markedly higher variation between replicate samples than the two lower concentrations, although the variation was still less than observed between water controls.

When examining the estimates for the presence of VNC *Salmonella* amongst the cells recovered following leaf washing, the results following hypochlorite treatment were striking. Whereas the number of culturable cells equalled the number of viable cells determined by DVC when eaves were washed in water, increasing the concentration of free chlorine caused a progressively higher proportion of the cells to become VNC. At 100 ppm chlorine, plate counts indicated zero viable *Salmonella*, whereas DVC counts did not, showing that 100 % of the viable *Salmonella* cells recovered were in a VNC state. Conversely, water-washed samples analysed during Citrox experiments did not contain 100 % culturable cells; more than half the *Salmonella* population entered the VNC state without the addition of Citrox. Despite this, Citrox can still be seen to have generated more VNC *Salmonella* cells with increasing concentration. At the highest Citrox concentration examined, almost 100 % of *Salmonella* recovered were in a VNC state.

6.3.3 Chlorine and stomatal closure

EDIC microscopy was used to determine the numbers of open and closed stomata on spinach phylloplanes following immersion in hypochlorite solutions of differing concentrations. Figure 43 presents open and closed stomata as they are typically encountered when viewing spinach leaf surfaces using EDIC microscopy. The resolution of surface imaging achieved by the microscope ensured clear distinction between open and closed stomata; the open aperture in Figure 43A is typical of the degree of opening observed, on occasion stomatal apertures up to twice as wide were observed. When comparing stomatal closure in response to hypochlorite solutions, only stomata which were fully closed along the entire length of the aperture, as in Figure 43B, were recorded as ‘closed’; any degree of partial opening was recorded as ‘open’.

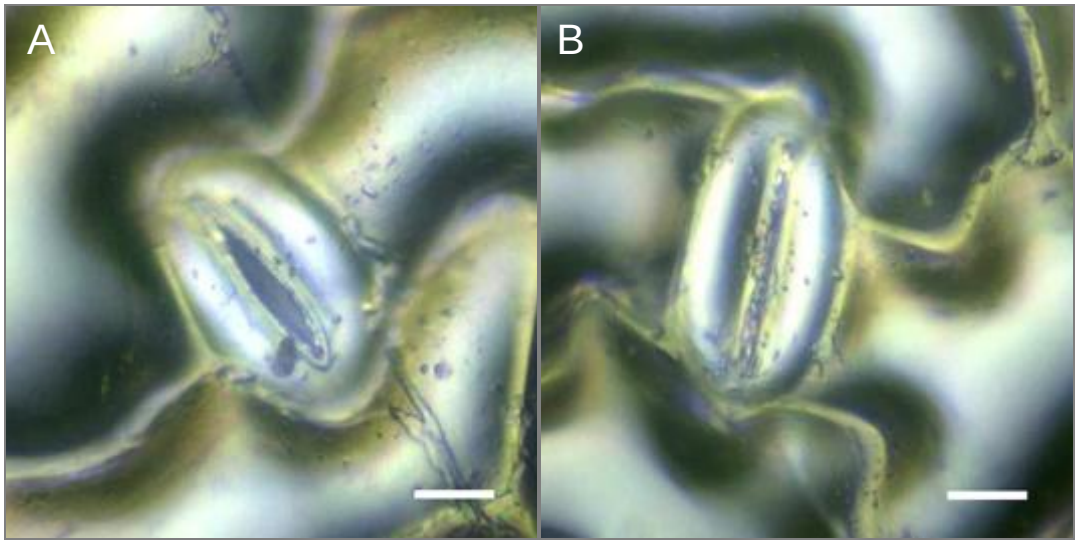


Figure 43: EDIC micrographs of open (A) and closed (B) stomata observed on spinach lower leaf surfaces. Bar = 10 µm.

Before application of hypochlorite solutions, on average 12.7 % of stomata were observed to be closed. The data in Table 9 clearly indicated that the proportion of closed stomata increased as hypochlorite solutions containing both 50 and 100 ppm chlorine were applied for increasing lengths of time.

A concentration of 50 ppm produced the greatest initial change in stomatal closure: an increase to 44.1% compared to 27.8 % with 100 ppm chlorine. After incubation for 60 min, significantly more stomata were closed in response to 100 ppm than 50 ppm (two way ANOVA, $p < 0.05$, d.f. = 6,35), although at 5 and 30 min the two solutions did not produce significantly different results from one another. The higher chlorine concentration produced an initially smaller, yet more consistent, increase in stomatal closure in the first 30 min of the time course before appearing to plateau between 30 and 60 min; stomatal closure appeared to fluctuate in the 50 ppm samples at 5 and 30 min, before increasing significantly between 30 and 60 min (two way ANOVA, $p < 0.05$, d.f. = 6,35).

Table 9: Percentage of stomata on spinach leaf surfaces observed to be closed following immersion in hypochlorite solutions of either 50 or 100 ppm free chlorine. Values in parentheses indicate ± standard error of the mean of three experiments.

Exposure time (min)	Percentage of stomata closed	
	50 ppm chlorine	100 ppm chlorine
0	12.7 (±2.3)	
5	44.1 (±4.2)	27.8 (±3.1)
30	34.8 (±4.4)	49.5 (±3.8)
60	45.9 (±5.0)	58.7 (±6.4)

6.4 Discussion

6.4.1 Efficacy of biocides against phylloplane-associated *S. thompson*.

DVC counts of viable *Salmonella* recovered from spinach after washing in chlorine indicate that chlorine is not a significantly more effective biocide than water for decontaminating spinach leaves, even at a concentration of 100 ppm. Other studies have reported that chlorine is ineffective against *Listeria monocytogenes* (Beuchat 2002) as well as mixed spoilage bacteria on lettuce (Garcia *et al.* 2003). Percentage viability of recovered cells determined by DVC reduced with an increase in chlorine concentration; viable counts themselves did not differ significantly. This suggests that increasing chlorine concentrations improved the recovery of dead cells from leaf surfaces. From a food safety perspective this is not beneficial; however, it suggests that the act of rendering cells non-viable has the additional effect of reducing their adhesion to leaf surfaces. If the primary method of attachment was physical adhesion by exopolymer secretion, then this would be unlikely to occur as chlorine is not effective at breaking down biofilm (De Beer *et al.* 1994; Lapidot *et al.* 2006). What EDIC microscopy did show was that hypochlorite treatment did not seem to inflict damage upon the leaf; trials have indicated that ozone solutions caused structural damage and discoloration to watercress leaves (Sihota 2008), both reducing the shelf life of leaves and making them visually unappealing.

A greater number of non-viable *Salmonella* were recovered from leaves as the chlorine concentration of the wash water was increased; this caused a decrease in the percentage viability of the recovered cells. This, despite the fact that the absolute numbers of live cells recovered did not change across the treatment groups. It is possible that the attachment of dead cells to leaf surfaces differs at the molecular level from that of live cells, or is stronger in metabolically active cells, and that the oxidising power of chlorine is able to weaken the attachment of non-viable cells, aiding their removal using the Pulsifier. As yet, the exact mechanism of *Salmonella* attachment to leaf surfaces is currently unknown, though mechanisms have been proposed. Barak *et al.* (2005) discovered that virulence genes were necessary for attachment to plant tissue

Citrox was shown by DVC count to be an ineffective biocide, even at twice the concentration used in industry to sanitise salad leaves. Washing in water was demonstrated, in this investigation, to be at least as effective as either Citrox or hypochlorite at reducing viable *Salmonella* on spinach. In addition to the lack of any strong biocidal effect demonstrated both in this study and in previous studies (Foley *et al.* 2002; Koivunen and Heinonen-Tanski 2005), the use of chlorine solutions on an industrial scale forms by-products which can be hazardous to produce workers and the environment (Hery *et al.* 1998; Komulainen 2004). This study therefore suggests that if produce is grown to high microbiological standards before processing, it is unnecessary to add chemicals to wash water during processing particularly if such chemicals pose a risk to workers and require removal from wastewater in order to protect the surrounding environment.

6.4.2 Induction of the VNC state by chemical biocides

The proportion of *Salmonella* estimated to be VNC following chlorine or Citrox washing of spinach was not only greater than washing in water alone, but also increased with increasing chlorine concentration. Rambach agar is a chromogenic medium designed to selectively allow the growth of *Salmonella* species recovered from environmental samples. However, following disinfection of spinach leaves with 100 ppm chlorine, no *Salmonella* grew on Rambach agar plates, whilst DVC counts of the same sample revealed 1×10^4 viable cells/ml. This has extremely serious implications for the food industry as a false negative result from *Salmonella* quality control procedures poses a risk to consumers. The

use of DVC in conjunction with GFP reporter technology in this study is an emerging technique which is proving instrumental in studying the VNC state of pathogens in complex environmental samples (Cho and Kim 1999; Na *et al.* 2006). *S. thompson* GFP produced a consistent degree of elongation in live cells, allowing for manual discrimination of live versus dead cells; control samples also showed no spontaneous elongations, increasing the accuracy of the assay. DVC is increasingly being used in a variety of systems to detect VNC populations; cell elongation can be combined with fluorescent nucleic acid stains (Kogure *et al.* 1979; Besnard *et al.* 2000) and also in combination with FISH techniques (Armisen and Servais 2004; Moreno *et al.* 2007).

The UK Health Protection Agency's standard methods for the recovery and enumeration of *Salmonella* from environmental samples specify a selective enrichment step involving incubation of *Salmonella* on filters with Tetrathionate-novobiocin broth (Health Protection Agency 2007b). The guidelines do not specify how many *Salmonella* species were used to validate the methods, nor do they specify any known exceptions. When resuscitation was applied to samples during this investigation, fewer viable *Salmonella* were enumerated per ml than when it was not used. Therefore, either the enrichment medium or the protocol had a negative effect upon the growth of *Salmonella thompson*. This calls into question the validity of a Government–produced standard method described as suitable in general for enumerating *Salmonella* strains. Tetrathionate broth enrichment has previously been independently validated for a number of clinical *Salmonella* serovars including *Salmonella typhimurium* and *Salmonella enteritidis* (Carli *et al.* 2001), however no published *Salmonella thompson* validation data currently exists.

The ability of zoonotic pathogens to become VNC on the salad leaf phylloplane, especially when stressed by the very sanitisers recommended to kill them, creates a public health concern. There have been numerous outbreaks of foodborne disease linked to the consumption of fresh produce, in particular the recent outbreaks of *Salmonella*, including serotype Thompson, in Western Europe and verocytotoxigenic *E. coli* O157 in the USA (CDC, 2006a,b; Takkinen *et al*, 2005; Nygard *et al*., 2008). It is not unreasonable to assume that in the majority of cases the produce was properly washed with appropriate sanitisers during processing and bagging procedures. In many outbreaks, tracing the source of the outbreak required professional epidemiological surveillance, as the pathogen was undetectable in suspected batches of produce. It was considered that the numbers of bacterial cells present in a sample were either too low for the detection method employed or that the contamination was non-homogeneously distributed and only 'clean' samples

were examined. However, the present study indicates that a third potential reason for this inability to detect the pathogenic agent could be the presence of pathogenic VNC material which was not discovered using standard isolation procedures.

In order make detection of pathogens in food more rapid and accurate, many have advocated moving to molecular detection methods such as PCR and qPCR (Hill 1996; Malorny *et al.* 2003). However, these techniques suffer serious disadvantages such as insensitivity and inhibition in complex environmental samples, along with their inability to resolve viable from non-viable cells (Keevil 2001). The DVC method used here with the GFP reporter is ideal for laboratory studies; additionally, while the modification of the DVC method using FISH to enhance detection of metabolically active elongated cells has proven successful to detect VNC *E. coli* in complex environmental samples (Juhna *et al.* 2007a). These technologies should be seriously considered for routine diagnostic use by the food industry to help avoid future foodborne outbreaks of disease.

6.4.3 Chlorine and induction of stomatal closure

Stomatal penetration of fresh leafy produce by enteric pathogens poses a definite risk to consumers as the leaf endosphere offers both a refuge from many stresses and in many ways a more favourable environment for population growth than the phylloplane (Rosenblueth and Martinez-Romero 2006). In Chapter 5, stomatal penetration by *S. thompson* GFP was observed; this chapter considered whether biocide could influence stomatal closure and hence potentially alter the dynamics of endosphere-phylloplane bacterial migration.

Bacterial cells contained within the endosphere are shielded from many environmental stresses, most notably drought stress (Hardoim *et al.* 2008). In addition, virulent plant pathogens are known to form reservoirs of cells in the leaf interior prior to pathogenic attack (Beattie and Lindow 1999). During fresh produce processing, stomatal penetration is likely to afford pathogens a high degree of protection from both commercial biocides and shear stress induced mechanical detachment. This protection would likely be enhanced by the closure of stomata during such operations as it would lead to effective sealing of pathogenic bacterial cells inside the leaf. The data presented here clearly indicates that immersion of spinach leaves in hypochlorite solutions significantly increased the proportion of closed stomata on the leaf surface. However, 30-60 minutes of incubation

were needed to achieve this change – far longer than leaves would be sanitised for in industrial food-processing practice. Due to the relative size of bacteria cells, around 1-2 µm, and stomatal openings, approximately 20 × 5 µm, for the purposes of this study partial closure of stomata were designated as 'open'; only a completely sealed aperture represents a barrier to bacterial entry/egress. Stomata are known to close as part a plant's inducible defences against pathogenesis (Melotto *et al.* 2006) and also in response to drought (Neill *et al.* 2008) and to oxidative stress (McAinsh *et al.* 1996). The weak response to the presence of free chlorine may be due to the fact that plants require an endogenous oxidative signal to activate the stomatal closure pathway via abscisic acid signalling (Neill *et al.* 2002a). Alternatively, it may be a further indicator of the weakly biocidal effect of hypochlorite when applied to the leaf phylloplane, in turn suggesting that chlorine's oxidative properties are lessened on contact with the leaf ecosystem.

An interesting finding of this study was that a physiological change in stomatal closure activity was observed in cut leaves over and above that which would be expected from normal spoilage or senescence. Such a finding is a reminder that the phylloplane differs markedly from abiotic surfaces and that generalisations cannot be made when investigating microbial surface life. In addition, the producers of processed fresh produce should be aware that consideration of the physiological changes in fruit and vegetable products should not be limited to consideration of spoilage and shelf life; the responsiveness of cut leaves to external conditions may also impact the survival and growth of immigrant enteric pathogens.

6.4.4 Conclusions

Chlorine-based sanitisation of fresh produce is widely applied by industry throughout the world, yet this study has demonstrated two compelling arguments against its use. Firstly, hypochlorite, the most popular chlorine formulation, is an ineffective biocide against leaf-associated *Salmonella*; in fact, washing in water alone produced the same level of bacterial destruction as the levels of hypochlorite examined. Secondly, hypochlorite was demonstrated to cause *Salmonella* on spinach to enter a VNC state, to the point where detection of viable *Salmonella* was entirely masked using standard diagnostic culture techniques.

The novel fruit-acid based sanitiser Citrox has been marketed as an alternative to chlorine-based disinfectants for use on both surfaces associated with handling food produce and on produce itself. Unlike chlorine-based disinfectants, Citrox claims not to produce by-products hazardous to workers; however, as observed with hypochlorite, Citrox solutions were not more effective at killing *Salmonella* than water alone. More concerning still was the ability of Citrox to induce the VNC state, which masked 99.5 % of viable *Salmonella* from detection on selective agar at the highest concentration examined.

The induction of the VNC state in pathogenic bacteria on salad leaves as caused by two biocides with differing modes of action has serious implications for the food industry; this study has shown that it can be induced by of the very sanitising procedures designed to ensure safe produce. Pathogens in the VNC state escape detection by conventional methods, so foods could falsely be declared free of viable pathogens, and hence could a risk to consumers. Viability determination by DVC count, as employed during this study, has the potential to provide a more accurate alternative to plate counts for routine quality control in the food industry and for the evaluation of novel sanitising agents used to sanitise fresh produce.

This study suggests that until an effective biocide which acts against enteric pathogens on leaf phylloplane is discovered, it is likely to be safer to wash produce in water alone. A process involving only water-washing of leaves would eliminate the masking of viable cells on agar media due to VNC, and additionally would result in fewer hazards to workers and the environment from chemical breakdown products. Better detection methods are urgently needed to guarantee product safety; until such methods become routine the use of substances which are shown to become VNC should be avoided.

CHAPTER 7

GENERAL DISCUSSION

Though EDIC microscopy has been used to study microbial surface life in a variety of systems, this investigation has been the first to evaluate the technology for the study of plant leaf surfaces. EDIC and EDIC/EF are already known to be rapid and non-destructive techniques for examining complex, opaque samples without the need for sectioning, fixation or dehydration; attributes ideally suited for studying the dynamic and fragile phylloplane environment.

In contrast to SEM, leaf samples required no preparation prior to examination using EDIC microscopy. Using the EDIC system allowed images to be captured within just a few minutes of commencing sample analysis, allowing a higher sample throughput than is typically associated with more complex SEM and CLSM techniques. Use of an EDIC microscope enables more rapid surface imaging than both SEM and CLSM; in addition, it benefits from lower capital outlay and running costs than either of the latter two systems. Sample preparation is typically minimal or absent prior to EDIC microscopy due to its use of non-contact lenses removing the need for mounting or coverslips. The ability to perform multiple image capture in the *z*-plane was highly beneficial for resolving the complex, uneven topography typical of plant leaves. EDIC microscopy has also shown to be useful in assessing the effects of chemical biocides on leaf physiology, as demonstrated by the assessment of stomatal closure in spinach in response to chlorine washing. Being able to monitor the condition of the leaves themselves following sanitising treatments could lead to improvements in produce shelf life through reduction of tissue damage, which can otherwise promote the growth of spoilage microorganism in response to nutrient leakage.

Whilst SEM can attain a level of magnification far in excess of the capabilities of EDIC microscopy, for the study of microbial ecology of the phylloplane it is useful to be able to put bacterial colonisation patterns into context using key leaf structural features such as veins, trichomes and stomata. SEM is necessary for the visualisation of the wax crystalline structure of the leaf cuticle; however, at higher magnification such topographical points of reference become less evident. EDIC microscopy can resolve single microbial cells more rapidly and is perhaps more applicable than SEM for the ecological analysis of colonisation and survival within the phylloplane ecosystem. Critically, microbial biofilms can be observed *in situ* without the creation of structural artefacts through dehydration, as occurs prior to SEM (Keevil 2003). A growing body of evidence suggests that biofilms are an important part of phylloplane microbial communities (Morris and Monier 2003; Aruscavage *et al.* 2006), and so EDIC microscopy is well suited to further study in this field.

The development of EDIC/EF techniques for the specific identification of microbes on leaf surfaces was a complex task due to the nature of the leaf as a surface. Nucleic acid staining, a popular technique in microbiology, had to be carefully optimised to minimise staining of the nucleic acids in the leaf itself, which produced a strong background fluorescence signal obscuring the presence of surface microorganisms. In addition, fluorescence emission by leaf pigments, most notably red fluorescence produced by chlorophyll, necessitated careful selection of fluorophores. Despite such potential obstacles, leaf surface bacteria were successfully stained with DAPI and resolved to the single-cell level on five different types of salad leaf, demonstrating a robust technique for the *in situ* quantitation of total bacterial load on leaf phylloplanes. Previous studies have used Acridine Orange to stain phylloplane bacteria both *in situ* on leaves (Morris *et al.* 1997) and following recovery using conventional epifluorescence microscopy; however, the resolution achieved was markedly lower that achieved during this investigation using DAPI staining viewed with EDIC/EF. Key to the success of DAPI staining was its reproducibility; during the evaluation of potential viability indicators for *in situ* use of leaves, SYTOX Orange at times showed excellent resolution but the low reproducibility the technique suggested that a great deal of further optimisation would be necessary before a reliable viability assay could be produced.

The chief limitation of the EDIC/EF system is that unlike CLSM it cannot be used to detect subsurface cells. However, given the complexity of the leaf matrix, the resolution attainable when examining endophytic bacteria is limited due to the difficulty in differentially labelling plant and microbial cellular material. In a study by Brandl and Mandrell (2002), leaf structural element were visualised by laser excitation of chlorophyll, allowing spatial localisation of subsurface *Salmonella* relative to their environment. For the purposes if this study, however, subsurface penetration by *Salmonella* was demonstrated using EDIC/EF of stomatal opening inside which immigrant *Salmonella* could be clearly observed. Thus, EDIC and EDIC/EF are sufficient to demonstrate whether stomatal penetration is taking place as part of bacterial leaf colonisation, an important ecological consideration given the favourability of subsurface penetration as a survival strategy.

Having developed an *in situ* assay for quantifying total phylloplane bacteria, this project aimed to demonstrate the value of *in situ* detection in phylloplane microbiology by comparing the staining protocol developed in this study with a traditional culture-recovery method. R2A medium designed for the water industry, but its design for resuscitation of stressed microorganisms makes it a suitable medium. Culture media typically used for the

isolation of clinically significant bacteria may not be appropriate for use in phylloplane microbiology due to their being nutrient rich and designed for bacteria to be grown at higher incubation temperatures than phylloplane bacteria would typically encounter. As expected, *in situ* quantification produced significantly higher estimates of phylloplane populations than recovery and culture, highlighting the inaccuracies inherent in the latter through inefficient recovery and limited culturability of bacterial cells. However, in order to quantify the extent of the inaccuracy of culture more precisely, an *in situ* viability assay would be needed to enable like-for-like comparison of viable populations.

EDIC and EDIC/EF were used to examine the spatial pattering of leaf-associated microflora. Despite its hostility as a habitat, the plant leaf surface is host to dense populations of bacteria; when leaves are examined using EDIC microscopy, contamination can be identified on all parts of the leaf surface, particularly leaf veins, epidermal cell margins, stomata and, where present, trichomes. Similar observations have previously been made using SEM and epifluorescence techniques (Morris *et al.* 1997; Monier and Lindow 2004; Rayner *et al.* 2004), validating the observations made in this study. When performing this analysis it is important to be able to distinguish bacterial cells on leaves from the surrounding debris; this was achieved using DAPI staining. These techniques were also employed to assess the efficiency of the Pulsifier for the detachment of bacteria from leaf surfaces. Previous work had shown the Pulsifier to be at least as effective as the laboratory standard Stomacher blender (Fung *et al.* 1998; Sihota 2008), whilst producing clearer suspensions of microorganisms and causing less leaf damage (Sihota 2008). Previous studies have focussed on efficiency of the Pulsifier at removing pathogens or indicators species such as faecal coliforms from food matrices; by examining the effect on total bacterial removal, this study has also considered the potential of the technology of ecological studies such as microbial community analysis. Efficiency of cell removal was improved when single leaves were pulsified, so studies not limited by the standard 1:10 (w/v) ratio of sample to diluent (International Organisation for Standardization 2007) may benefit from enhanced efficiency of the Pulsifier. Given, though, that recovery from leaves was shown to still be typically only 50 % of surface bacteria when leaves were treated singly, there may be potential for aurther optimisation of the technology for ecological applications.

A further bacterial survival strategy characterised in other systems is aggregation and the formation of biofilms (Donlan 2002). Three dimensional aggregates of bacteria observed in this study using EDIC/EF microscopy, as were putative secreted exopolysaccharides possessing a slimy appearance; both of these features are indicative of biofilm formation (Donlan 2002; Dunne 2002). Biofilms on leaves have been inferred from population size and stability (Jacques *et al.* 2005), while dehydrated biofilms have been directly visualised on leaves using SEM (Carmichael *et al.* 1999). In addition to providing physical adhesion to the leaf surface, biofilms could be key in bacterial resistance to desiccation stress in the phyllosphere (Morris and Monier 2003), and may potentially harbour non-indigenous microflora such as human enteric pathogens.

It was interesting to note that different leaf species grown under similar conditions displayed significantly different total bacterial loads. The two species which have the strongest flavours, rocket and watercress, also had the lowest bacterial loads, supporting the notion that plant volatiles may have an antimicrobial effect; this has been demonstrated *in vitro* using a number of plant essential oils (Gutierrez *et al.* 2008). A novel way to complement existing processes used reduce the risk of foodborne pathogens may therefore be careful choice of leaf species which are used to make up pre-packaged salad products. However, when this was explored in relation to anthocyanin content in red and green cos leaves, there was no significant difference in bacterial load, despite anthocyanins having known antimicrobial activity *in vitro* (Kahkonen *et al.* 1999). Compartmentalisation of such compounds within the plant cell vacuole is likely to protect epiphytic microorganisms from antimicrobial compounds located within the leaf; further study using artificially induced wounding would demonstrate whether vacuolar contents had an antimicrobial effect when leached onto the surface.

This PhD project incorporated within it the first use of GFP reporter technology in conjunction with EDIC/EF microscopy and as such led to the first published study combining these techniques. GFP-labelled *Salmonella thompson* produced a useful assay for pathogen tracking within the phyllosphere in this and previous studies (Brandl and Mandrell 2002), as there was no need to use fluorescent stains or dyes which may have cross-reacted with the leaf itself. When *S. thompson* GFP cells were inoculated onto spinach leaves, they showed a strong association with leaf microflora, appearing to prefer the cell margins and veins where the highest concentration of indigenous bacteria were already resident. Association with existing microflora may improve the survival of immigrant *Salmonella*; immigrant syringes cells show enhanced survival if they colonise

regions already occupied by resident bacteria aggregates (Monier and Lindow 2005a). Opportunistic incorporation into an existing biofilm could provide shielding from environmental stresses, particularly desiccation, which accounts for a great deal of bacterial mortality in the ours following immigration onto a leaf (Hirano and Upper 2000).

Stomatal penetration by *Salmonella* was observed during this study; inoculated *S. thompson* GFP was identified within open stomatal pores. This conclusion is in agreement with a previous study using CLSM to examine the interior of cilantro leaves (Brandl and Mandrell 2002), which also identified bacterial cells within the substomatal cavity. EDIC micrographs of leaf surfaces revealed putative bacterial cells and exopolysaccharide secretion present on stomatal guard cells and within stomatal openings; their proximity to the stomatal pore was indicative of stomatal penetration. Penetration of the leaf interior provides physical shielding from solar UV radiation; in addition the internal environment provides a higher level of relative humidity and greater nutrient availability than the leaf surface (Hirano and Upper 2000). It has previously been proposed that for some plant-pathogenic bacteria the endophytic population could be the primary reservoir of inoculum (Manceau and Kasempour 2002). However little is known about the incidence of stomatal penetration amongst non-pathogenic phyllosphere bacteria.

Curli played a prominent role in attachment of *S. typhimurium* to polystyrene ; the correlative effect between curli production and surface attachment suggests that curli production is the predominant method of attachment. However, no such correlation was present during attachment to spinach leaves. Clearly bacterial perception of the surface occurs differently following arrival on a leaf compared to polystyrene, leading to different phenotypic states in each system. Expansion of this work to include other abiotic and biotic surfaces would determine whether the difference is a broad distinction between perception of abiotic and biotic surfaces, or whether a spectrum of highly tailored responses are produced in response to perception of the nature of the surface.

The cell elongation assay was originally developed for direct microscopic analysis of cell viability in marine bacteria (Kogure *et al.* 1979). The technique has since been applied in other contexts, such as monitoring of potable water distribution systems (Juhna *et al.* 2007b) and, in this project, to the monitoring of pathogens on a food matrix.

This study demonstrates that *Salmonella thompson* becomes VNC on spinach leaves in response to chlorine washing; in addition, the proportion of the population that enter a VNC state increases with chlorine concentration. This has serious implications for food

safety, as industrial quality control of fresh produce is reliant on culture-based detection and enumeration of enteric pathogens. DVC-based experimentation indicated that, when attached to spinach leaves, *Salmonella thompson* is resistant to sanitisation using water, sodium hypochlorite and Citrox. This suggests that the use of chlorine in food disinfection may appear to be effective, when in fact its biocidal effects are exaggerated by induction of the VNC state. Citrox was also found to be an ineffective biocide against spinach-associated S. *thompson* but, more importantly, was also found to induce a VNC state in the cells. This work suggests that current techniques for washing pre-packaged, ready-to-eat salads are not completely effective, and their evaluation is hindered by a reliance on culture-based detection and enumeration. Leaf-attached enteric pathogens such as *Salmonella* on leaves may interact with biofilms indigenous microflora to receive protection from both chemical and mechanical disinfection. Chemical induction of the VNC state causes plate counts to under-report the number of viable *Salmonella* recovered from leaves, posing a potential risk to consumers of chlorine washed fresh salads. DVC is an effective technique as it determines viability of sublethally stressed cells without the need for the high growth rate needed to form visible colonies on agar media, yet still uses routine reagents and equipment to achieve this sensitivity, so has more potential for use in high throughput analysis than more expensive and technically challenging molecular methods.

Use of cell elongation and DVC *in situ* on leaves difficult to interpret due to shielding effects of leaf surface topography, soil particles or by 3D aggregations, all of which could cause elongated cells to overlap and intertwine. In addition, within biofilms the extent of elongation may be limited by biofilm matrix components. Consequently, the determination of viability in leaf-associated *S. thompson* GFP in this study necessitated recovery from the leaf surface. However, even within the limits of recovery DVC was demonstrated to be significantly more sensitive than plate counts at enumerating stressed cells. Recovery-based methodologies are likely to remain a prominent part of food analysis; in addition to being necessary for plate counts, recovery is required for molecular applications where organic matter may inhibit the reaction or affect the results. Similarly it greatly increases the amount of material that can be sampled in a single test, an important consideration in routine monitoring.

Further work

Techniques for the tracking of *Salmonella* using GFP reporter technology and EDIC/EF microscopy could be applied to the field of plant pathology to track the progression of pathogenesis in foliar bacterial plant pathogens. could be expanded to the tracking the progression of bacterial plant pathogens during infection, data which could benefit the agricultural industry by enabling disease control measures to be more efficiently targeted.

This study has developed an inexpensive and rapid assay for the laboratory validation of biocidal activity in novel produce sanitisers, using a combination of DVC and conventional plate count techniques, to include the likelihood of causing bacteria to become VNC. This could be now be applied to new sanitising methodologies entering the food industry. EDIC microscopy techniques used in the project could also rapidly screen for damage to leaf tussle of the crop itself, at the cellular level which is invisible to the naked eye but could have a detrimental effect on shelf life by causing nutrient leakage promoting the proliferation of spoilage bacteria and improving the chances of any surviving pathogens.

Further work to optimise an *in situ* viability assay for use on phylloplanes would have a variety of applications. Firstly, it would enable fundamental questions to be answered concerning the relationship between spatial localisation and survival in the phylloplane. In addition, efficacy of biocides against leaf-associated pathogens and the protective effect of the leaf topography could be elucidated.

Having established significant variation in total microbial load on salad leaves grown under the same conditions, it would be interesting to examine the variation in bacterial community structure amongst different salad leaf species. Differences in community diversity between leaf species could be indicative of differences between the physiochemistry of the leaf surface or could indicate differences in host response to bacterial colonisation. Community profiling using PCR-DGGE has previously been used for analysis of phylloplane communities of a number of crops, but at present little data exists for raw salad products.

The limitations of crop growing seasons forced the use of field-grown crops from multiple countries of origin in order to maximise experimentation time during the project. An interesting further study would be to compare the phenotypic characteristics of the same leafy cultivars grown in different parts of the world, particularly with respect to their ability to support total microbial load and also the growth of key enteric pathogens. This

may ultimately allow modelling of microbial load in relation to local climate conditions and help determine whether produce of differing countries of origin has a variation in quality and safety beyond that caused by differences in farming practice.

Two further projects have arisen in connection with the work undertaken during this PhD project. The first was a BBSRC Knowledge Transfer Partnership in conjunction with Vitacress salads Ltd, exploring the potential to exploit the processes of the Pulsifier on a larger scale to produce energised wash water system for chemical-free sanitisation of leafy salad crops prior to packaging. Secondly, a BBSRC PhD studentship has commenced exploring the role of nitric oxide, obtained on the strength of the techniques developed during this project. The project will also further consider the methods of attachment of salmonella to salad leaf surfaces, including assessment of the role of flagella.

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APPENDIX 1

Table A.1 Reported fresh-produce related outbreaks of Salmonella, Escherichia coli *and* Campylobacter, *1996 onwards and viral gastroenteritis, 1990 onwards (Heaton and Jones 2008).*

Year	Pathogen	Vehicle	Reference
1997	*Salmonella* Enteritidis	Cauliflower	(Anon. 2005a)
1997	*S.* Enteritidis PT4	Pepper	(Anon. 2005a)
1997	*S.* Meleagridis	Sprouted seeds (alfalfa)	(Taormina *et al.* 1999)
1997	*S.* Infantis/S. Anatum	Sprouted seeds (alfalfa and mung bean)	(Taormina *et al.* 1999)
1997	*S.* Saphra	Cantaloupe	(Sivapalasingam *et al.* 2004)
1997–8	*S.* Senftenberg	Sprouted seeds (clover and alfalfa)	(Taormina *et al.* 1999)
1998	S. Havana/*S.* Cubana/*S.* Tennessee	Sprouted seeds (alfalfa)	(Taormina *et al.* 1999)
1998	*S.* Oranienburg	Cantaloupe	(Anon. 2001a; Anon. 2001b)
1998–99	*S.* Baildon	Tomatoes	(Anon. 2001a; Anon. 2001b)
1999	*S.* Muenchen	Unpasteurized orange juice	(Anon. 2001a; Anon. 2001b)
1999	*S.* Thompson	Cilantro	(Sivapalasingam *et al.* 2004)
1999	*S.* paratyphi B var. java	Sprouted seed products	(Stratton *et al.* 2001)
2000	*S.* Enteritidis	Unpasteurized citrus juice	(Anon. 2001a; Anon. 2001b)
2000	*S.* Poona	Cantaloupe	(Centers for Disease Control 2002a)
2000	*S.* Typhimurium	Lettuce	(Horby *et al.* 2003)
2000	*S.* Typhimurium DT104	Lettuce	(Anon. 2005a)

Year	Pathogen	Vehicle	Reference
2000	*S.* Typhimurium DT204B	Lettuce	(Anon. 2005a)
2000	*S.* Enteritidis 11b	Mung bean sprouts	(Harb *et al.* 2003)
2001	*S.* Kottbus	Sprouted seeds (alfalfa)	(Centers for Disease Control 2002c)
2001	*S.* Newport	Mixed bagged salad	(Anon. 2005a)
2001	*S.* Virchow	Salad items	(Anon. 2005a)
2001	*S.* Poona	Cantaloupe	(Centers for Disease Control 2002a)
2001	*S.* Enteritidis	Mung bean sprouts	(Honish and Nguyen 2001)
2002	*S.* Javiana	Tomatoes	(Centers for Disease Control 2002b)
2002	*S.* Poona	Cantaloupe	(Centers for Disease Control 2002a)
2004	*S.* Newport	Lettuce	(Gillespie 2004)
2004	*S.* Thompson	Rocket salad	(Nygard *et al.* 2004)
2004	*S.* Braenderup	Tomatoes	(Centers for Disease Control 2005)
2004	*S.* Javiana	Tomatoes	(Anon. 2005a)
2005	*S.* Typhimurium DT104	Spanish lettuce	(Takkinen *et al.* 2005)
2005	*S.* Typhimurium DT104	Lettuce	(Anon. 2005a)
2005	*S.* Enteritidis	Bean sprouts	(Anon. 2005b)
2006	*S.* Newport	Tomatoes	(Anon. 2007)
2006	*S.* Typhimurium	Tomatoes	(Centers for Disease Control 2006c)
2007	*S.* Senftenberg	Basil	(Pezzoli *et al.* 2007)
1997	*E. coli* O157:H7	Sprouted seeds (alfalfa)	(Sivapalasingam *et al.* 2004)

Year	Pathogen	Vehicle	Reference
1997	*E. coli* O157:H7	Salad	(Anon. 2005a)
1998	*E. coli* O157:H7	Salad	(Anon. 2001a; Anon. 2001b)
1998	*E. coli* O157:H7	Fruit salad	(Anon. 2001a; Anon. 2001b)
1998	*E. coli* O157:H7	Coleslaw	(Anon. 2001a; Anon. 2001b)
1998	*E. coli* O157:H7	Sprouted seeds (clover/alfalfa)	(Taormina *et al.* 1999)
1998	*E. coli* O157:H7	Unpasteurized apple juice	(Anon. 2001a; Anon. 2001b)
1998	*E. coli* O157:H7	Parsley	(Sivapalasingam *et al.* 2004)
1999	*E. coli* O157:H7	Coriander (cilantro)	(Campbell *et al.* 2001)
1999	*E. coli* O157:H7	Unpasteurized apple juice	(Anon. 2001a; Anon. 2001b)
2003	*E. coli* O157:H7	Cucumber	(Duffell *et al.* 2003)
2003	*E. coli* O157:H7	Lettuce	(Anon. 2005a)
2005	*E. coli* O157:H7	Lettuce	(Soderstrom *et al.* 2005)
2006	*E. coli* O157:H7	Spinach	(Centers for Disease Control 2006b)
2006	*E. coli* O157:H7	Lettuce	(Centers for Disease Control 2006a)
1996	*Campylobacter jejuni*	Salad items	(Mandrell and Brandl 2004)
1996	*C. jejuni*	Lettuce*	(Centers for Disease Control 1998)
1996	*C. jejuni*	Lettuce	(Anon. 2005a)

Year	Pathogen	Vehicle	Reference
1997	*C. jejuni*	Sweet potatoes[†]	(Harriman 1998)
1997	*C. jejuni*	Cucumber[‡]	(Kirk *et al.* 1997)
2000	*C. jejuni*	Lettuce	(Anon. 2005a)
2001	*C. jejuni*	Orange juice	(Anon. 2005a)
1990	Norovirus	Fresh cut fruit	(Herwaldt *et al.* 1994)
1990	Hepatitis A	Iceberg lettuce	(Seymour and Appleton 2001)
1992	Norovirus	Lettuce/tomato	(Anon. 2005a)
1992	Norovirus	Melon	(Anon. 2005a)
1992	Hepatitis A	Strawberries	(Seymour and Appleton 2001)
1994	Hepatitis A	Tomatoes[§]	(Anon. 2001a)
1994	Norovirus	Salad	(Anon. 2005a)
1994	Norovirus	Carrot	(Anon. 2005a)
1994	Norovirus	Salad	(Anon. 2005a)
1995	Norovirus	Salad	(Anon. 2005a)
1995	Norovirus	Salad	(Anon. 2005a)
1995	Hepatitis A	Diced tomatoes	(Seymour and Appleton 2001)
1997	Hepatitis A	Frozen strawberries[§]	(Anon. 2001a)
1997	Unknown virus	Orange juice	(Anon. 2005a)
1998	Norovirus	Fruit salad	(Anon. 2005a)
1998	Norovirus	Fruit salad	(Anon. 2005a)
1998	Hepatitis A	Salad items	(Seymour and Appleton 2001)

Year	Pathogen	Vehicle	Reference
1999	Norovirus	Salad items	(Anon. 2005a)
1999	Norovirus	Salad items	(Anon. 2005a)
2001	Norovirus	Raspberries	(Le Guyader *et al.* 2004)
2002	Norovirus	Salad items	(Anon. 2005a)
2002	Norovirus	Fruit salad	(Anon. 2005a)
2004	Hepatitis A	Spring onion[§]	(Josefson 2003)
2005	Norovirus	Raspberries	(Korsager *et al.* 2005)
2005	Norovirus	Raspberries	(Cotterelle *et al.* 2005)
2006	Norovirus	Raspberries	(Hjertqvist *et al.* 2006)

*Cross-contamination from chicken juices.

†Cross-contamination either with meat juices or kale garnish.

‡Contamination during storage or processing.

§Thought to be due to contact with an infected food handler.

APPENDIX 2

Table A.2: Formulation used to prepare CRI agar

Ingredient	Quantity (g per litre dH_2O)
Bacto-tryptone	10
Yease Extract	5
Agar	15
Congo Red	0.04

Table A.3: Formulation used to prepare LB0 agar

Ingredient	Quantity (g per litre dH_2O)
Bacto-tryptone	10
Yease Extract	5
Agar	15

Table A.4: Formulation used to prepare CFA broth

Ingredient	Quantity (g per litre dH_2O)
Casamino acids	10
Yeast Extract	1.5
Masgnesium sulphate	0.05
Manganese (II) chloride	0.05

Table A.5: Formulation used to prepare R2 broth

Ingredient	Quantity (g per litre dH_2O)
Yeast extract	0.5
Proteose peptone	0.5
Casein hydrolysate	0.5
Glucose	0.5
Starch	05
Di-potassium phosphate	0.3
Magnesium sulphate	0.024
Sodium pyruvate	0.3

APPENDIX 3

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Use of episcopic differential interference contrast microscopy to identify bacterial biofilms on salad leaves and track colonization by *Salmonella* Thompson

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Summary

Zoonotic pathogens such as *Salmonella* can cause gastrointestinal illness if they are ingested with food. Foods such as salads pose a greater risk because they are consumed raw and have been the source of major outbreaks of disease from fresh produce. The novel light microscopy methods used in this study allow detailed, high resolution imaging of the leaf surface environment (the phyllosphere) and allow pathogen tracking. Episcopic differential interference contrast microscopy coupled with epifluorescence was used to view the natural microflora *in situ* on salad leaves and their topographical distribution. Fluorescent nucleic acid staining was used to differentiate between bacterial colonists and inorganic debris. *Salmonella enterica* serovar Thompson expressing green fluorescent protein was inoculated onto individual spinach leaves for 24 h at 22°C in order to observe spatial and temporal patterning of colonization on the two surfaces of each leaf under different osmotic conditions. The results obtained show that salad leaves are host to high numbers of bacteria, typically 10^5 per square millimetre. Cells are present in complex three-dimensional aggregations which often have a slimy appearance, suggesting the presence of biofilms. Washing of the leaves had little effect on the number of adherent pathogens, suggesting very strong attachment. Episcopic differential interference contrast microscopy is a rapid alternative to both scanning electron microscopy and confocal laser scanning microscopy for visualizing leaf topography and biofilm formation in the natural state.

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Introduction

The global plant leaf surface habitat (the phyllosphere) is estimated at 2×10^8 km^2 (Morris *et al*., 2002), a vast expanse available for bacterial colonization. Despite this, a great deal remains unknown about the population and community-level processes which occur on leaf surfaces. Bacterial surface life has been studied extensively on abiotic surfaces such as stainless steel, and clinically important situations such as dental plaques and catheters. In such ecosystems bacterial biofilms are ubiquitous, characterized by ordered three dimensional aggregations within an exopolysaccharide matrix. Bacteria within biofilms coordinate some processes in a density-dependent manner using diffusible signal molecules, a process known as quorum sensing (Waters and Bassler, 2005). Biofilms are also important sites of nutrient cycling and horizontal gene transfer, and the exopolysaccharide matrix offers protection from chemical challenges including antibiotics and from drought stress. However, these systems are usually at least partly water-saturated, which raises the question of how far these studies relate to the more hostile phyllosphere. Leaf surfaces have extremely limited surface water availability, which fluctuates daily along with rainfall, humidity, ambient temperature and solar intensity.

The characterization of biofilms on leaf surfaces is in its infancy. Very little published work currently exists which conclusively demonstrates their presence, yet determining their ubiquity and abundance has enormous implications for both plant disease management and the food industry. Similarly, there has been extensive work published on the interaction of phytopathogens and leaf surfaces in the phyllosphere (Hirano and Upper, 2000) but much less on human enteric pathogen survival or interaction on leaf surfaces. Biofilms have been demonstrated to provide a safe haven for a variety of bacterial, protozoal and viral pathogens in aqueous environments (Rogers *et al*., 1996; Lehtola *et al*., 2007). It might be expected therefore that if complex communities of microorganisms can exist as true biofilms on leaf surfaces then fresh

produce food crops such as salads may harbour foodborne human pathogens and protect them from detachment and chemical sanitizers (Carmichael *et al.*, 1999). It has been suggested that these pathogens contact the phyllosphere through contaminated manures recycled to agricultural land contaminating the emerging leaves in the soil, run off of rain water from adjacent contaminated land, contaminated irrigation water or direct faecal contamination from wild animals and birds. Food crops can also be contaminated post harvest by infected field and factory operatives. Pathogen/biofilm interaction in the phyllosphere may in part account for the various recent outbreaks of foodborne disease from ingestion of fresh produce in Europe and North America (Center for Disease Control, 2005; 2006; Health Protection Agency, 2005), despite claims that appropriate washing and disinfection procedure were being used prior to sale. If biofilms account for the majority of bacterial life on leaves, this may help to explain how communities of such high density are able to survive in a hostile environment which, being itself made of living tissue, not only detects but is capable of responding to colonization events with both specific and non-specific defence mechanisms when required. Adaptive traits which improve stress tolerance may be passed within and even between epiphytic species by horizontal gene transfer (Bailey *et al.*, 2002).

The dynamic nature of the leaf surface, along with its fragility, mean that many standard microbiological methods need to be carefully adapted if they are to be applied to study of the phyllosphere. It is also currently not possible to reproduce phyllosphere life using model culture systems, as often happens in studies of biofilm communities on abiotic surfaces. When studying phyllosphere bacteria *in situ*, any staining techniques used for microscopy must take into account that the leaf surface is made up of cellular material and may easily be stained along with the bacteria. Ideally the more rapid and non-destructive the techniques are, the higher the quality of data obtained.

In this study we present a detailed visual description of adherent microbial cells on plant leaf surfaces, including the development of ordered communities forming microcolonies and biofilms. The rapid light microscopy techniques allow direct visualization of biotic surfaces without the need for dehydration or fixation which destroy the three-dimensional structure of potential biofilms; epifluorescence techniques can be used simultaneously to visualize fluorescent stains and reporters on surfaces. GFP reporter technology has been used to quantitatively track the colonization of salad leaves by the human pathogen, *Salmonella enterica*, to determine how it interacts with the leaf surface and/or biofilms. This knowledge is key to understanding phyllosphere ecology and ensuring food safety.

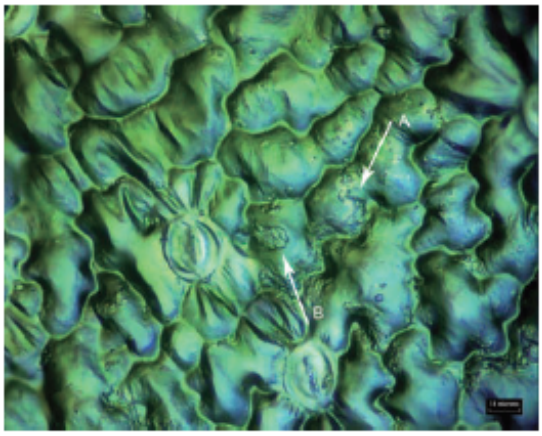

Fig. 1. EDIC micrograph of the upper surface of a processed watercress leaf obtained using a *z*-plane scan of the surface. Solitary rod-shaped structures are visible (arrow A), of appropriate size to be bacterial in origin. A potential bacterial aggregate (arrow B) can be seen to the right of the central stoma, spreading out from the margin and over the main part of the epidermal cell.

Results

Visualization of leaf surfaces using EDIC/EF microscopy

Spinach and watercress leaves were observed using episcopic differential interference contrast (EDIC) microscopy without the need for fixation or staining allowing up to ×1000 magnification with a longer working distance to remove the need for coverslips or oil. All fields of view on all leaves had some degree of surface contamination. Generally spinach leaves had a greater proportion of the surface covered by debris with an appearance of individual microbial cells and clusters, whereas on watercress the contamination was confined almost exclusively to the margins between epidermal cells. This debris was present whether the leaves were unwashed or processed and packaged as 'ready-to-eat', suggesting that it was tightly attached. In watercress the epidermal cells are much smaller than those in spinach, and the margins between them less deep. The large aggregation of debris visible in Fig. 1, reminiscent of a microbial microcolony associated with the early stage of biofilm development, measures 11 µm in diameter and is located near a stoma. Although no bacterial cells can be clearly distinguished within the aggregate it has a slimy appearance which may be due to bacterial exopolysaccharide synthesis. Figure 2 shows a single open stoma on a spinach leaf, the guard cells of which are completely covered by similarly slimy contamination. This material continues in an unbroken layer into all the adjacent cell margins.

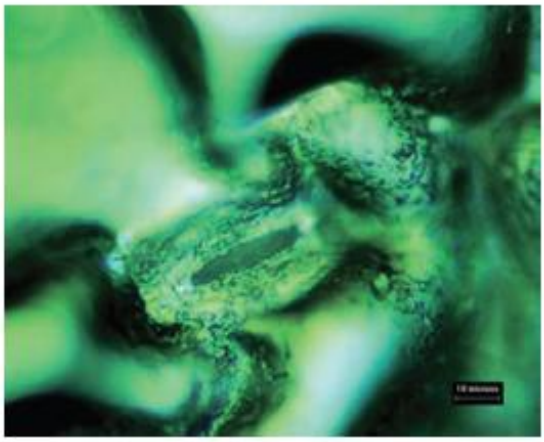

Fig. 2. EDIC micrograph of the upper surface of an unwashed spinach leaf, showing detail of contamination around an open stoma. Solitary bacterial cells are not apparent. The contamination has a slimy appearance and a three dimensional structure.

Nucleic acid staining and enumeration of total phyllosphere bacteria

4,6-diamidino-2-phenylindole (DAPI) staining was used to differentiate between inorganic soil debris and cellular material attached to leaf surfaces. This produced high resolution fluorescence micrographs, when viewed using the EF channel of the microscope, in which the blue fluorescence of DAPI was visible for single bacterial cells at high magnification. The fluorescence images could be overlaid onto the corresponding EDIC bright field image to distinguish between organic and inorganic surface contamination of the leaf. Figure 3 shows a heavily contaminated spinach leaf on which a large aggregate of cellular material is present. This aggregate is larger than those more frequently observed, being 41 µm in length at its longest point, and unusually it is situated in the centre of a leaf epidermal cell, rather than in the intercellular margin. It is not a monolayer of cells; the resolution of the EDIC image clearly shows a three dimensional structure. The ability to use lenses with a long working distance means that structures like this can be viewed intact; the structure will not have been altered by potential artefacts, such as dehydration or fixation, associated with other microscopy procedures.

When total bacteria were enumerated by direct counting on the leaf surface and by plate counts, similar counts were obtained per square millimetre (Table 1). However, when scaled up to the actual size of the leaves used, the log estimated counts detected via DAPI staining were significantly higher than those from the plate counts (*t*-test; $t = 4.97$ d.f. = 16, $P < 0.001$).

Inoculation of spinach leaves with Salmonella enterica *serovar Thompson*

The EDIC/EF microscopy can be used as a rapid technique for tracking inoculated GFP-expressing pathogens in the phyllosphere. In order to examine the attachment of *Salmonella* to spinach leaves, individual leaves were inoculated with a high concentration of *Salmonella* Thompson RM2311, which constitutively expresses GFP. Inoculation by prolonged immersion allowed the bacteria access to all colonization sites without being governed by water currents across the leaf surface. *Salmonella* were suspended in either sterile distilled water or phosphate-buffered saline (PBS), and under both conditions the majority of bacteria were observed to attach in cell margins and around stomata, which is where the majority of native bacteria and biofilm microcolonies were found

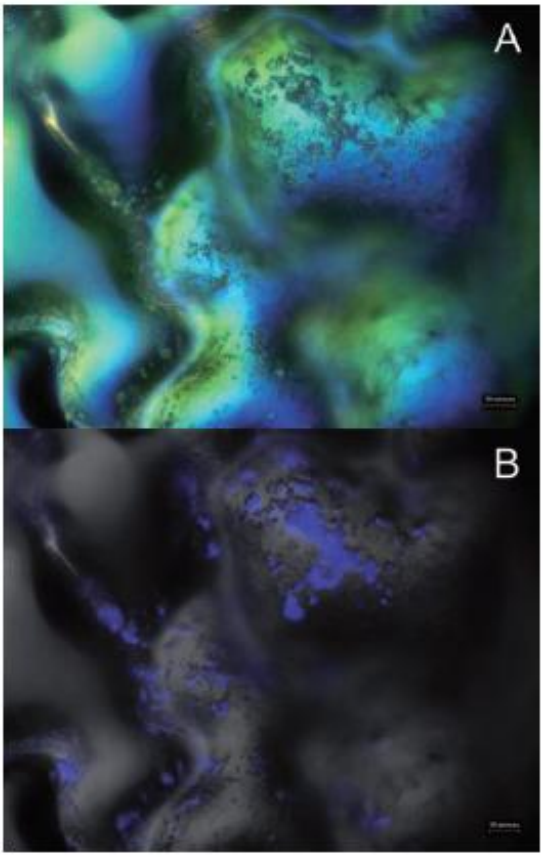

Fig. 3. EDIC micrograph (A) of the lower surface of a spinach leaf, showing heavy bacterial contamination in the centre of an epidermal cell as well as in the margins. The same image overlaid with imaging of the fluorescent DNA stain DAPI (B) shows that the majority of the debris on the leaf surface is in fact cellular material and is microbial colonization of the leaf.

Table 1. Total bacterial counts of individual baby spinach leaves obtained using either direct microscopic counting of DAPI stained bacteria on leaves or recovery and culture of bacteria on R2A medium.

	Direct microscopic count	R2A plate count
Bacteria per cm^2 upper surface	$5.19\ (\pm 0.26) \times 10^5$	ND
Bacteria per cm^2 lower surface	$5.12\ (\pm 0.34) \times 10^5$	ND
Average bacteria per cm^2 leaf	$5.12\ (\pm 0.21) \times 10^5$	$1.38\ (\pm 0.52) \times 10^5$
Total bacteria on a single leaf	$1.58\ (\pm 0.08) \times 10^7$	$4.77\ (\pm 1.82) \times 10^6$

Data are the mean of 12 leaves for microscopy and six leaves for plate counts. Values in parentheses indicate log standard error of the mean. ND, not determined.

(Fig. 4). There was strong interaction between the *Salmonella* and the biofilms, with little evidence of removal following manual water washing of the leaf such as might take place in the home (Fig. 5). Washing produced only a 0.21 log reduction in *Salmonella* from the water-incubated samples and 0.14 log reduction when PBS was used.

The two diluents were chosen to represent environments hypertonic and isotonic, respectively, to the interior of the bacterial cell. This provides two different levels of osmotic pressure, and also compares the presence and absence of free salt in the immediate environment. When the *Salmonella* on both leaf surfaces were counted, the average number of adherent bacteria per square millimetre of leaf area was only 0.1 log higher for PBS than water (Fig. 6). When each leaf surface was considered separately, a slight preference for attachments for the lower leaf surface was observed with both diluents (Fig. 6); however, a *t*-test did not show this to be statistically significant.

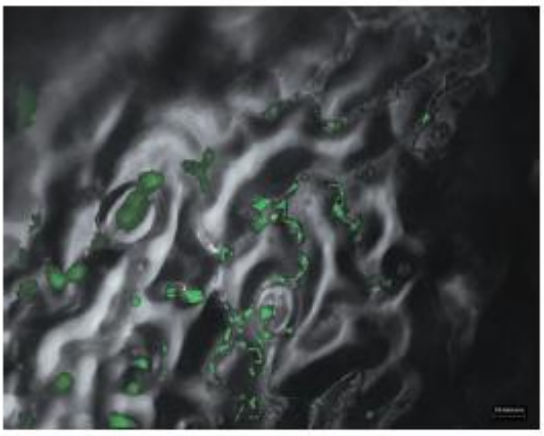

Fig. 4. GFP–expressing *Salmonella* Thomson on a spinach leaf. A fluorescence micrograph of the GFP expression (green fluorescence) obtained using the EDIC microscope fitted with a FITC filter set has been overlaid onto the corresponding EDIC bright field image. *Salmonella* are found almost exclusively in the intercellular margins on the leaf or in and around stomatal openings.

Discussion

Examining leaf surfaces using EDIC microscopy

The observations of natural leaf surface contamination made using EDIC microscopy are similar to published work using other microscopy techniques, such as scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Bacteria are predominantly found in epidermal cell margins, around stomata and on leaf veins. In addition to these locations, trichomes are popular colonization sites in leaf species which possess them (Monier and Lindow, 2004; Yadav *et al.*, 2005). The salad leaf species used in this study do not have trichomes, so an assessment of their colonization has yet to be made using

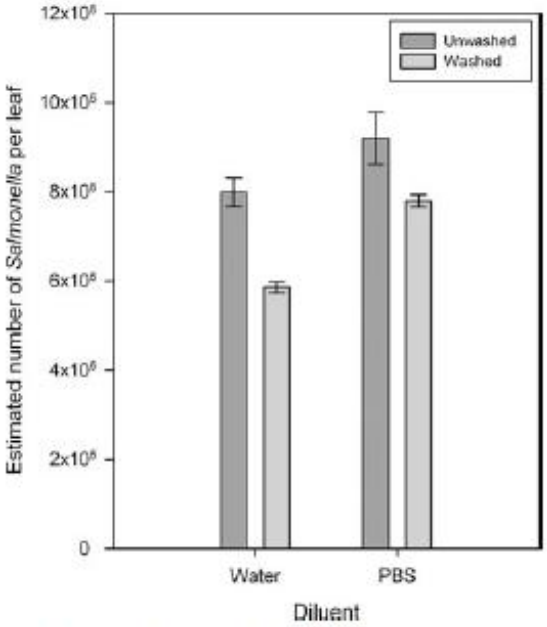

Fig. 5. Mean *Salmonella* counted per leaf on individual leaves inoculated in either PBS or sterile distilled water for 24 h at 22°C then either left unwashed or washed in tap water. Error bars represent ±1 standard error of the mean for 3 leaves per treatment.

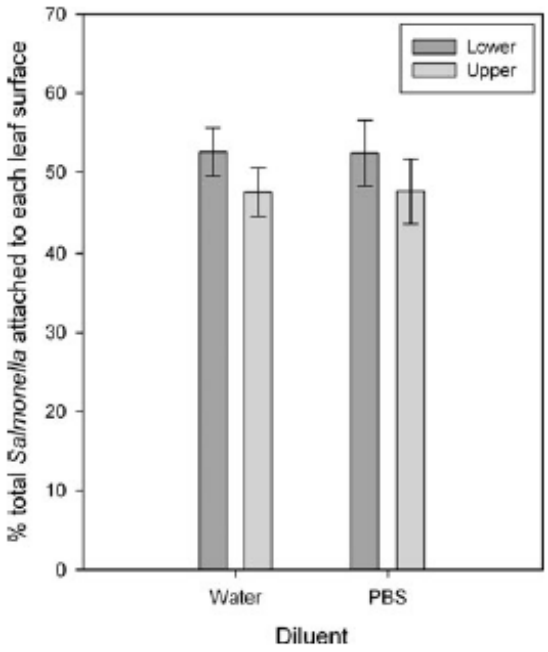

Fig. 6. Mean percentage of total *Salmonella* counted on each leaf surface following inoculation in either PBS or sterile distilled water for 24 h at 22°C. Error bars represent ±1 standard error of the mean percentage from 9 leaves for water and 8 leaves for PBS.

the EDIC microscope. In a recent review, Aruscavage and colleagues (2006) note that leaf structural damage plays a major role in determining patterns of colonization and survival. Even damage to a singe leaf cell dramatically increases nutrient availability at the leaf surface. Reducing leaf cuticular wax density increases bacterial survival by improving surface water movement (Marcell and Beattie, 2002). Plant pathogens also modify the leaf surface increasing water availability (Schreiber *et al.*, 2005). The EDIC microscope is well suited to rapidly assessing leaf damage at the cellular level, which is important as structural damage greatly reduces product shelf life in foods.

Despite the continuing advancement of microscopic techniques, direct imaging of leaf surfaces still does not form a major part of many phyllosphere studies, particularly those primarily concerned with food safety. As the leaf surface is a biotic and therefore dynamic environment, much of the microscopy which does take place involves fixation and/or dehydration to stabilize the samples. Unfortunately, this causes extensive loss of spatial data by flattening the samples, so that structural and topographical features become unclear. Confocal laser scanning microscopy is popular because its ability to scan through the leaf surface to the interior, albeit to only a 100 µm or so, avoids the issues of uneven surface topography. Unfortunately, the laser excitation requires that fluorescent staining is used on the leaves, although some leaf features can be distinguished from their chlorophyll autofluorescence (Brandl and Mandrell, 2002). By contrast, the EDIC micrographs used in this study required neither staining nor fixation to view the leaf surface, and images could be obtained in a matter of minutes. More and more studies are using epifluorescence microscopy to observe bacteria on leaves, but without being able to obtain a high-resolution image of the leaf surface itself, it is harder to explain spatial patterns of colonization.

Many published studies have also used recovery techniques, enabling phyllosphere bacteria to be studied microscopically on filters or cultured (Morris *et al.*, 1997; 1998; Mercier and Lindow, 2000; Barak *et al.*, 2002). This raises two issues: first, no recovery method exists which is guaranteed to remove all of the bacteria on a leaf, and second that not all phyllosphere bacteria are culturable. Yang and colleagues (2001) were the first to undertake phyllosphere community profiling using culture-independent molecular methods. Their study showed that a far higher number of bacterial species were detected using the molecular methods than would grow in culture.

The data presented here indicate that although significantly more bacteria are detected by direct observation on leaves, growth on R2A medium can actually account for around 1% of the total population. Not all bacterial strains are culturable; in addition, stressed cells may enter the viable but non-culturable state and consequently not grow in culture media, which may further reduce the accuracy of culture-based detection. However, the lower nutrient concentration, longer incubation times and lower incubation temperatures used with R2A in this study and others (Reasoner and Geldreich, 1985) are designed to aid culturability of stressed bacteria. Total bacterial enumeration using DAPI does not provide any assessment of bacterial viability; therefore a comparison is in fact being made between a method of obtaining total cell numbers using DAPI and one for determining the total culturable population. This may also partially account for the lower bacterial counts obtained in culture. DAPI staining combined with EDIC/EF microscopy demonstrates the potential for quantitative, *in situ* detection of bacterial epiphytes on leaf surfaces, providing insight into the spatial patterning of phyllosphere colonization.

A Pulsifier was used instead of a Stomacher as it has previously been shown to provide cleaner bacterial suspensions with less sample debris while recovering at least as many bacteria as a Stomacher (Fung *et al.*, 1998). Instead of paddles, the sample is prepared in a bag as for stomaching and the bag is placed inside a metal ring which moves backwards and forwards at high frequency. This method is capable of detaching bacteria while leaving even soft food samples, such as raisins, undamaged.

Bacterial aggregations were frequently observed on all leaves using the microscopy techniques presented here. Their size, three dimensional appearances and the frequent occurrence of slimy deposits on the leaves are all strongly indicative of biofilm. Biofilms, or strictly speaking the presence of attached cells, have been demonstrated previously *in situ* on plant aerial surfaces using SEM (Gras *et al*., 1994), CLSM (Carmichael *et al*., 1999) and epifluorescence microscopy (Morris *et al*., 1997) although these techniques do not readily visualize exopolymeric substance production characteristic of biofilm slime. As the leaf surface is a dynamic and hostile environment, biofilms may play a vital role in the survival of epiphytic bacteria, offering protection from fluctuating environmental stresses (Molina *et al*., 2003; Morris and Monier, 2003). They may also have important implications for the survival of non-native bacterial immigrants, including human pathogens such as *Escherichia coli* and *Salmonella*.

Pathogen tracking in the phyllosphere using GFP

When GFP-expressing *Salmonella* Thompson was placed in both an isotonic and a hypertonic osmotic environment, it readily attached to leaves at 22°C. A short wash in tap water, such as might take place in the home before consumption of salad products, removed very few of the *Salmonella*, indicating that the attachment is very strong. The presence or absence of salt in the diluent had little effect on how many *Salmonella* attached or where on the leaf they could be found following attachment. A slight preference for the lower leaf surface was observed in both an isotonic and hypertonic inoculum. This preference was not found to be statistically significant, but it has previously been noted in other plant species that the two surfaces of a leaf differ not only in their environmental conditions but also in molecular features, particularly wax crystal structure and composition (Leigh and Coplin, 1992; Marcell and Beattie, 2002). In this experiment both leaf surfaces were exposed to the same conditions, which is not the case in field grown plants.

As the first uses of GFP-based reporter systems (Prasher, 1995), transgenic GFP-expressing bacteria have become a popular tool across microbiology. Bacterial strains such as the *Salmonella* used in this study, which stably and constitutively express GFP, are easily tracked and quantified in a variety of systems. This means that differences in colonization behaviour and survival can be compared under diverse conditions such as the intestinal tract versus the leaf surface (Brandl *et al*., 2005). An innovative use of GFP technology in the phyllosphere is as an indicator of active cellular processes. A sucrose/fructose bioreporter was used in one study to monitor sugar utilization by bacterial colonists on leaves (Leveau and Lindow, 2001). Coupled with detailed leaf imaging using EDIC, this would be an extremely powerful tool to spatially map bacterial processes in the phyllosphere.

Understanding the factors which affect bacterial attachment in the phyllosphere is important in both plant and microbe evolution, microbial ecology and food safety. The data presented here is an example of how EDIC/EF microscopy is a rapid alternative to the quantitative techniques used in other studies, such as CLSM (Brandl and Mandrell, 2002), and provides a more comprehensive picture of the patterns of colonization. As bacteria can be quantified *in situ*, there are no inaccuracies introduced by inefficient recovery or loss of culturability. There is enormous potential to use *in situ* pathogen tracking to study the effects of altering anything from environmental conditions to bacterial or even leaf phenotype.

Conclusions

The EDIC microscopy is a technique well suited to *in situ* examination of the phyllosphere. The bright field images provide a rapid alternative to SEM which does not require fixation or dehydration to give a detailed three-dimensional image of the leaf surface. This greatly reduces the likelihood of forming artefacts, thereby giving a more accurate representation of the leaf surface. Coupled with epifluorescence, this technique can be used to track and quantify inoculated pathogens using GFP reporters, or potentially to examine the composition of the phyllosphere community using fluorescent stains and probes.

Experimental procedures

Bacterial strains and culture media

Salmonella enterica serovar Thompson RM2311 containing plasmid pWM1007 was kindly provided by R. Mandrell (Brandl and Mandrell, 2002). pWM1007 contains the gene encoding the *Aequorea victoria* GFP while pWM10012 contains the gene for CFP, the cyan derivative of GFP. Both plasmids contain genes required for replication and a kanamycin resistance gene (*kan*), which acts as a selectable marker (Brandl and Mandrell, 2002).

Salmonella strains were stored at −80°C on Protect™ beads in glycerol and routinely grown in Tryptone Soya Broth (TSB) (Oxoid, UK) supplemented with 50 μg ml^{-1} kanamycin; unless otherwise specified, cultures were incubated at 37°C with shaking at 200 r.p.m. overnight (~20 h) prior to use. Total bacteria recovered from leaves were grown on R2A medium at 22°C to improve detection of sublethally stressed cells. Selective enumeration of *salmonellae* recovered from leaves was performed by spread plating of serial dilutions of the inoculum in sterile PBS onto Rambach agar plates (Merck, UK), which are chromogenically selective for *Salmonella* species. Rambach plates were counted after incubation at 27°C for 24 h.

EDIC imaging of naturally occurring bacteria on salad leaf surfaces

The EDIC microscopy is a modified light microscopy technique which uses reflected rather than transmitted light to allow visualization of curved and opaque surfaces (Keevil, 2003). Lenses with a long working distance allow magnifications of up to ×1000 without need for coverslips or oil. An EDIC/EF microscope (Best Scientific, Swindon, UK) was used to study the surfaces of field-grown spinach, rocket and watercress leaves to study key topological features of the leaf surface along with the amount and localization of bacterial colonization.

Leaf pieces measuring 1 × 1 cm were cut and placed onto glass slides. The leaf pieces were viewed under the EDIC/EF microscope using the ×50 and ×100 objective lenses. The lenses had sufficient working distance to eliminate the need for coverslips and oil, allowing bright field images to be captured. Multiple image capture in the *z*-plane was used to study some regions with particularly uneven topography in order to bring a greater portion of the field of view into focus.

Enumeration of total bacterial cells on leaves

The nucleic acid-binding fluorescent dye, DAPI, was used on leaves to stain all bacterial cells found on the leaf surface. The total leaf areas of individual field-grown, unwashed baby spinach leaves were first obtained by creating a leaf outline on 1 cm graph paper. Leaf disks 1 cm in diameter were then cut from the leaves with a cork borer; one upper surface disk and one lower surface disk were prepared per leaf. DAPI solution (10 μg ml^{-1}; Sigma) was pipetted onto the leaf pieces which were incubated in the dark for 20 min and then washed in sterile distilled water. The slides were then viewed using the EDIC/EF microscope fitted with a DAPI filter set. Total bacteria were counted in a minimum of 10 fields of view to give at least 200 bacterial cells.

Total culturable bacteria were determined by growth on R2A medium (Oxoid, UK). Individual unprocessed baby spinach leaves were placed in 50 ml of sterile PBS and shaken for 30 s in a Pulsifier (Microgen Bioproducts ltd, Camberley, UK). Serial dilutions of the PBS were inoculated in triplicate on R2A agar plates and incubated at 22°C. Colonies were counted after 4 days.

Attachment of Salmonella to spinach leaf surfaces

Salmonella Thompson GFP in suspension were allowed to attach to individual spinach leaves in the presence or absence of osmotic stress to compare the effect on the degree and spatial positioning of bacterial attachment. Single unprocessed baby spinach leaves were suspended vertically in 125 ml of bacterial inoculum, consisting of ~10^8 CFU ml^{-1} *Salmonella* GFP in either sterile distilled water or PBS. The leaves were incubated at 22°C for 24 h. Control samples of the inoculum with no leaf were also included to monitor the level of inoculum growth during the incubation period. This was achieved using plate counts on both TSA and Rambach agars. In addition, Rambach agar plate counts of the inoculum for each individual leaf were obtained at the end of the incubation period. The leaves were then removed and 1 cm diameter leaf disks were cut with a cork borer for microscopy analysis. For each leaf, one disk of the upper surface and one of the lower surface were prepared. Bacteria were counted directly using the EDIC microscope fitted with a FITC filter set to visualize the GFP fluorescence. A minimum of 10 fields of view per disk were counted to give a total of at least 200 bacterial cells. In addition, some leaves were subjected to a tap water wash to simulate home washing of salads prior to consumption. Following inoculation, leaves were placed in sterile bags containing 50 ml of tap water and manually agitated for 2 min before being removed and analysed as described for unwashed leaves.

Analysis

Image capture from the microscope was performed using a CCD camera (Roper Industries, UK) with the aid of ImagePro Plus software (MediaCybernics), which was also used to perform *z*-plane scanning where required, and to take measurements from micrographs. Fluorescence images with a high degree of background coloration were processed using Picasa™ image processing software v2.1 (Google) to decrease the background fluorescence. The same software was used to increase the contrast of the pseudo-3D images created by EDIC microscopy and emphasize the heterogeneity in leaf topography.

Bacterial counts were square-rooted to normalize them for parametric statistical analysis. *t*-tests were used to compare bacterial counts for the two leaf surfaces with one another, to compare plate counts with DAPI-stained microscope counts, and also to compare washed versus unwashed leaves. All statistical analyses were performed using SigmaStat v3.5 (Systat Software).

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