Escherichia coli and Rhizobium leguminosarum response mechanisms to sub-lethal 2,4-dichlorophenoxyacetic acid

A Thesis

Submitted to the Faculty of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

in

Biochemistry

University of Regina

by

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Regina, Saskatchewan

February, 2017

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Supriya Venkatesh Bhat, candidate for the degree of Doctor of Philosophy in Biochemistry, has presented a thesis titled, *Escherichia coli and Rhizobium leguminosarum response mechanisms to sub-lethal 2,4-dichlorophenoxyacetic acid*, in an oral examination held on November 16, 2016. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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*Via Skype*
ABSTRACT

The chlorophenoxy herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is used extensively worldwide despite its known toxicity and our limited understanding of how it affects non-target organisms. To determine the global effects of 2,4-D at sub-lethal levels on *Rhizobium leguminosarum* bv. *viciae* 3841 (*Rlv*) and *Escherichia coli* BL21, I used a novel combination of methods involving advanced microscopy and metabolomics. *Rlv* showed an oxidative stress response, but showed adaptive capabilities with changes to specific metabolic pathways and consequent changes to its phenotype, surface ultrastructure, and physical properties during 2,4-D exposure. Interestingly, auxin and 2,4-D, its structural analogue, induced common morphological changes *in vitro*, which were similar in shape to bacteroids isolated from plant nodules, implying that these changes may be related to bacteroid differentiation required for nitrogen fixation.

In *E. coli*, 2,4-D altered biofilm formation and induced a filamentous phenotype in the lab strain and a selection of genotypically diverse strains isolated from the environment. The phenotype was observed at concentrations 1000 times below field application levels, and was reversible upon supplementation with polyamines, implicating DNA damage. Cells treated with 2,4-D had more compliant envelopes, significantly remodelled surfaces that were rougher and more hydrophobic and altered vital metabolic pathways. Most of the observed effects could be attributed to oxidative stress, consistent with increased reactive oxygen species as a function of 2,4-D exposure.

The characteristic filamentous phenotype and metabolic changes with 2,4-D exposure implicated impact on cell division. I developed correlative atomic force microscopy-
quantitative imaging and laser scanning confocal microscopy to simultaneously probe cell surface alterations at the pico-nanoscale with details of molecular changes inside live cells in real-time. At the mechanistic level, 2,4-D at >1 mM altered FtsZ, FtsA and SulA localization within seconds accompanied by DNA damage resulting in immediate inhibition of Z-ring formation and arrest of cell division. There were simultaneous changes to cell surface roughness, elasticity and adhesion in a time-dependent manner. I propose that 2,4-D rapidly blocks cell division in *E. coli* likely by affecting the higher order assembly of the divisome complex with inhibition of Z-ring formation resulting from oxidative stress and DNA damage.
ACKNOWLEDGEMENTS

I am genuinely grateful to Dr. Tanya Dahms for being an incredible supervisor and a great mentor in many aspects of my life. She has challenged me to be independent, hardworking, have a positive attitude and her constant encouragement has increased my confidence significantly. She is a wonderful person and the best supervisor I could have hoped for.

I would like to thank my colleagues, especially Andrea Jun Dong and Biplab Paul for being good examples. I learned a lot from their experience which helped me greatly to complete this research on time. Of course, without my summer students Erik Vantomme, Seamus McGrath and Belma Kamencic this research wouldn’t have been complete on time, for which I am deeply thankful. A special thanks to Dr. Dinah Tambalo for providing valuable advice whenever needed.

I am sincerely indebted to our collaborators, Sean Booth, Dr. Ray Turner, Dr. Aalim Weljie, Dr. Andre Kornig, and Dr. Heiko Hashke; their technical expertise and scientific knowledge have been a great contribution to this research. I would also like to thank Dr. Rod Kelln for enzyme assays, Dr. Ian Coulson, Dr. Tzu-Chiao Chao and IECS for providing technical expertise with the SEM and confocal facilities and Dr. William Margolin for providing GFP strains crucial for this research. My sincere gratitude to my graduate committee members, Dr. Mohan Babu, Dr. Renata Raina-Fulton, Dr. Dae Yeon Suh and Dr. Chris Yost for providing valuable guidance and support throughout the program. I would like to acknowledge the Faculty of Graduate Studies and Research, the Department of Chemistry and Biochemistry, NSERC and Government of Saskatchewan for helping to fund this research.
DEDICATION

I would like to dedicate this thesis to my mother. She is the most hardworking and the strongest woman I have ever known. You single-handedly took care of me during some of the most difficult times in life and I attribute all my success to the moral, intellectual and physical education I received from you. I have no words to describe how grateful I am for everything that you have done for me.

A special dedication to my dear husband, Venkatesh Bhat, without your love and support this thesis wouldn’t have been possible.
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<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>2,4-dichlorophenol</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CM</td>
<td>confocal microscopy</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-enzyme A</td>
</tr>
<tr>
<td>CPS</td>
<td>capsular polysaccharide</td>
</tr>
<tr>
<td>CV-ANOVA</td>
<td>seven-fold cross validation analysis of variance</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCFDA</td>
<td>dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNPH</td>
<td>dinitrophenylhydrazine</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ECU</td>
<td>electron control unit</td>
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<tr>
<td>EDTA</td>
<td>ethidium diamine tetra-acetate</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polysaccharide</td>
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<tr>
<td>ESI</td>
<td>electron spray ionization</td>
</tr>
<tr>
<td>FM</td>
<td>fluorescence microscopy</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescent protein</td>
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<tr>
<td>FS</td>
<td>force spectroscopy</td>
</tr>
<tr>
<td>FtsZ</td>
<td>filamenting temperature-sensitive mutant Z</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<td>GC</td>
<td>gas chromatography</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>high performance liquid chromatography</td>
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<tr>
<td>IAA</td>
<td>indole acetic acid</td>
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<tr>
<td>ICL</td>
<td>isocitrate lyase</td>
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<tr>
<td>KEGG</td>
<td>Kyoto encyclopedia of genes and genomes</td>
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<tr>
<td>KNN</td>
<td>k-means nearest neighbor</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LCD</td>
<td>liquid crystal display</td>
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<td>LM</td>
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<tr>
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<td>lipopolysaccharide</td>
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<tr>
<td>LSCM</td>
<td>laser scanning confocal microscopy</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>MD</td>
<td>metabolite detector</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MOPS</td>
<td>(3-(N-morpholino) propanesulfonic acid)</td>
</tr>
<tr>
<td>MS</td>
<td>malate synthase</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSTFA</td>
<td>trimethylsilyltrifluoroacetamide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>partial least squares and discriminant analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
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</table>
PLL       poly-L-lysine
pN        pico-newton
PTS       glucose–phosphotransferase system
QI        quantitative imaging
RI        retention index
Rlv       *Rhizobium leguminosarum* bv. *viciae*
ROS       reactive oxygen species
RT        room temperature
SD        standard deviation
SDS       sodium dodecyl phosphate
SEM       scanning electron microscopy
SOS       singlet oxygen stress
SUS       shared and unique structures
st        streptomycin
TCA       trichloroacetic acid
TY        tryptone yeast extract
UMP       uridine monophosphate
VIP       variable influence on projection
CHAPTER 1 – GENERAL INTRODUCTION

1.1 Introduction

Over the last few decades the synthesis of manmade chemicals has increased exponentially. Over 109 million organic and inorganic chemicals are currently registered in the CAS REGISTRY database (www.cas.org), with about 12,000 new chemicals added each day. This list includes toxic chemicals, pesticides and other chemicals generated during human activities. New chemicals are synthesized with an intention to simplify our life but with a disregard for the environmental consequences, often greater than what is intended by manufacturers. For example, in 1998 a US Environmental Protection Agency report indicated that there was no toxicity information available for 43% of High Production Volume chemicals produced in the US, which amount to more than 1 million tons per year, and full toxicity data sets were available for only 7% (for review see Binetti et al., 2008). The overall pesticide sales for Canada reported in 2008 were over 87 million kg active ingredient (Health Canada), 60% of which were used in the agricultural sector. This statistical data provides an idea of how countless new chemicals are synthesized and released into the environment prior to sufficient knowledge of their toxic effects on microorganisms, animals and humans.

The application of modern agricultural and landscaping practices has led to the widespread use of pesticides, some of which persist in the environment. The effects of exposing organisms to ever increasing combinations of pesticides and other xenobiotics at sub-lethal levels remain largely understudied, yet have implications in human disease and environmental dysfunction. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one such example.
1.2 2,4-Dichlorophenoxyacetic acid (2,4-D)

The herbicide 2,4-D is one of the most extensively used man-made agrochemicals currently in use, with over 205 registered products containing 2,4-D in Canada (Barnekow et al., 2001). This herbicide is highly effective against broad leaf plants with applications to weed control in lawns and gardens around homes, cereal crops in agriculture and pastures, and defoliants in forestry (Bukowska, 2005; Garabrant and Philbert, 2002). This herbicide belongs to the class of phenoxy herbicides, with deleterious hormonal effects to DNA, RNA and protein synthesis in the meristematic tissues of the target species, the broad leaf plants. Various 2,4-D commercial formulas are available including acidic forms, water soluble amine forms, and the ester form designed to diffuse efficiently across the organic matrices of plants (Chinalia et al., 2007). The physical and chemical properties of 2,4-D are outlined in Table 1.1.

1.2.1 Fate of 2,4-D in the environment

2,4-D enters the air mainly through aerosols during spray applications, and the use of improper spray techniques leading to highly volatile esters which enter air drift (Howard, 1989). Small amounts of 2,4-D thus enter into the air, become photooxidized by free radicals and are degraded with a half-life of 1 day or deposited into the soil through precipitation (Howard, 1989).
Table 1.1 Chemical properties of 2,4-dichlorophenoxyacetic acid

<table>
<thead>
<tr>
<th>Properties</th>
<th>Data</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>221.04 g/mol</td>
<td>Hartley and Kidd, 1983</td>
</tr>
<tr>
<td>Melting point</td>
<td>135–142 °C</td>
<td>Hartley and Kidd, 1983</td>
</tr>
<tr>
<td>Boiling point</td>
<td>160 °C</td>
<td>Cessna et al., 1988</td>
</tr>
<tr>
<td>Water solubility</td>
<td>677 ppm at 25°C</td>
<td>Yalkowsky et al., 2010</td>
</tr>
<tr>
<td>Hydrolysis half life</td>
<td>10–40 days</td>
<td>DeLaune and Salinas, 1985</td>
</tr>
<tr>
<td>Photolysis half life</td>
<td>400 days</td>
<td>Howard, 1989</td>
</tr>
<tr>
<td>Aqueous half life</td>
<td>13 days</td>
<td>Cessna et al., 1988</td>
</tr>
<tr>
<td>Biodegradation half life</td>
<td>20–200 days</td>
<td>Pazmino et al., 2011</td>
</tr>
<tr>
<td>Soil partition coefficient (Koc)</td>
<td>19.6–109.1</td>
<td>Davidson et al., 1980</td>
</tr>
</tbody>
</table>
In aqueous environments, the 2,4-D amine salt and ester formulations dissociate into their respective anions. The anionic form has relatively high persistence and can enter groundwater, rivers and or irrigation channels; runoff from soils; or can leach through the soil column (DeLaune and Salinas, 1985). Leaching into groundwater also depends on soil type, with coarse-grained sandy soils having low organic content expected to leach more efficiently (Boivin et al., 2005). Degradation of the anion is mainly due to microbial and photo conversion. In water, 2,4-D will biodegrade at a rate dependent upon the nutrient levels, temperature, oxygen availability, and whether or not the water was previously contaminated with 2,4-D or other phenoxyacetic acids due to the presence of 2,4-D degrading microbes (Howard, 1989).

When applied to soil, 2,4-D is a moderately persistent herbicide with a half life varying from 20–200 days, depending upon the soil constituents and complex processes of sorption, mineralization and microbial interactions (Boivin et al., 2005). Once in the soil, this herbicide can undergo physical, chemical or biological transformation, photodecomposition, volatization, adsorption, leaching, microbial degradation or plant uptake (Kashyap et al., 2005). Sorption and mineralization are the main factors that determine the persistence and transport of 2,4-D in soil. The rate of microbial degradation depends on soil properties such as organic content, type of soil (clay, loam or sandy), pH, temperature, soil depth and water potential (Veeh et al., 1996). A number of soil microbial communities such as fungi and bacteria are capable of 2,4-D biotransformation (Vroumsia et al., 2005; Zabaloy et al., 2010). The esters and salts of 2,4-D are first converted to their acid forms prior to mineralization and microbial degradation, the main routes of 2,4-D degradation in soil (Han and New, 1994). The most common degradation
pathway involves removal of the acetic acid side chain to yield 2,4-dichlorophenol (2,4-DCP), followed by ring cleavage and degradation to produce aliphatic acids such as succinate.

1.2.2 2,4-D toxicological effects

Even with a short half-life, 2,4-D is highly toxic to non-target species (Figure 1.1). Despite safety claims by manufacturers, there is a bulk of evidence suggesting that 2,4-D is linked to slow growth rates, reproductive problems, changes in behavior and death in non-target species such as earthworms, cattle, birds, horses and fishes (Bukowska, 2005). 2,4-D can disrupt normal biochemical reactions in the cell by affecting enzymes, phospholipids, nucleic acids and can induce free radical formation that leads to apoptosis (Bongiovanni et al., 2007). Several investigations provide evidence for cytotoxicity (Tuschl and Schwab, 2003), genotoxicity (Bortolozzi et al., 2004), teratotoxicity (Bortolozzi et al., 2004), hepatotoxicity (Charles et al., 2001; Ozaki et al., 2001), embryotoxicity (Charles et al., 2001), neurotoxicity (Venkov et al., 2000) and immunosuppressive characteristics (Pistl et al., 2003). Exposure to 2,4-D has been linked to non-Hodgkin’s lymphoma and soft sarcoma among farmers (Bukowska, 2005).

Due to the wide-spread use of 2,4-D in Saskatchewan and around the globe, it is one of the most frequently detected pesticides in Canadian wetlands, sediments, forests and ground water systems (Paasivirta et al., 1990). This herbicide can easily seep into the ground water and run off into rivers and lakes and through precipitation can contaminate water reservoirs, 2,4-D is also capable of traveling long distances through the atmosphere from the initial area of application (Raina et al., 2011). Therefore, a wide variety of
Figure 1.1 The effects of 2,4-D on various parts of the ecosystem. Sections highlighted in dark grey show the effects on target plant populations, affecting the ethylene pathway and leading to accumulation of cyanide, which is toxic to plant cells. Overproduction of ethylene leads to accumulation of abscisic acid leading to premature aging and shedding of leaves. Sections highlighted in black and light grey show effects on microbes and mammals, respectively, which are non-target species. In microbes, this herbicide is known to passively enter the cell, affecting membrane fluidity and composition. Upon entering the cell it affects a series of specific and non-specific pathways, causes genetic alterations and eventually altering resident populations in the 2,4-D treated soil and aquatic ecosystems. In humans it is known to be absorbed through the skin, can be inhaled, or ingested causing oxidative stress-induced secondary reactions.
organisms in diverse ecosystems are exposed to this chemical, increasing its impact on the environment. As such, it is important to understand the mechanism of cellular effects on non-target organisms.

1.2.3 Effects of 2,4-D on microorganisms

There is a significant amount of research into the non-targeted effects of high levels of 2,4-D on microbial communities and on cultured microorganisms. Previous studies have shown that 2,4-D has toxic effects on various species of soil bacteria (Arias and Fabra, 1993; Fabra et al., 1992; Fabra et al., 1997; Zabaloya et al., 2010), human pathogenic E. coli (Balague et al., 2002) and Saccharomyces cereviciae (Teixeira et al., 2004) at higher concentrations. Table 1.2 summarizes the effects of 2,4-D on microbes, showing clear toxic effects on several microorganisms at higher concentrations and potential adverse effects at lower concentrations, but little or no information on the sub-lethal effects of 2,4-D. This herbicide is known to influence soil microbial communities by altering the balance between resident populations. The metabolic diversity of microbial populations is substrate defined and is crucial for the natural balance of the ecosystem; the application of xenobiotic chemicals shifts this balance by triggering specific degradation pathways (Baelum et al., 2006). Prado and Airoldi (2000) showed that certain microbial communities were capable of quickly assimilating 2,4-D as a carbon source in contaminated soil, shifting the metabolic activity of microbes away from naturally occurring organic matter. Thus 2,4-D applications directly affect organic matter turnover in the soil.
Table 1.2 Summary of the effects of 2,4-D on various bacterial species.

<table>
<thead>
<tr>
<th>[2,4-D] (mM)</th>
<th>Bacterial species</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 0.043 Azospirillum brasilence</td>
<td>1 mM completely diminished growth, 43 μM (crop application levels) inhibited attachment of the bacterium to maize roots, affected polyamine synthesis, protein biosynthesis, nucleic acids</td>
<td>Fabra et al., 1993; Rivarola et al., 1992</td>
<td></td>
</tr>
<tr>
<td>1 Rhizobium sp. M4</td>
<td>Completely diminished growth, changes in membrane adhesion, fluidity, transport, proteolysis, genotoxicity and bioaccumulation</td>
<td>Arias and Fabra, 1993; Fabra et al., 1992; Fabra et al., 1997</td>
<td></td>
</tr>
<tr>
<td>1 Escherichia coli</td>
<td>Diminished growth, total protein content, altered hydrophobicity, fimbriation, alteration in lipoperoxidation, lipid degradation and motility diminution</td>
<td>Balague et al., 2001</td>
<td></td>
</tr>
<tr>
<td>10 Pseudomonas putida KT2440</td>
<td>Disturbed cell respiration, repressed enzymes for energy consuming pathways and induced membrane transporters</td>
<td>Benndorf et al., 2006</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>Organism</td>
<td>Response</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>0.01-1</td>
<td><em>Deftia acidovorans</em> MC1</td>
<td>Induction of two chlorocatechol 1,2-dioxygenases</td>
<td>Benndorf and Babel, 2002</td>
</tr>
<tr>
<td>0.5-10</td>
<td><em>Burkholderia cepacia</em> YK-2</td>
<td>Induction of stress shock proteins DnaK and GroEL, wrinkled surfaces with perforations, shift in the cellular fatty acid composition, ability to degrade the herbicide at 2.25 mM concentration</td>
<td>Cho et al., 2000, Cho et al., 2002</td>
</tr>
<tr>
<td>0.45</td>
<td><em>Corynebacterium glutamicum</em></td>
<td>Changed protein levels related to energy metabolism, cell envelope synthesis and ABC transport system</td>
<td>Fanous et al., 2007</td>
</tr>
<tr>
<td>1 mg/g of soil</td>
<td>Microbial communities</td>
<td>Significantly decreased population number and shifted the microbial community structure</td>
<td>Zhang et al., 2010</td>
</tr>
</tbody>
</table>
Studies on the effects of 2,4-D are conflicting, with a large body of evidence showing negative effects and a number of reports showing non-toxic, harmless effects at low (< 1 mM) exposure levels (Garabrant and Philbert, 2002). According to a review by Garabrant and Philbert (2002), there is insufficient experimental evidence to support the association of 2,4-D to genotoxic, oncogenic effects, any form of cancer, adverse reproductive, or immune system effects, and 2,4-D causes damage to the kidney and liver only at overwhelming doses. Charles et al. (2001) reported that phenoxy-herbicides are non-carcinogenic, non-mutagenic, non-neurotoxic and of moderate toxicity after sub-chronic administration to bacteria and cultured mammalian cells, and Venkov et al. (2000) demonstrated the absence of genotoxicity to yeast and mammalian cells. Such conflicting conclusions can be attributed to the measurement of symptomatic responses, ignoring effects preceding most of the biological outcomes and primary responses at the molecular level. Differences in toxicity mechanisms are expected at the molecular level, controlled by distinct concentration thresholds in lower and higher doses, thus testing toxicity based on mortality is too coarse to determine low concentration and long-term chronic effects (Aardema and MacGregor, 2002).

As outlined in Table 1.2, 2,4-D has deleterious effects on diverse soil bacteria at higher concentrations (>1 mM), with potential effects even at lower levels. In rhizobia and E. coli, 2,4-D is known to affect membrane composition, cell envelope and macromolecule biosynthesis and cause macromolecular denaturation at higher concentrations (Arias and Fabra de Peretti, 1993; Balague et al., 2001; Balague et al., 2002; Chinalia et al., 2007; Fabra et al., 1997) which are considered secondary and symptomatic responses.
I hypothesized that 2,4-D has sub-lethal effects on microbes which may arise as direct or secondary responses leading to changes in morphology and behavioral characteristics such as biofilm formation and locomotion with underlying molecular mechanisms that are associated with stress resistance. I chose *Rhizobium leguminosarum* bv. *viciae* (*Rlv*), a beneficial soil bacterium, and *Escherichia coli* (*E. coli*), a diversly occurring, well studied bacterium as model organisms to explore my hypothesis.

1.3 *Rhizobium leguminosarum* bv. *viciae*

Rhizobia are a group of rod shaped, aerobic, Gram negative soil bacteria belonging to the class Alphaproteobacteria and family *Rhizobiaceae* that form a symbiotic relationship with leguminous plants. During the symbiotic relationship they form specialized structures called root nodules within which *Rlv* forms a differentiated phenotype called the bacteroid, capable of converting atmospheric nitrogen to ammonia available for plant uptake. *Rlv* are highly robust, with a large genome that provides a selective advantage in the highly heterogenous soil environment. This bacterium survives in the soil environment for long periods of time between symbiotic phases (Young et al., 2006). The cytoplasm of this Gram negative bacterium is surrounded by an inner membrane and then peptidoglycan consisting of disaccharide tetrapeptide subunits with GlcNAc, MurNAc, alanine, glutamic acid, and 2,6-diaminopimelic acid. The organism produces large amounts of LPS (lipopolysaccharide), associated with the outer membrane, and secreted polysaccharides consisting of CPS (capsular polysaccharide) and EPS (extracellular polysaccharide) (Skorupska et al., 2006). Cellulose microfibrils which extend to the
surface are covalently connected to the peptidoglycan. The cell envelope in *Rlv* therefore is robust, providing resistance to a wide variety of external stressors.

### 1.4 *Escherichia coli*

*E. coli* is also Gram negative, belonging to the class Gammaproteobacteria and family *Enterobacteriaceae*, a common inhabitant of the mammalian intestine. It is a morphologically simple rod-shaped bacterium and is used as a model prokaryote for a number of reasons. It is found in diverse ecological niches, is easy to genetically modify due to its simple genome and is widely used as a host for biotechnological applications (Clermont et al., 2000; Higgins et al., 2007; Touchon et al., 2009). *E. coli* consists of four distinct phylogroups based on their ecological niches, life history and propensity to cause disease, namely A, B₁, B₂ and D. Groups B₂ and D are mostly recognized as environmental strains, whereas A and B₁ are intestinal inhabitants (Gordon et al., 2008; Walk et al., 2007). *E. coli* BL21 has been widely used as a research model for phage sensitivity, restriction systems, and bacterial evolution in laboratories and as a major workhorse for protein expression in the biotechnological industry (Jeong et al., 2009) and therefore has been genetically modified to meet the desired applications.

The primary focus of this research was to explore the global effects of the herbicide 2,4-D at sub-lethal concentrations on *Rlv* and *E. coli* using advanced microscopy and metabolomics. I expected subtle morphological, ultrastructural and physical effects on these bacteria exposed to 2,4-D in a range of sub-lethal concentrations, indicative of first-line responses of the microbes or secondary effects as a result of intracellular molecular changes. I chose bright field light microscopy (LM) to reveal cell-cell interactions and
overall cellular morphology at low resolution, scanning electron microscopy (SEM) to highlight morphological effects at higher resolution with a wide field of view, and atomic force microscopy (AFM) to report on the surface ultrastructure, chemical and physical properties.

Metabolomics was used to determine the metabolite fingerprint associated with the biochemical impact of 2,4-D stress on cells, which may differ from first-line responses at the cell surface. Genomics (Touchon et al., 2009), proteomics (Han and Lee, 2006) and metabolomics (Rabinowitz, 2007) profiling platforms have been relatively well established for *E. coli* and the study had the added benefit of developing a suitable metabolite profiling technique for *Rlv*. Therefore working with *E. coli* and *Rlv* simultaneously helped facilitate the proposed work.

**1.5 Methods to assess the effects of xenobiotics at sub-lethal concentrations**

**1.5.1 Microscopy**

Microscopy has been a standard tool for determining morphological effects of xenobiotic compounds. Revolutionary progress in this field over the past several decades has led to a much wider resolution range.

As the oldest microscope design developed in the 16th century, LM uses light and a system of lenses with objectives and condensers to magnify microscopic objects. LM is routinely used to examine the overall morphology of cells at low resolution. Based on simple sample preparation and fast image acquisition rates, specimens can be examined in their native and preserved state. LM with its different modalities such as bright-field,
dark field and differential interference contrast find wide application in biology, but most of the conventional instruments are diffraction limited to 0.2 µm (Murphy and Davidson, 2013). LM is advantageous in requiring minimal sample preparation, while fluorescence microscopy (FM) uses a range of fluorophores, that when excited with a suitable wavelength of radiation, emit light of a longer wavelength. FM when used with appropriate fluorescent dyes, antibodies or protein conjugates can provide information on sample thickness, biomolecular localization, composition, and dynamics (Webb and Brown, 2013).

Confocal microscopy (CM) represents a major development from conventional wide-field FM, by using a suitably placed “pin-hole” that eliminates out-of-focus light from the image focal plane. Thus CM provides fluorescent images with exceptional resolution very close to the diffraction limit. CM has the ability to provide ‘optical sections’, to produce images in the Z-axis allowing one to visualize into cells and tissues, thus enabling 3-dimensional (3-D) image rendering (Mourilino-Perez and Roberson, 2015; Shaner, 2014). Initially CM used fixed specimens labelled with fluorescent dyes, but with the introduction of fluorescent proteins (FPs) and non-toxic dyes the imaging has been extended to live specimens, vastly increasing its applications in biology (Dailey et al., 2006). Diverse FPs with a wide range of excitation and emission spectra have been developed over the past decade allowing one to tag multiple proteins with multicolored FPs and CM to track their localization and dynamics in real-time (Shaner, 2014). With major improvements to the confocal system, CM is an excellent tool to determine chemical identity and localization of biomolecules, and their intermolecular interactions. Currently laser scanning confocal microscopy (LSCM) is the most popular confocal
mode. A suitably placed pinhole not only removes unwanted out-of-focus light, but also restricts the focal plane to micron size to produce optical sections while a laser beam scans the specimen point-to-point. Here, only the information from the plane of interest reaches the photodetector producing clear and crisp images (Paddock and Eliceiri, 2014).

SEM is a surface scanning method that allows the visualization of bacterial phenotypes over wide ranges in field of view, and has been widely used for the 3-D structural analysis of biofilms, mammalian tissue and abiotic surfaces (Duckett and Ligrone, 1995; Toyshiba et al., 2006). SEM uses a focused beam of electrons to generate a variety of signals through electron-sample interactions that provide information on sample morphology, topography and other physical properties. Topography and morphology are generated from secondary electrons emitted from the specimen, creating crisp images with high lateral resolution but inaccurate height resolution. Since vacuum is required for SEM to prevent electron scatter, sample features are preserved by fixation prior to critical point drying, which together retain the three dimensional structure (Minoura et al., 1995; Ferrer et al., 2006). SEM and cryoSEM had been the gold standard for examining the bacterial surface ultrastructure, appearance of biofilms and morphological properties at high resolution (Bozzola, 2007).

The more recently developed AFM offers data similar to SEM, with unprecedented surface ultrastructural resolution, including accurate height measurements with nm resolution for microbes. AFM also provides the opportunity to probe physical and biochemical surface properties in real-time, either for fixed samples or those under physiological conditions (Muller and Dufrene, 2011). AFM requires minimal sample preparation, reducing the chance of introducing sample preparation artifacts. The ability
to image and make measurements of biochemical and mechanical properties of live cells at nanometer (nm) and picoNewton (pN) resolution, respectively, sets AFM apart from conventional surface scanning microscopy (Frederix et al., 2009).

The central component of AFM consists of a cantilever with a sharp tip attached to its free end which raster scans the sample surface. Small deflections in the cantilever are often measured using an optical lever. A laser is reflected from the top of the cantilever onto an array of photodiodes, with vertical deflections indicating variations in sample topography and lateral movements of the cantilever indicating tip-sample interactions (Gupta et al., 2012; Kirmizis and Logothetidis, 2010; Zeng et al., 2014). Vertical bending of the cantilever yields vertical deflection and lateral bending or twisting is detected as horizontal deflection by the photodiode (Eaton and West, 2010). A piezoelectric-based feedback mechanism maintains a constant force between tip and sample throughout the raster scan. Force spectroscopy measures the force of tip-sample interaction with pN sensitivity as the tip is pushed towards and retracted from the sample with a desired force. The sample and the cantilever mounted on piezoelectric scanners ensure highly accurate 3D positioning (Figure 1.2) (Kellermayer, 2011; Kirmizis and Logothetidis, 2010; Muller and Dufrene, 2011; Shi et al., 2012).

Extremely sensitive (low force constant) cantilevers can be used to measure the force of indentation (sample stiffness) or image delicate biological samples in buffer or physiological conditions and if the tip is functionalized with a molecule of interest, detect specific unbinding events (Riener et al., 2003). Since AFM provides topographical images at high resolution, it is an invaluable tool to investigate the effects of xenobiotics.
Figure 1.2 Schematic of AFM components and data output. The AFM has an atomically sharp tip that raster scans across the sample surface. The force of tip-sample interaction is monitored with piconewton sensitivity and measured using the feedback control loop based on signal from the photodiode directed to the electronic control unit (ECU). The sample and the cantilever are mounted on piezoelectric tubes that ensure 3-D positioning of the tip for raster scanning (x, y) and tip-sample distance (z). The laser reflected from the back of the cantilever and projected onto the photodiode measures cantilever deflection, with the position of the laser indicative of sample topography (vertical) and tip-sample interactions (horizontal) registered and recorded by the computer. Cantilever deflection can also be plotted during force spectroscopy, wherein the tip is pushed into the sample with a desired force and then retracted. A force curve is generated using the spring constant of the cantilever which can be used to obtain the Young’s modulus (slope of b-c) and adhesion (f-e).
on cell surface ultrastructure. The cell envelope acts as the first point of contact to xenobiotics, which may be capable of remodeling the cell surface, and AFM is an excellent tool to quantify these effects. AFM can also be used as a controlled vehicle to probe the cell surface with pesticide-coupled AFM tips to deliver a limited stimulus to the cell exterior, and when coupled with CM, the cellular changes to the stimulus can be simultaneously monitored (Koenig et al, 2012). As such, AFM has become a valuable tool for characterizing cellular changes in response to toxic exposure at very low levels. However the method is limited to probing the outer most surface of the cell. LSCM and biochemical methods, such as metabolomics and traditional assays, are more suited to probing intracellular changes.

To simultaneously probe the inside and outside of live cells, I developed a novel high-content microscopy assay. AFM and LSCM are both powerful tools for probing different aspects of a cellular system, and when integrated for simultaneous data collection they provide an efficient platform for examining bacterial response mechanisms. AFM provides exceptional spatial resolution that is not diffraction limited, but as a surface scanning microscope its temporal resolution is limited by the need to raster scan. AFM generates high content data describing not only sample surface ultrastructure at the nm-scale but also physical properties (for detailed application notes, see Muller and Dufrene, 2011). On the other hand, LSCM is light based and so diffraction limited, but produces images with excellent temporal resolution (Kondra et al., 2009). Integrating AFM with LSCM offers nm-scale spatial and biochemical resolution, pN-scale mechanical resolution of surfaces and real-time imaging of intracellular physiology of live cells in situ. Live quantitative imaging (QI) by AFM is the least invasive of all surface imaging
methods, based on its superior control over vertical forces and minimal lateral forces (Chopinet et al., 2013), providing data rich images with force curves of pN sensitivity at each pixel that reflect cell surface adhesion and elastic properties. I have physically integrated AFM-QI-CLSM for simultaneous correlative imaging to examine the specific response mechanisms of *E. coli* during exposure to 2,4-D.

**1.5.2 Metabolomics**

Metabolomics, or metabolic profiling, is a newly emerged bioanalytical tool which enables the comprehensive monitoring of global metabolite networks in response to various external stimuli (Fiehn et al., 2000; Trethewey et al., 1999). Metabolic profiling techniques have the ability to measure small metabolic fluctuations in the entire metabolome that are important to answer a specific biological question but do not necessarily lead to a symptomatic change in phenotype (Van der Werf, 2005). The analysis of an entire metabolome is believed to be an impossible task due to the extreme diversity of metabolites. Therefore, the ultimate goal in metabolomics is to identify as many metabolites as possible, achievable using sensitive, high resolution metabolite separation instrumentation and identification platforms.

Sample preparation is the crucial step in metabolite profiling and the quantity of metabolites depends on the type of sample preparation. Reproducible measurements require homogeneous temperature, humidity and environment when growing the organism of interest (Moco et al., 2007). Metabolite quenching, the first step in the sample preparation procedure, provides a metabolic snapshot in time by quenching (stopping) reactions and preventing further enzyme and metabolic activity. The few
metabolite quenching strategies that have been applied to several different bacteria and mammalian cells are highly laborious. The lack of efficient quenching techniques is based on high metabolic reactivity and so the inability to inactivate metabolism faster than the rate of metabolic changes (Álvarez-Sánchez, et al 2010).

Metabolite extraction, a key step directly affecting the final data quality, is used to separate metabolites from other cellular components (i.e. nucleic acids, proteins, cell debris). An efficient extraction method should completely release metabolites from cells, concentrate the sample and remove compounds (i.e. salts, proteins) that interfere with the chosen analytical technique (Álvarez-Sánchez, et al., 2010). There are several extraction methods (Grivet et al., 2003; Mashego et al., 2007; Dunn and Ellis, 2005), but efficient metabolite quenching and extraction techniques must be tailored to target various classes of metabolites in specific organisms, so there is no “one-size fits all” strategy (Beger et al., 2010).

Selecting a sensitive analytical platform to identify diverse metabolites is crucial for accurate metabolite profiling. Mass spectrometry (MS), which separates molecules based on the mass-to-charge ratio \(m/z\), is a well-established tool for the analysis of diverse chemicals and biomolecules (Dunn and Ellis, 2005). The advent of liquid chromatography-MS (LC-MS), LC-electron spray ionization-MS (ESI-MS), gas chromatography MS (GC-MS), matrix assisted laser desorption ionization-time of flight-MS (MALDI-TOF-MS), along with a growing interest in capillary electrophoresis-MS (CE-MS) has provided a blend of rapid, sensitive and selective qualitative and quantitative analyses of metabolite profiles (Dunn and Ellis, 2005). The applications of various MS platforms have been reviewed (Aliferis and Jabaji, 2011; Issaq et al., 2009;
GC-MS is the most commonly used of all MS modes based on its robustness, availability of metabolite libraries, high sensitivity, automatic spectral deconvolution, metabolite identification and affordability. GC-MS yields extensive and highly reproducible fragmentation due to the standardized use of electron ionization, identification of metabolites by matching their Kovats indices and fragmentation patterns available from the library databases (e.g. NIST) (Garcia et al., 2008). A major drawback of GC-MS is that metabolites need to be volatile and so analytes must be derivatized. Moreover, thermally labile compounds such as phosphorylated metabolites can degrade easily at high temperatures inside the GC oven (Wittmann, 2007). Varying affinities of metabolites for derivatizing agents can lead to inaccurate identification, and derivatized chemical standards and strategies are required to correct the bias (Kanani and Klapa, 2007).

Metabolomics produces rich data that does not make it possible to directly draw conclusive results. A strong chemometrics support component is used to develop statistical pattern recognition models, achieve optimal characterization of the samples and detect biomarkers from diverse, highly dimensional data sets for a high level functional interpretation (Beger et al., 2010). Supervised and unsupervised tests such as the principal component analysis (PCA) and partial least squares and discriminant analysis (PLS-DA) respectively are widely applied to the multidimensional and inherently complex metabolomics data. Such methods not only simplify the data by reducing dimensionality but also offer a visual representation (Masoum et al., 2006; Scholz et al., 2005).
Various software and web-based tools are available for metabolite pathway and enrichment analysis (see review (Booth et al., 2013)). MBrole (http://csbg.cnb.csic.es/mbrole/) is a free, powerful and easy to use web-based platform that takes a list of metabolite KEGG IDs and provides a tabulated output with enriched pathways. MBrole’s metabolite libraries for diverse organisms make pathway investigation convenient.

1.6 Research Objectives

Bacteria have evolved several adaptation systems to survive external stresses. With the increased daily disposal of new anthropogenic chemicals into the environment, it is important to understand how they impact microbes, one of the main components of our ecosystem. In this research *E. coli* and *Rlv* were used as model bacteria to characterize their adaptation mechanisms to 2,4-D stress. The herbicide 2,4-D was chosen as it is one of the most commonly used agrochemicals in Saskatchewan and world-wide, therefore the results of this study will have broad environmental implications. The overall aim of this research was to characterize the specific stress response mechanisms of *E. coli* and *Rlv* exposed to 2,4-D at sub-lethal levels. The specific objectives of this research include;

(I) exploring the general effects of 2,4-D at sub-lethal concentrations in *Rlv* and *E. coli* and to help uncover the associated mechanism(s) (Chapter 3 and 4 respectively)

To fulfill this objective, I used a combination of microscopy and metabolomics to determine the sub-lethal effects of 2,4-D on the overall morphology, surface ultrastructure, physical properties and physiology of *Rlv*
and *E. coli*. For metabolomics, I collaborated with Sean Booth (Ph.D. candidate, U. Calgary) who assisted with the treatment and statistical analysis of the GC-MS data.

(II) characterizing the specific mechanisms of 2,4-D impact in *E. coli* (chapter 5)

To do so, I developed a high content microscopy assay to assess the impact of 2,4-D in live bacteria. An integrated microscopy assay using correlative AFM-CLSM was used to simultaneously codify surface ultrastructural, physical and biochemical changes along with internal cellular physiology in real-time. The technique physically integrated AFM-QI with CLSM to simultaneously probe alterations in cell surface properties along with molecular changes inside the cells. This technique overcomes the limitations of each individual technique and provides high content data of cell response mechanisms with broad application to other cellular systems and xenobiotics. In this report I describe the AFM-QI-CLSM technique, which I successfully applied to characterizing sub-lethal 2,4-D response mechanisms in *E. coli*.

1.7 Bibliography


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CHAPTER 2 - GENERAL MATERIALS AND METHODS

Parts of this chapter are published or currently under review in open access journals,


Bhat, S. V.; Kamencic, B.; Kornig, A.; Dahms, T. E. S. Live cell imaging of real-time cell division and envelope remodeling in *Escherichia coli* exposed to 2,4-dichlorophenoxyacetic acid, *App. Env. Micro*. Manuscript # AEM01511-16 (In revision)
This chapter describes the methods and materials that are relevant to all chapters in the thesis.

2.1 2,4-dichlorophenoxyacetic acid

The 2,4-D amine formulation (w/w- 84.21 % 2,4-D, 0.5 % Triton-X-100, 1.5 % EDTA, 1.41 % of 60% dimethylamine solution, and 12.38 % water; analysis by Interprovincial Cooperative Limited (Agri Products Department, Winnipeg, Canada)) was purchased from Viterra, Canada. All other chemicals were analytical grade and purchased from Sigma-Aldrich, unless otherwise noted. Sterile deionized water (18 MΩ, Barnstead Nanopure, Thermo Scientific) was used for media and sample preparation.

2.2 HPLC analysis of 2,4-D

2,4-D was analyzed by reverse phase HPLC to confirm its stability in the context of the formulation and in water used for media preparation. The 2,4-D formulation was diluted in water (5 mM), incubated (24 h) and analyzed by reverse phase HPLC (HP/Agilent 1050 series) using a C18 (4.6 mm i.d. × 150 mm length, 3.5 µm particle size) column (Agilent) to assess chemical stability under culture conditions. Standard sample and test samples (100 µL; 0.0001 ppm) were injected, separated by gradient elution (water: 0-15 min to 100% acetonitrile over 30 min) at a flow rate of 1 mL/min and detected by UV absorption (λ = 228 nm).

HPLC analysis indicated that 2,4-D (RT = 43.3 min) within the formulation is stable, but after a 24 h incubation in water, approximately 89% of the 2,4-D had been dissociated into its more hydrophilic anion (RT = 16.1 min) at neutral pH.
2.3 Strains and growth conditions

All the strains and their sources used in this study are listed in Table 2.1.

*Rhizobium leguminosarum* bv. *viciae* 3841 (*Rlv*) was maintained on Ca$^{2+}$ rich tryptone-yeast-streptomycin (TY-st) media (Beringer, 1974) (5 g/L Tryptone, 3 g/L yeast extract and 0.5 g/L CaCl$_2$$\cdot$2H$_2$O) at 30 °C. A 24 h culture was used as a stock for all inoculations.

*E. coli* BL21 (DE3) and environmental strains NECD 1, 2, 3 and 4 were maintained on lysogeny broth-ampicillin (LB-amp, 100 µg/ml) and LB agar alone, respectively, at 37 °C. A 24 h overnight LB/LB-amp broth culture was used as a stock for all inoculations.

The *Escherichia coli* wild type parent strain WM1074 and its mutants WM2026 (WM1074+stable chromosomal fusion FtsZ-GFP), WM2760 (WM1074 containing pWM2760) and WM2739 (WM1074+stable chromosomal fusion SulA-GFP) were a kind gift from Dr. William Margolin (Geissler et al., 2007). WM2026, WM1074 and WM2739 were routinely grown on LB broth at 32 °C and WM2760 was grown under the same conditions but with 100 µg/mL ampicillin. WM2026 and WM2760 were induced with 40 µg/mL and 10 µg/mL of IPTG respectively, two hours prior to harvest. An overnight culture was used as a stock for inoculating into all the test samples. A formula control, consisting of all formulation ingredients except 2,4-D, and sample controls containing deionized water in place of formulation, were tested in parallel.

2.4 MIC assays

The MIC (minimum inhibitory concentration) of the 2,4-D formulation (Viterra, Canada) for *Rlv* and *E. coli* strains was determined by growing strains in their respective liquid
Table 2.1 List of strains used in this study.

<table>
<thead>
<tr>
<th>Name of the strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium</em> <em>leguminosarum</em> bv. <em>viciae</em> 3841</td>
<td>Spontaneous streptomycin-resistant derivative of <em>R. leguminosarum</em> bv. <em>viciae</em> strain 300</td>
<td>Johnston and Beringer, 1975</td>
</tr>
<tr>
<td>E. coli BL23 (DE3) pET21b</td>
<td>Laboratory strain with pET21b plasmid</td>
<td>Stavrinides et al., 2010</td>
</tr>
<tr>
<td>E. coli NECD1</td>
<td><em>E. coli</em> isolated from water samples collected from Wascana Lake, Regina</td>
<td>Afroj, 2014</td>
</tr>
<tr>
<td>E. coli NECD2</td>
<td><em>E. coli</em> isolated from water samples collected from Wascana Lake, Regina</td>
<td>Afroj, 2014</td>
</tr>
<tr>
<td>E. coli NECD3</td>
<td><em>E. coli</em> isolated from water samples collected from Qu’appelle, near Lumsden, Regina</td>
<td>Afroj, 2014</td>
</tr>
<tr>
<td>E. coli NECD4</td>
<td><em>E. coli</em> isolated from water samples collected from Qu’appelle, near Lumsden, Regina</td>
<td>Afroj, 2014</td>
</tr>
<tr>
<td>E. coli WM1074</td>
<td>Parent environmental strain</td>
<td>Geissler et al., 2007</td>
</tr>
<tr>
<td><strong>E. coli WM2760</strong></td>
<td><strong>E. coli WM1074 with</strong>&lt;br&gt;plasmid pDSW209-FtsA&lt;br&gt;encoding FtsA-GFP</td>
<td><strong>Geissler et al., 2007</strong></td>
</tr>
<tr>
<td>-------------------</td>
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<td>--------------------------</td>
</tr>
<tr>
<td><strong>E. coli WM2026</strong></td>
<td><strong>E. coli WM1074 with</strong>&lt;br&gt;chromosomally tagged&lt;br&gt;FtsZ-GFP with an IPTG&lt;br&gt;inducible promoter</td>
<td><strong>Geissler et al., 2007</strong></td>
</tr>
<tr>
<td><strong>E. coli WM2739</strong></td>
<td><strong>E. coli WM1074 with</strong>&lt;br&gt;chromosomally tagged&lt;br&gt;SulA-GFP</td>
<td><strong>Geissler et al., 2007</strong></td>
</tr>
</tbody>
</table>
media culture for 24 h containing increasing concentrations (0-10 mM) of filter sterilized 2,4-D formulation. The lowest concentration showing complete absence of growth (OD 600 nm = 0) after 24 h incubation indicates the MIC. A formula control, consisting of all the formulation ingredients except 2,4-D, and negative controls containing deionized water in place of formulation, were tested in parallel.

2.5 Microscopy

Microscopy was used to examine different phenotypes, with bright field LM showing overall phenotypic changes at low resolution, epifluorescence and confocal for tracking fluorescently labeled molecules in different phenotypes of live and fixed cells. SEM was used examine fixed cell phenotypes at high resolution along with their 3D architecture and AFM for phenotypic imaging at ultra-high resolution while also probing the physical properties and surface topography of live and fixed cells.

2.5.1 Sample preparation for microscopy

Standard sized coverslips (22 mm × 22 mm) were cleaned (by sequentially immersing in 1 M HCl for 1 min, piranha solution -3:1 v/v of H2SO4:H2O2, for 1 h, rinsed with deionized water, immersed in methanol and acetone for 2 min and air dried) and coated with poly-L-Lysine (PLL, incubated 10% diluted PLL on cleaned glass coverslip for 5 min, air dried) (Dong et al., 2011) onto which approximately 500 μL of a suitable concentration of 2,4-D-treated Rlv and E. coli and control overnight broth cultures (OD ~ 1) were pipetted, incubated (30 min), rinsed with water and stained for optical microscopy (crystal violet 0.1 mg/ml). Samples were imaged by light microscopy.
Dose-response relationships at higher structural resolution were based on suitable sub-lethal concentrations chosen by LM. Coverslips were incubated with culture, rinsed (0.5 M, pH 7 phosphate buffer) and fixed (3.7% formaldehyde, 0.2% Triton-X-100 in phosphate buffer pH 7, 10 min), rinsed with water and air dried. Samples were substituted (20–100 % EtOH, v/v in water) for 10 min, mounted on stubs with carbon tape, copper strips attached and sputter coated with gold (SC7620 mini sputter coater, Emitech, 120 s) for SEM imaging. Sample preparation for AFM was similar to that for SEM but without substitution, drying and coating. Following fixation, coverslips were dried overnight.

**2.5.2 Bright field microscopy**

Samples prepared on glass coverslips or slides were imaged by light microscopy (Olympus BX51) at 10 ×, 40 × and under oil immersion (1000 ×) and digitally captured (Olympus DP70). Images were collected for at least ten fields of view for each sample and processed with Image J. A minimum of three replicates were imaged for each test sample.

**2.5.3 Epifluorescence microscopy**

Samples labeled with a suitable fluorescent dye or FP marker were mounted on high precision glass coverslips (Zeiss). The specific excitation and emission spectra were chosen using the Zen software (Zeiss) based on fluorophore emission and excitation maxima. The transmission illumination was used to bring the sample into focus followed
by illumination with excitation light. The gain and exposure time were adjusted to obtain crisp images with the least amount of background signal. Images saved in Zen format were processed to reduce background by adjusting the intensity profile and then saved as tif files.

2.5.4 Laser scanning confocal microscopy

Confocal image collection was similar to that of epifluorescence, but with more options such as Z-stacking and time lapse. Z-stacks of sample height (Z-dimension), especially useful for 3D and thick samples, are made possible by the pin-hole technology which also eliminates far-field signal (background) unlike epifluorescence. Z-stacks were collected by choosing the highest and the lowest point of interest in the sample using the fine focus knob and by defining the number of desired sections (Zen software, Zeiss). Time lapse was used to examine changes in fluorophore localization over a user-defined period of time and selected number of images.

2.5.5 Scanning electron microscopy

Samples were imaged using SEM (JEOL JSM-6360) at 8 keV in secondary electron mode. Samples were mounted on the SEM sample stage and the sample chamber was evacuated to 10^-6 Torr. Once the vacuum level in the chamber dropped below 7.5×10^-5 Torr, the high voltage electron beam was established. The sample stage was positioned using the X and Y adjustment screws or joystick, such that the beam was directed on the substrate using the LCD monitor window. The sample was scanned manually at low magnification to determine the desired area on the sample, and subsequently at high
magnification or focused using the ‘autofocus’. The desired scan area, contrast and brightness, scan speed and voltage were set to optimize imaging. Contrast determines sample edge definition for sharp, high contrast images, and increasing the scan speed does the opposite and therefore the two parameters were balanced for the best results. Images were captured and stored as tif or JPEG image files.

2.5.6 Atomic force microscopy

Samples were imaged (minimum of 3 different samples, 2 areas on each sample and 10 bacteria) using an Explorer™ AFM in contact mode equipped with cantilevers (k ~ 0.05–0.5 N/m; v ~ 15–30 kHz) having silicon nitride (Si₃N₄) tips (MLCT-EXMT-A1, Veeco, Inc.). Samples were sometimes fixed to expedite imaging of clearly defined surface features (Kaminskyj and Dahms, 2008; Ma et al., 2005; Paul et al., 2011). Contact mode was used to obtain high resolution topography images of fixed bacteria. Glass coverslips of samples were mounted on the AFM stage and a desired area was chosen by viewing the sample through the optical microscope on which the AFM was mounted. The optical display was also used to precisely position the AFM cantilever on the desired area of the sample and to focus the laser onto the cantilever cross hairs. The laser was subsequently positioned to the center of the photodiode using the vertical and lateral deflection knobs controlling the mirror, which was centered to obtain the optimal output (sum). The cantilever was calibrated using the thermal noise method (Lubbe et al., 2013). Briefly, the free vertical fluctuations of the cantilever due to its natural thermal environment are plotted against the frequency, while the area under the curve is used for calculating the cantilever spring constant. Imaging was initiated after determining a suitable scan area,
scan speed, set point and gains. The line scan function that allows the scanner to scan the same line continuously was used to detect noise and optimize parameters accordingly, while monitoring the set point and adjusting the feedback gains to minimize noise. The sample was imaged at low magnification (i.e. 50 × 50 µm; 200 × 200 pixels) and a desired area was chosen for a high resolution image (i.e. 1 × 1 µm; 500 × 500 pixels).

Force spectroscopy (FS) and QI data was collected using a NanoWizard® 3 AFM (JPK, Germany) in contact mode with silicon nitride (Si$_3$N$_4$) cantilevers (HYDRA2R-50NG-10, AppNano, $k = 0.092 \pm 0.03$ N/m, nominal tip radius <10 nm). For FS, the cantilever approached the sample from a height of 1 µm, using a constant approach velocity (0.1 µm/s) in triplicate at three spots on the center axis of at least 30 bacterial cells.

2.5.7 Image processing and analysis

LM and SEM images were processed for sharpness and contrast while average cell lengths ± standard deviation (SD) were determined using ImageJ (http://rsb.info.nih.gov/ij/) from 100 cells per sample. Histograms were plotted using GraphPad Prism (Version 5.0, GraphPad, La Jolla, CA, USA). FM images were processed to remove background and adjust contrast and intensity (Zen image processing software, Zeiss).

AFM images were leveled and adjusted for optimal contrast, while the shadowing effect was used only for visual clarity. Subunit size was measured from full width at half maximum (FWHM) of peak height, and surface roughness calculated (SPMLab Version 5.01 software) from ten different bacterial surfaces for at least three different samples based on the following equation,
\[ R_a = \frac{1}{n} \sum_{i=0}^{n} |Z_i - \bar{Z}| \] ;

where \( \bar{Z} \) is the average height of surface features and \( Z_i \) is the height of each surface feature.

Cantilever deflection was converted to force (JPK image processing software) using the cantilever spring constant, and surface adhesion values were plotted using GraphPad Prism.

Young’s modulus was calculated using the Hertz model (JPK software) (Lin et al., 2007) for a tetrahedral tip,

\[ F = \frac{E}{1-\nu^2} \frac{\tan \alpha}{\sqrt{2}} \delta^2 \quad \alpha = \frac{\tan \alpha}{\sqrt{2}} \delta \] ;

where \( E \) is the Young’s modulus, \( \delta \) is the indentation, \( \nu \) is Poisson’s ratio (0.5 for biological samples) and \( \alpha \) is the face angle of the cantilever.

A paired student’s t-test (GraphPad Prism) was used to assess differences between treated and control samples.

### 2.6 Reactive oxygen species assay

Intracellular reactive oxygen species (ROS) was measured using the ROS-sensitive probe 2, 7-dichlorodihydrofluorescein diacetate (DCFDA) according to Perez et al. (2008)

Briefly, \( Rl/v \) cells were grown for 24 h in the presence of 0–0.4 mM of 2,4-D to an OD ~ 1, harvested (12,000 × g, 10 min), washed and suspended in 0.1 M potassium phosphate buffer containing 10 μM DCFDA in DMSO, and then incubated for 30 min in the dark at
30 °C in a rotary shaker (200 rpm). Cells were harvested, washed and lysed by sonication on ice for 2 min in 30 s/1 min on/off cycles in the dark, 200 µL of the supernatant was transferred to a 96 well plate and the fluorescence measured using a BioTek microplate reader (Winooski, VT, US; $\lambda_{\text{ex}} = 485$ nm; $\lambda_{\text{em}} = 528$ nm). The assay was repeated a minimum of three times.

### 2.7 Metabolite quenching, extraction and analysis

Six replicates of *Rlv* or *E. coli* BL21 (DE3) treated with formula control, control and 2,4-D were grown overnight in respective media. Cells were quenched and extracted according to Booth et al. (2011). Briefly, *Rlv* cells were grown to ~ 1 OD and 50 mL of the culture was quenched with 150 mL of precooled 60 % methanol (-20 °C) in centrifuge bottles that were balanced, and centrifuged (12,000 × g; -20 °C) for 15 min. The supernatant was discarded and the pellet was washed, weighed and stored at -80 °C. Samples were maintained at ≤20 °C using a cold ethanol bath during the entire quenching procedure.

Pellets were thawed on ice and resuspended by pipette into 300 µL methanol-chloroform (2:1). Samples were transferred to fresh microfuge tubes and cells lysed by sonication for 1 min with 10 s on/off cycles. To this, 100 µL of each layer from a chloroform/water solution was added and mixed gently by inversion. The aqueous and organic layers were separated by centrifugation in a refrigerated microfuge (10,000 × g 4 °C) for 7 min. The top aqueous layer was transferred to a clean tube and centrifuged again to remove any remaining organic phase. Aqueous phase (100 µL) was placed in a separate tube, dried (SpeedVac, (Savant-Thermoquest, DDATM)) and stored at -80 °C.
Samples were thawed, treated with methoxylamine hydrochloride (50 µL; 20 mg/mL), incubated (2 h; 37 °C, 25 rpm), silylated (Booth et al., 2011) using N-methyl-N-trimethylsilyl trifluoroacetamide (50 µL MSTFA), incubated on a shaker (45 min, 37 °C, 25 rpm), diluted (400 µL hexane), centrifuged (7 min, 14,000 × g) and 200 µL transferred to a gastight vial. Sample and alkane standards (1 µL) were injected (splitless injection; 275 °C) onto a GC-MS (Waters GCT premier MS) equipped with a DB5-MS column (30 mm × 0.25 mm, i.d. 0.25 µm) for separation (80 °C for 1 min, increased by 12 °C min⁻¹ to 320 °C, held 8 min) and analysis (range = 50–800 m/z), with helium used as the carrier gas (1.2 mL/min).

2.8 Spectral processing and multivariate statistical analysis

The GC-MS spectra were converted to net-CDF files, peaks detected and compounds identified using the metabolite detector (MD) software tool (Hiller et al., 2009). The raw GC-MS spectra in net-CDF format can be directly imported into MD. The baseline can be analyzed and shifted for each spectrum, as necessary. Deconvolution is used to extract the mass spectra which are used to calculate the Kovat’s retention index (RI) for each compound based on the reference spectrum of a standard alkane mixture (C_{10}–C_{30}). A standard reference library was imported, the RI calibrated, metabolites identified and batch quantified. The integrated tabulated data with RI, peak area and identified compounds was exported. Metabolites found in at least 60% of the replicates were subsequently analyzed, with remaining missing values imputed by k-means nearest neighbor (KNN) (Hrydziuszko and Viant, 2012). Tabulated data files were imported to Simca (Simca-12, Umetrics), and the variables (treatments, metabolites) and observations
(metabolite quantity) were defined. The data was normalized using median fold change, where the observations were normalized into the interval given by the median start and endpoints of the batches. The data was centered and unit-variance scaled for multivariate statistical analysis, i.e. each variable is divided by its standard deviation so that all variables are given equal weight. General clustering trends and metabolite differences were assessed with unsupervised PCA. PCA finds trends and correlations in the data set and reduces dimensionality in the data by finding components in the order of decreasing importance. In this case, the metabolite data sets were grouped based on their similarity and the resulting data projected into a plane geometric plot with co-ordinates showing data trends and outliers.

The differences in the control and treated sample data sets were further examined using supervised orthogonal partial least squares discriminant analysis (OPLS-DA). OPLS is a prediction method that uses known information about the data for analysis, i.e. here the data is pre-labeled into groups. Models are generated showing correlations between the data sets and good models were confirmed using model diagnostics ($R^2$ and $Q^2 \sim 1$) (Wishart, 2009). The reliability and significance ($p < 0.05$) of the OPLS models were tested with seven-fold cross validation analysis of variance (CV-ANOVA). OPLS-DA also provides correlation coefficients ($p_{corr}$) showing how the variables (in this case metabolites) vary between sample sets, whether they increase in both or increase in one while decreasing in the other. The correlation is represented with a positive or a negative sign and lies in the range -1 to +1. Variable influence on projection (VIP) values indicate the importance of the variable in generating the model, i.e. higher values represent increased importance, therefore metabolites with VIPs > 1 were used for data
interpretation. OPLS analysis output included filtered tabulated data showing metabolites and correlations for different models (control vs. treated, control vs. formula and treated vs. formula), VIP scores and scores plots for visualization. Shared and unique structures (SUS) plots showing common and unique trends were plotted using Graph Pad Prism 5.

KEGG (Kyoto Encyclopedia of Genes and Genomes) IDs were obtained for all the metabolites and uploaded to MBrole (csbg.cnbc.csic.es/mbrole/), and metabolic pathways most representative of detected metabolites were determined (MBrole pathway enrichment analysis) (Chagoyen and Pazos, 2011).

2.9 Bibliography


Dong, J.; Signo, Karla, S, L; Vanderlinde, E., M; Yost, Christopher, K. and Dahms, Tanya, E, S. Atomic force microscopy of a ctpA mutant in Rhizobium


CHAPTER 3 – RHIZOBIUM LEGUMINOSARUM BV. VICIAE ADAPTS TO 2,4-DICHLOROPHENOXYACETIC ACID WITH “AUXIN-LIKE” MORPHOLOGICAL CHANGES, CELL ENVELOPE REMODELING AND UPREGULATION OF CENTRAL METABOLIC PATHWAYS

The majority of this chapter is published in PLoS One.


GC-MS of the metabolomics samples and part of the statistical analysis were performed by Sean Booth in the Turner laboratory at the University of Calgary.
3.1 Introduction

Environmental disturbance both through anthropogenic and natural sources is a global issue, transforming habitats and creating stress for a wide variety of organisms. It is increasingly recognized that there is a need to assess the effects of chemical pollutants at the molecular level to better understand its impact on the environment (Moore et al., 2004). The herbicide 2,4-D, as one of the most widely applied herbicides in the world, is one example of an anthropogenic chemical pollutant. As a structural analogue of indole acetic acid (IAA, auxin), a naturally occurring plant hormone which induces cell growth, elongation and division, 2,4-D mainly targets dicotyledonous weeds. Unlike auxin, 2,4-D is stable and resistant to plant auxin degradation pathways and so accumulates in plant cells causing oxidative-induced damage, loss of membrane integrity, senescence, foliar damage, accumulation of abscisic acid and ethylene, and eventually plant death in sensitive dicots (Grossmann, 2010; Pazmino et al., 2012). This herbicide is known to cause carcinogenic free radical reactions, mutagenicity, birth defects, tissue defects, DNA damage and apoptosis, a wide range of negative impacts in mammals, fishes, birds and humans (Bukowska, 2005). However, the impact of 2,4-D at the molecular level in soil bacteria is only beginning to be understood (Chinalia et al., 2007).

Rhizobia are beneficial soil bacteria belonging to the family of α-proteobacteria, which fix atmospheric nitrogen by forming a symbiotic relationship with leguminous roots. Organisms of this genera are crucial for soil fertility as they are capable of infecting plant roots and forming nodules within which they convert atmospheric nitrogen to ammonia available for plant uptake (Mylona et al., 1995). This key symbiotic relationship can be affected by a number of factors, including abiotic chemical stresses such as fertilizers and
pesticides (Fox et al., 2007), making the study of such stressors on rhizobial physiology extremely important. Auxin is produced by free living rhizobia and is thought to play a role in nodule development based on its increased transport and accumulation at the site of nodule formation (Pii et al., 2007). Auxin and 2,4-D are known to induce nodular outgrowths, also called para-nodules on monocot roots with increased colonization of *Azospirillum brasilense* accompanied by a general increase in nitrogenase activity post treatment (Elanchezhian and Panwar, 1997; Jofre et al., 1996; Katupitiya et al., 1995). Evidence regarding the effects of 2,4-D on rhizobial nodulation is limited and controversial (Akao et al., 1991; Eberbach and Douglas, 1989) and does not examine the role of plant and rhizobia derived auxin on rhizobial physiology.

Rhizobia can be used as model soil bacteria which are capable of persisting for years in the soil environment between its symbiotic phases. Rhizobia are known to produce excess amounts of surface and exopolysaccharides as one of the main stress adaptation mechanisms to desiccation and other abiotic stresses (Janczarek, 2011). *Rhizobium leguminosarum* bv. *viciae* 3841 is a spontaneous streptomycin resistant mutant of a soil isolate for which the complete genome sequence became available during the past decade (Young et al., 2006). While this strain is possibly one of the most well studied of its genera, very few studies have investigated its stress signaling pathways (Corticeiro et al., 2006; Pereira et al., 2006).

Studies exploring the impact of 2,4-D on soil microbes indicate that it is capable of causing toxic responses in *Escherichia coli* (Balague et al., 2001; Balague et al., 2002), *Corynebacterium glutamicum* (Fanous et al., 2007), *Deftia acidovorans* (Benndorf and Babel, 2002), *Burkholderia sp.* (Cho et al., 2000), *Saccharomyces cerevisiae* (Simoes et
al., 2003; Teixeira et al., 2004; Viegas et al., 2005), *Pseudomonas putida* (Benndorf et al., 2006) and *Azospirillum brasilense* (Castro et al., 1996; Fabra et al., 1993; Jofre et al., 1996; Rivarola et al., 1992; Rivarola et al., 1992), but the molecular basis of its mechanism is not completely understood. In rhizobial species 2,4-D affects growth, protein content and membrane fluidity (Arias and Fabra de Peretti, 1993; Fabra de Peretti et al., 1992; Fabra et al., 1997). This study identifies the specific cellular pathways targeted by 2,4-D and the consequent phenotypic changes to *Rlv*.

For unicellular bacteria, biofilm formation and swarming are the two multicellular behaviors which enhance their survival in competitive environments. Chemicals such as (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone and resveratrol (3,5,4-trihydroxy-trans-stilbene) are known to inhibit this multicellular behavior by preventing quorum sensing in swarming bacteria (Ren et al., 2001; Wang et al., 2006). Swarmers are also known to be hyper-resistant to antibiotics and other xenobiotics (Tambalo et al., 2010). Therefore, swarming serves as a phenotypic indicator of the effects of 2,4-D on *Rlv*.

Metabolomics, a method that unbiasedly determines differences in metabolite profiles between treated and control sample sets, combined with AFM which determines changes in phenotype at ultra-high resolution, together constitute a novel approach for characterizing toxicological effects. Metabolomics detects the specific pathways altered by any given stimulus, establishing metabolic fingerprints of external stress factors (Zhang et al., 2010), while AFM can determine cell structure from mm to nanometer resolution and physical properties down to the pico-newton scale (Muller and Dufrene, 2011). AFM has been widely used to characterize the surface ultrastructural changes,
mechanical properties and single molecule force mapping in biological systems (Dong et al., 2011; Gupta et al., 2012; Kurland et al., 2012; Nan et al., 2013; Paul et al., 2011).

In agriculture, amine salts, alkali or esters of 2,4-D are applied at 0.2-2 kg ha\(^{-1}\) of active ingredient, while granular herbicides are used as aquatic herbicides at 1-122 kg ha\(^{-1}\) (Chinalia et al., 2007). While environmentally relevant levels of 2,4-D have been determined to be 5 mg kg\(^{-1}\) of soil (Zabaloy et al., 2010), bacterial exposure will depend on several environmental factors such as moisture, organic content and the presence of 2,4-D assimilating organisms (Boivin et al., 2005; Chinalia et al., 2007), such that soil bacteria are exposed to a range of sub-lethal concentrations.

In the present study, \(Rlv\) 3841 was exposed to sub-lethal levels of 2,4-D to determine its impact on the surface ultrastructure, physical properties and metabolism. This is the first study of its kind in this bacterium, providing useful insights into the mode-of-action of this herbicide and specific \(Rlv\) stress response pathways to 2,4-D exposure. Modifications in \(Rlv\) during 2,4-D exposure indicate a remarkable adaptation capability, changes to vital pathways of cell metabolism and consequent changes to the cell envelope and phenotype. 2,4-D induced phenotypic and ultrastructural changes were similar to those induced by auxin \textit{in vitro} and bacteroids directly isolated from pea root nodules, indicating that 2,4-D likely induces auxin-like non-target responses in \(Rlv\).

\subsection*{3.2 Materials and methods}

The 2,4-D amine formulation source, composition and HPLC analysis are described in chapter 2.
3.2.1 RiLV growth conditions and MIC assays

The RiLV media, growth conditions and MIC assays are described in detail in chapter 2.

3.2.2 Microscopy

RiLV were treated with 2,4-D (0–0.4 mM), IAA (0.4, 0.9 mM) and benzoic acid (0.4, 1 mM) and imaged using SEM and AFM to observe changes in phenotype, surface ultrastructure and physical properties. Force spectroscopy and QI imaging were done using AFM to quantify the Young’s modulus and adhesion, respectively. The SEM and AFM sample preparation, data collection and analysis are described in chapter 2.

3.2.3 Swarming assay

The swarming assay followed the method described by Tambalo et al. (2010a). Briefly, different amounts of filter sterilized 2,4-D was added to autoclaved swarming media containing 0.01% K₂HPO₄; 0.01% NaCl; 0.02% MgSO₄·7H₂O; 0.04% KH₂PO₄; 0.4% yeast extract; 0.1% mannitol and 0.7% Bacto agar, which was poured into plates and allowed to set for 24 h at room temperature. A 24 h TY stock culture of RiLV was used to stab inoculate the center of a plate which was incubated at room temperature for 3–4 weeks. After formation of a completely developed swarming colony, a suspension of swarmer cells was removed using two loops (~ 1 μL) of culture taken from the edge of a swarming colony. The swarmers were added to 1 mL of VMM (Vincent’s minimal medium (VMM; Vincent, 1970)) buffer (0.1 M, pH 7), mixed and deposited on coverslips, fixed using the procedure described in chapter 2, section 2.5 and imaged by AFM.
3.2.4 Plant assays

*Rlv* bacteroids were isolated from pea root nodules and imaged by AFM for phenotypic and ultrastructural comparison.

Nodulation assays were carried out with peas (*Pisum sativum* cv. Trapper) as the host legumes. Seeds were surface sterilized, germinated and planted as described previously (Yost et al., 1998). Briefly, peas were surface sterilized by washing in 50% bleach for 5 min, then with 70% ethanol, followed by three rinses in sterile distilled water. The seeds were germinated on water agar plates for 3 d and then transferred to modified magenta jars with pre-sterilized vermiculite substrate. After the transfer, the germinating peas were inoculated with 500 µL of *Rlv* culture (OD ~1). The inoculated peas were then grown for 5 weeks in the plant growth chamber, after which the nodules were harvested and surface sterilized to be used for microscopy.

To observe the morphology of bacteroids at high resolution they were imaged by AFM. Samples were prepared by crushing clean nodules onto PLL-coated coverslips between two clean, glass slides. After 5 min RT incubation, nodule debris was removed by a gentle rinse with buffer and coverslips were prepared and imaged by AFM as for the other samples.

3.2.5 Metabolite extraction and analysis

Six replicates of *Rlv* treated with formula control, control and 0.4 mM 2,4-D were used for metabolite quenching, extraction, GC-MS analysis. The associated protocols and statistical analyses are described in detail in chapter 2, section 2.9 and 2.10.
3.2.6 Intracellular reactive oxygen species assay

Intracellular ROS was measured using the procedure described in chapter 2, section 2.8.

3.2.7 Protein carbonylation assay

The carbonylated proteins were detected using the dinitrophenylhydrazine (DNPH) assay according to Semchyshyn et al. (2005). *Rlv* cells treated with 0–0.4 mM 2,4-D were grown for 24 h and the nucleic acid free cell extracts were treated with four volumes of 4,4’-dinitrophenylhydrazine (dissolved in 2 M HCl) and incubated at room temperature for 1 h, vortexing every 25 min. Protein was precipitated using 20 % TCA and pelleted by centrifugation (12,000 × g, 10 min). The pellet was washed three times using ethyl acetate:ethanol (1:1) to remove unreacted DNPH and then dissolved in 450 µL of 50 mM dithiothreitol (DTT) in 6 M guanidine HCl. Carbonyl content was determined spectrophotometrically at 370 nm.

3.2.8 MS and ICL enzyme assays

Malate synthase was assayed according to Ramirez-Trujillo et al. (2007) with minor changes. Briefly, *Rlv* was grown in 50 mL cultures in the presence of 0–0.4 mM 2,4-D until an OD of ~1 was reached. Cells were harvested by centrifugation (12,000 × g), the pellet washed with 100 mM Tris HCl and suspended in the same buffer containing protease inhibitors (protease inhibitor cocktail tablets, Roche) and sonicated on ice for 2 min in 30 s/1 min on/off cycles. Extracts were isolated by centrifugation (20,000 × g, 10 min) and MS activity determined by the glyoxylate-dependent release of free co-enzyme (CoA) from acetyl-CoA. The assay mixtures (0.2 mL, 96 well plate) contained 100 mM Tris HCl (pH
7.5), 10 mM MgCl₂, 2.5 mM glyoxylic acid, and *Rlv* extract. The reaction was initiated by addition of acetyl-CoA to a final concentration of 0.43 mM, incubated (room temperature, 10 min), terminated, color developed by the addition of Ellman’s reagent, 5,5’-dithiobis(2-nitrobenzoic acid) (Life technologies), and its absorbance measured (412 nm) using a BioTek microplate reader. Negative controls did not contain glyoxylate.

ICL activity was measured as described previously (Ramirez-Trujillo et al., 2007). The reaction buffer containing 50 mM MOPS, 5 mM MgCl₂, 5 mM cysteine HCl, 1 mM EDTA and 4 mM phenyldrazine HCl was added to *Rlv* extract, and isocitric acid added to a final concentration of 12.5 mM to initiate the reaction. The increase in the level of the phenylhydrazone derivative of glyoxylate was measured as absorbance at 324 nm. Negative controls lacked isocitrate.

Absorbance values for protein carbonylation, MS and ICL assay were normalized with protein concentrations determined with the Bradford method, and were repeated a minimum of three times for statistical validity. Statistical analysis was done using GraphPad 5.

### 3.3 Results

#### 3.3.1 Sub-lethal levels of 2,4-D alters phenotype, surface ultrastructure and elasticity

*Rlv* exposed to 2,4-D had a MIC of 0.6 mM and were able to grow at 0.4 mM 2,4-D with sufficient cell density to be used as the highest sub-lethal concentration for microscopy, enzyme assays and metabolomics studies. LM did not show observable morphological differences between the control and treated *Rlv*, however SEM images of *Rlv* cells
exposed to 0.4 mM 2,4-D exhibited a Y-shaped phenotype (Figure 3.1 C, E, F, G) some of which appeared to branch (Figure 3.1 B) at a frequency of 32 ± 11% compared to the formula (4.6 ± 1.1%) and control (3.8 ± 0.8%) samples (p < 0.0001, n ≥ 200) (Figure 3.2). These cells were much larger than the untreated rod-shaped phenotype.

*Rhizobia* grew in broth containing a maximum of 0.42 mM 2,4-D but had the capability to form well developed swarming colonies in media supplied with up to 1.47 mM 2,4-D. Preliminary data indicates that the unexposed swarmers took 3–4 weeks to develop a fully developed swarming colony, however the cells treated with more than 0.42 mM 2,4-D took 5–6 weeks. Exposed swarming colonies had completely different characteristics compared to those of the unexposed swarmers. The swarmers treated with 1.47 mM 2,4-D produced relatively smaller colonies with convoluted patterns, while unexposed swarmers produced smooth, nearly circular colonies (Figure 3.3). The treated swarmers also had rougher surfaces with irregular patterns when compared to the unexposed swarmers (Figure 3.4).

AFM indicated that *Rlv* treated with 0.4 mM 2,4-D had a two fold increase in surface roughness (12.12 ± 4.29 nm) compared to the formula (6.52 ± 1.5 nm) and sample controls (6.65 ± 1.5 nm) (n = 30, p < 0.0001). *Rlv* treated with 0.4 mM 2,4-D also exhibited extracellular material around the cells by SEM, which clearly appeared as a matrix by AFM (Figure 3.1 D, F and G). The e–f segment of the force retraction curve collected by force spectroscopy and quantitative imaging indicates adhesion between the cell surface and hydrophilic AFM tip, while the b–c segment of the approach curve was used to calculate Young's moduli representing envelope compliance (Figure 3.5 I, II). Cells treated with 0.4 mM 2,4-D showed significantly more (n ≥ 60, p < 0.0001) adhesion (15.9 ± 8 nN) compared to the formula (10 ± 3 nN) and the sample control (8.25 ± 3.16),
Figure 3.1 SEM (A-E) and AFM (F, G) images highlight the branched phenotype in 2,4-D treated *Rlv*. Many of the untreated (A) and treated (D) *Rlv* appeared normal but ~32% of the 2,4-D (0.4 mM) treated cells appear ‘Y’ shaped (C, E, F, G) and some of those appeared to branch (B). F and G show one (top) of the four adherent cells with clear demarcation for the initiation of the ‘Y’ shaped phenotype. Arrows indicate extracellular matrix on the substrate surface. Scale bar, A–E = 2 µm, F and G = 1 µm. (Taken from Bhat et al., 2015a)
Figure 3.2 Frequency of cells exhibiting an altered phenotype with each treatment.

Cells treated with 0.9 mM IAA, 0.4 mM IAA and 0.4 mM 2,4-D had significantly higher frequency of altered phenotypes compared to formula, control and benzoic acid controls (p < 0.0001, n ≥ 200). (Taken from Bhat et al., 2015a)
Figure 3.3 Preliminary work showing effect of 2,4-D on *Rlv* swarming colony. Image of a completely developed *Rlv* swarming colony on swarming media plate containing (A) 1.47 mM 2,4-D and (B) no 2,4-D after incubating for 5–6 weeks and 3–4 weeks respectively. 2,4-D treated swarmers formed irregular lobed and relatively smaller colonies compared to the unexposed swarmers producing smooth colonies.
Figure 3.4 AFM images showing the changes in surface ultrastructure of swarming *Rlv* in the presence of 2,4-D. Swarming *Rlv* in the presence of 0.42 mM 2,4-D (B, D) showed remarkable changes in surface characteristics when compared to unexposed swarmers (A, C). (A, B- 200 × 200 and C, D- 400 × 400 pixels) Scale bars, A, B = 2 µm C, D = 0.5 µm
Figure 3.5 AFM and QI images with force curves illustrate cell surface remodeling with 2,4-D exposure. *Rlv* treated with 0.4 mM (a, A) and 0.2 mM (b, B) 2,4-D have markedly altered surfaces compared to those of formula (c, C) and sample control (d, D). Arrows indicate extracellular matrix on the substrate surface, only observed for *Rlv* treated with 2,4-D. Images a–d are low resolution (300 × 300 pixels) and A–D are high resolution (500 × 500 pixels). Scale bars, a–d = 1 µm, A–D = 0.5 µm. I and II show quantitative imaging data and representative force curves corresponding to one pixel at the center of the cell surface adhesion map for treated and control samples, respectively. From the force curves (a–c extend, c–g retract) the e-f segment is used to measure the adhesion force and the slope of b-c used to calculate Young’s moduli. QI adhesion maps indicated that treated cells had a much higher (lighter pixels) adhesion to the hydrophilic tip than to the control (darker pixels). (Taken from Bhat et al., 2015a)
and had a significantly higher (n ≥ 60, p < 0.0001) Young’s modulus (87 ± 24 MPa) compared to the formula (64 ± 9 MPa) and sample control (61 ± 16 MPa).

### 3.3.2 Auxin plays a role in rhizobial differentiation in vitro.

Since 2,4-D is a structural analogue of IAA which has been presumed to play a role in rhizobial differentiation, _Rlv_ were treated with 0.4 and 0.9 mM IAA. _Rlv_ showed a unique phenotype at all concentrations, with some cells appearing to branch and others producing bud-like extensions with a clear demarcation from which the daughter cell appeared to separate (Figure 3.6 and 3.7). These differentiated cells appeared at a frequency of 77 ± 15% for the 0.4 mM IAA and 79 ± 10% for the 0.9 mM IAA treated cells compared to the formula (4.6 ± 1.1%) and control (3.8 ± 0.8%) samples (p < 0.0001, n ≥ 200) (Figure 3.2). Benzoate treated samples, used as a negative control, had frequencies at 1 mM (3.8 ± 0.9%) and 0.4 mM (2.6 ± 0.2%) comparable to the control and formula samples (Figure 3.2). _Rlv_ treated with 2,4-D and IAA had similar phenotypes to differentiated bacteroids isolated from legume root nodules (Figure 3.7 c, C).

### 3.3.3 2,4-D causes ROS accumulation and protein carbonylation

ROS, a direct indicator of oxidative stress, measured using the fluorescent probe DCFDA showed a concentration dependent increase in oxidative stress (Figure 3.8) with 2,4-D exposure (p < 0.005). Protein carbonylation, a possible consequence of oxidative stress, was higher in 2,4-D treated _Rlv_ cell extracts (Figure 3.9) and exhibited a concentration dependent increase in absorbance values (p < 0.0001).
Figure 3.6 Representative SEM images of an altered phenotype for IAA treated cells. The majority of Rlv cells treated with 0.9 mM (A) and 0.4 mM (B) IAA showed an altered phenotype, with some cells appearing to be ‘Y-shaped’ and others branching or budding. Rlv treated with the benzoic acid negative control were identical to controls (C). Scale bar, A-C = 1 µm. (Taken from Bhat et al., 2015a)
Figure 3.7 Representative AFM images of an altered phenotype for IAA treated cells. The majority of Rlv cells treated with 0.9 mM (a, A) and 0.4 mM (b, B) IAA showed an altered phenotype, with some cells appearing to be ‘Y-shaped’ and others branching or budding. This phenotype was similar to the bacteroids isolated from pea root nodules which also exhibited branching cells (c, C). Rlv treated with the benzoic acid negative control (d, D) did not exhibit such a phenotype. Images a-d are low resolution (300 × 300 pixels) and A-D are high resolution (500 × 500 pixels). Scale bars, a-d = 1 µm, A-D = 0.5 µm. (Taken from Bhat et al., 2015a)
Figure 3.8 Plot showing an increase in reactive oxygen species as a function of 2,4-D exposure. Increase in ROS is an indication of increased oxidative stress and was measured as an increase in the ROS-sensitive DCFDA fluorescence intensity (528 nm) (p<0.005). (Taken from Bhat et al., 2015a)
Figure 3.9 Plot showing an increase in carbonylated protein concentration as a function of 2,4-D concentration. Carbonylated proteins were (p < 0.0001) measured as an increase in absorbance of dinitrophenyl hydrazine (370 nm) resulting from the oxidation of diphenylhydrazine. (Taken from Bhat et al., 2015a)
3.3.4 2,4-D alters the metabolic profile of Rlv

Optimized analytical conditions and statistical models achieved a good separation between the treated and control metabolic profiles. PCA analysis showed distinct clustering of the treated and the two controls, indicating good separation between control and the treated groups. OPLS-DA supervised pairwise models had strong \( R^2 \) and \( Q^2 \) values, indicative of good cross-validation (Table 3.1) (Wiklund et al., 2008), with significantly different metabolite profiles for the treated samples compared to the two controls (Figure 3.10 A). A total of 175 metabolites were detected in all replicate sample sets, out of which only 87 were used for further statistical analysis in a manner comparable to similar approaches (Wang et al., 2013). A total of 60 metabolites having a VIP score of greater than 1, indicating above average influence on the model, were chosen for downstream pathway analysis. SUS (shared and unique structures plot) were created to determine the common general trend in the significant and non-significant metabolites. VIP greater than 1 indicates metabolites that are significantly changed with treatment and the VIP SUS plots (Figure 3.10 B) indicate that the formula-treated and control-treated models show similar trends with significantly changed metabolites following treatment, indicating that the changes in the metabolome were mainly due to 2,4-D exposure. The correlation coefficient, \( p(corr) \), is positive for increased metabolite levels or negative for reduced metabolite levels in response to 2,4-D treatment. \( p(corr) \) SUS plots also showed a similar type of correlation between the control-treated and formula-treated models, with very few metabolites showing a change unique to the formula ingredients (Figure 3.10 C).
Table 3.1 OPLS-DA model statistics. (Taken from Bhat et al., 2015a)

<table>
<thead>
<tr>
<th>Model</th>
<th>$R^2_Y$</th>
<th>$Q^2$</th>
<th>CV-ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>All class</td>
<td>0.446</td>
<td>0.358</td>
<td>0.0005</td>
</tr>
<tr>
<td>Control-treated</td>
<td>0.982</td>
<td>0.976</td>
<td>3.05E-07</td>
</tr>
<tr>
<td>Formula-treated</td>
<td>0.985</td>
<td>0.980</td>
<td>2.30E-08</td>
</tr>
<tr>
<td>Control-formula*</td>
<td>0.864</td>
<td>0.464</td>
<td>0.367*</td>
</tr>
</tbody>
</table>

$R^2_Y$ and $Q^2$ values close to 1, for control-treated and formula treated models indicate significant differences in the metabolite profiles between the sample sets. Absence of statistically significant differences between control and formula samples is shown by low $R^2_Y$ and $Q^2$ values for the all class and control-formula models.

* The high CV-ANOVA value for control-formula model indicates lack of a good OPLS-DA model for the separation of control and formula sample sets due to the lack of a statistically significant difference.
Figure 3.10 OPLS-DA scores plot and SUS plots for the multivariate statistical analysis of the metabolomics data. The OPLS-DA (orthogonal partial least square discriminant analysis) scores plot (A) shows clustering of the metabolite profiles for control, formula and treated samples, indicating good models with strong separation. SUS
(Shared and Unique Structures) plots constructed for VIP (variable influence on projection) (B) and p(corr) values (C) show significant metabolites and metabolite correlation, respectively, with shared features. VIP and p(corr) values from formula and control samples were compared with those from treated samples. The diagonally aligned metabolites (highlighted with an ellipse) in both the plots show the similarity between the control and formula OPLS profiles indicating that the metabolic effects are mainly due to 2,4-D rather than other components of the formulation. (Taken from Bhat et al., 2015a)
3.3.5 Secondary analysis shows major pathways altered during adaptation to 2,4-D stress

All 60 significant metabolites were assigned a unique KEGG ID. We used Rlv libraries in MBrole (Chagoyen and Pazos, 2011) to study the affected pathway networks and biological interactions from 2,4-D exposure. Only pathways containing metabolites with a VIP value > 1 and a p-value of < 0.05 (as determined by MBrole) were considered to represent effects attributable to 2,4-D exposure. Out of the 60 positively and negatively correlated metabolites, the majority were positively correlated with 2,4-D stress, which included a number of amino acids (i.e. Pro, Glu, Thr, Asp, Met, Asn, Ala, Lys, Ser, Arg) and metabolites of TCA cycle, oxidative phosphorylation, ABC transport, the glyoxylate pathway and inositol phosphate metabolism (Figure 3.11). Fewer metabolites were negatively correlated, for which the majority belonged to pyrimidine metabolism and peptidoglycan biosynthesis (Table 3.2). The total number of metabolites altered represented approximately 66% of the Rlv metabolic pathways (MBRole).

3.3.6 2,4-D induces malate synthase activity

An increase in the metabolites associated with the glyoxylate pathway was rather intriguing, and therefore enzyme assays were used to assess this effect. Malate synthase activity was higher in Rlv exposed to 2,4-D compared to control samples (p < 0.05) (Figure 3.12). This effect was concentration dependent, indicative of 2,4-D directly targeting the enzyme. Interestingly, there was no statistically significant difference in the isocitrate lyase activity between treated and control samples (Figure 3.13).
Figure 3.11 Metabolic pathways affected during adaptation to 2,4-D-induced stress.

Metabolites and pathways colored in red and green are higher and lower, respectively, in 2,4-D stressed *Rlv* compared with formula and sample controls. Metabolites colored in black were unchanged. Metabolite enrichment analysis indicated that the majority of the metabolites identified were positively correlated with 2,4-D exposure, belonging to glycolysis, the TCA cycle, oxidative phosphorylation, ABC transport, the two component system and glutathione metabolism. Several pathways associated with pyrimidine and peptidoglycan metabolism were negatively correlated with 2,4-D exposure. (Taken from Bhat et al., 2015a)
Table 3.2 Metabolomics data summary. Pathways altered with associated p-values, % of altered metabolites, KEGG IDs, and metabolites. Pathways indicated in bold are upregulated and those in regular font are down regulated. (Taken from Bhat et al., 2015a)

<table>
<thead>
<tr>
<th>Pathway affected</th>
<th>p-value</th>
<th>Percentage</th>
<th>KEGG IDs</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
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<td>Citrate cycle (TCA cycle)</td>
<td>2.54E-05</td>
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<td>C00158</td>
<td>Citric acid, oxoglutaric acid, phosphoenol pyruvate, succinate</td>
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<td></td>
<td></td>
<td></td>
<td>C00026</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>C00092</td>
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<td>Oxidative phosphorylation</td>
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<td>--------------</td>
<td>--------------</td>
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<td>Glycolysis / Gluconeogenesis</td>
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<td>6.1</td>
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<td>C00137</td>
</tr>
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<td>Arginine and proline metabolism</td>
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<td>10.7</td>
<td>C00134</td>
<td>C00064</td>
</tr>
<tr>
<td>Galactose metabolism</td>
<td>0.039</td>
<td>7.1</td>
<td>C00137</td>
<td>C00243</td>
</tr>
</tbody>
</table>
Figure 3.12 Plot showing an increase in the activity of malate synthase enzyme as a function of 2,4-D concentration. Malate synthase activity was measured as an increase (p < 0.05) in absorbance of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (412 nm). (Taken from Bhat et al., 2015a)
Figure 3.13 ICL assay showing no difference in activity as a function of 2,4-D treatment. Increase in the levels of a phenylhydrazone derivative was measured as absorbance at 324 nm for 30 min. There was no difference in activity as a function of treatment type, as indicated by the close parallel lines for all sample sets (p > 0.05). (Taken from Bhat et al., 2015a)
3.4 Discussion

Rhizobium is a beneficial soil bacterium having both a symbiotic and free living lifestyle which requires a complex genome capable of altering its physiology for both the heterogeneous soil environment and the more predictable environment of plant nodules (Young et al., 2006). Consequently, rhizobia have a robust and versatile metabolism, well suited to surviving external stress factors such as environmental pollutants. Herbicide exposed Rlv had a remarkable ability to adapt to 2,4-D-induced stress, with changes to surface ultrastructure, physical properties and changes to integral pathways of cellular metabolism. As a structural analogue of auxin, it was not surprising that 2,4-D induced an auxin-like response in rhizobia in vitro which included an altered phenotype. This study provides valuable insights into 2,4-D sub-lethal effects and the associated adaptation of metabolic networks.

3.4.1 2,4-D and IAA alter the morphology of Rlv in vitro

SEM indicated that a about a third of 2,4-D treated Rlv cells were abnormally shaped, some appearing to branch whereas others appeared similar to the ‘Y’-shaped bacteroids (Figure 3.1 C, E, F, G) normally found only in root nodules. This observation is interesting but difficult to explain as this phenotype is normally associated with symbiosis during nitrogen fixation. Interestingly 2,4-D is a structural analogue of auxin, the plant hormone which is known to play a role during nodule development (Pacios-Bras et al., 2003). A proteomic study of Medicago trunculata shows strong overlap between protein changes during early nodulation and in roots treated with auxin (van Noorden et al., 2007), and auxin synthesized by rhizobia promotes nodulation and host root growth in
plants bearing indeterminate nodules (Pii et al., 2007). *Rlv*, in which the indole acetamide biosynthetic pathway proteins had been introduced and expressed, produced root nodules in *Vicia hirsuta* containing up to 60-fold more auxin and a higher nitrogen fixing capacity than nodules invoked by the wild-type strain (Camerini et al., 2008). In an *Azospirillum ipdC* mutant producing lower amounts of auxin than the wild type strain there was reduced nodulation and nitrogen fixation, demonstrating the role of bacterial auxin production in nitrogen fixation (Remans et al., 2008). However, whether auxin plays any role in bacteroid differentiation, which is crucial for nitrogen fixation, remains unclear.

To explore this idea and whether the 2,4-D induced phenotype is a result of an auxin-like response, *Rlv* were exposed to a range of auxin concentrations. Indeed auxin has the capability to induce such morphological changes, with some cells appearing ‘Y-shaped’ like bacteroids, while others appeared to branch and bud (Figure 3.6 A, B and 3.7 a, A, b, B). The frequency of uniquely shaped cells was significantly higher (p < 0.0001) with auxin treatment compared to 2,4-D (Figure 3.2), whereas treatment with benzoic acid did not induce such phenotypic changes (Figure 3.6 C and 3.7 d, D). Bacteroids isolated from pea root nodules showed a phenotype (Figure 3.7 c, C) similar to that of auxin and 2,4-D treated cells, further evidence that this herbicide likely mimics auxin, thus activating nodule-like signaling in *Rlv* and causing differentiation *in vitro*.

### 3.4.2 2,4-D alters the surface ultrastructure and physical properties of *Rlv*

*Rlv* exposed to a range of 2,4-D levels showed a dose-dependent change in surface features such as roughness, elasticity and adhesion (Figure 3.5). Changes in surface ultrastructure indicate cell wall macromolecular remodeling, which is consistent with
increased amino acids in the metabolite pool during 2,4-D exposure. *Acinetobacter radioresistens* S13 exposed to benzoic acid and phenol upregulated several proteins related to the biogenesis and repair of the cell wall and others involved in the envelope stress response (Mazzoli et al., 2011), indicating that aromatic organics stimulate the rearrangement of surface architecture and composition consistent with this study.

Rhizobia produce excessive amounts of diverse surface polysaccharides that are crucial for increased adhesion to abiotic surfaces and biofilm formation (Rinaudi and Giordano, 2010). Continuous units of monosaccharides such as D-glucose, D-galactose, D-mannose, L-rhamnose, D-glucuronic acid and D-galacturonic acid, substituted with non-carbohydrate residues (e.g., acetyl, pyruvyl, succinyl and 3-hydroxybutanoyl groups) and their exposed –OH groups induce adhesion and alter surface physical properties (Bhat et al., 2012; Dong et al., 2011; Janczarek, 2011; Skorupska et al., 2006). Increased adhesion and elastic modulus in *Rlv* post 2,4-D treatment is a direct indication of their propensity towards biofilm formation due to 2,4-D adaptation which is consistent with the extracellular material observed by SEM and AFM. Consistent with its lipophilic nature, 2,4-D is capable of entering the cell passively, changing the membrane fluidity and permeability barriers as demonstrated for *Rhizobium sp*. M4 (Arias and Fabra de Peretti, 1993). Surface ultrastructural perturbations could also reflect underlying membrane perturbations as observed for similar aromatic organics having uncoupling properties (Gage and Neidhardt, 1993; Lambert et al., 1997; Loffhagen et al., 2003; Lupi et al., 1995).

**3.4.3 2,4-D alters the swarming behaviour of *Rlv***
*Rhizobia* are known to have one or two sub-polar flagella which help the bacterium move on soft agar plates (Tambalo et al., 2010b) and hyper-flagellation is a common characteristic of swarvers. *Rhizobia* move as coordinated assemblages on the semisolid surface and the process is known to be independent of chemotaxis (Tambalo et al., 2010a).

Previous studies indicate that 3–4 weeks are required for the development of a complete swarming colony, and that swarvers are resistant to several antibiotics (Tambalo et al., 2010a). In this study, *Rlv* swarvers had more resistance to 2,4-D compared to their non-swarmer counterparts. Our preliminary data indicates that the convoluted patterns of the 2,4-D treated *Rlv* swarvers were distinct from the relatively circular colonies of the control cells and may represent a colony front containing cells that have channeled out forming convoluted patterns, having acquired resistance to 2,4-D. AFM imaging of 2,4-D treated swarvers also show distinct surface characteristics, similar to those of treated non-swarvers (Figure 3.4).

**3.4.4 2,4-D causes oxidative stress induced damage in *Rlv***

*Rlv* exposed to 2,4-D showed high levels of ROS (Figure 3.8) known to cause oxidative damage to envelope macromolecules, and a dose-dependent increase in carbonylated proteins (Figure 3.9) as a direct consequence of oxidative damage. Carbonyl (CO-) groups, produced upon oxidation of peptide side chains, are chemically stable and known to be more sensitive and direct indicators of oxidative stress (Dalle-Donne et al., 2003). Similarly, the majority of aromatic and chlorinated aromatic hydrocarbons induce oxidative stress in bacteria (Benndorf et al., 2001; Chavez et al., 2004; Palanisami et al., 2009; Santos et al., 2004; Teixeira et al., 2005), which has been associated with their
lipophilicity, reactivity and uncoupling capabilities. The decrease in pyrimidine metabolites such as thymidine, uridine 5-phosphate and uracil is also a direct indication of DNA damage resulting from oxidative stress as the ROS can directly damage the ribose and bases of DNA (Imlay, 2013). Down-regulation of nucleotide metabolism has been observed for *E. coli* in response to cold, heat, oxidative stress and during the stationary phase (Jozefczuk et al., 2010).

Metabolite profiling indicated an increase in several polyamine metabolites such as cadaverine and putrescine involved in glutathione metabolism, that are known to serve as biomarkers of oxidative stress (Hayes and McLellan, 1999; Schneider et al., 2013; Tkachenko et al., 2012). Increased polyamine biosynthesis has been associated with increased growth rate and increased protection from DNA damage due to oxidative stress (Johnson et al., 2012). Indeed, polyamine catabolism is initiated by the stationary phase stress transcription factor, σ^S_, producing a core metabolic stress response for a variety of environmental stresses (Schneider et al., 2013). Therefore, we propose that increased levels of polyamines is an adaptive modification to the metabolism to cope with 2,4-D induced oxidative stress.

3.4.5 2,4-D alters peptidoglycan and damages intracellular protein

The majority of significantly altered metabolites were positively correlated with 2,4-D treatment, including a large group of amino acids which could indicate either proteolysis and protein denaturation (Jenal and Hengge-Aronis, 2003) or a consequent upregulation of amino acid biosynthetic pathways to replace damaged proteins in the cell. Amino acid accumulation was observed in *E. coli* exposed to alcohols, heat, cold and oxidative stress,
all likely due to protein denaturation and degradation (Jozefczuk et al., 2010). A proteomic analysis of _Acinetobacter radioresistens_ exposed to benzoate and phenol showed upregulation of a majority of the proteins associated with periplasmic proteases, chaperones, enzymes catalyzing peptidoglycan biogenesis, proteins involved in outer membrane integrity, cell surface properties and cellular redox homeostasis (Mazzoli et al., 2011), all consistent with this study. The reduction in peptidoglycan components along with accumulation of alanine, lysine and glutamate directly implicates 2,4-D in cell wall damage, consistent with _C. glutamicum_ which expresses cell wall biosynthetic enzymes (Fanous et al., 2007) during 2,4-D exposure. Peptidoglycan damage would also be reflected by changes to envelope compliance and possibly roughness, observed in response to 2,4-D exposure.

3.4.6 Adaptation to 2,4-D stress is an energy consuming process

Interestingly, _Rlv_ grown in the presence of 2,4-D showed increased levels of glycolytic and TCA cycle metabolites, including citrate, phosphoenol pyruvate (PEP), glucose 6-phosphate (G-6-P), oxoglutarate and succinate, contrary to several studies using short exposure time to stressors. For example in _E. coli_, reduced amounts of these intermediates were observed during short time exposure to heat, cold and oxidative stress (Jozefczuk et al., 2010), whereas long term exposure experiments (Keum et al., 2008; Seo et al., 2013) are consistent with this study in which TCA cycle intermediates increased with 2,4-D exposure. _Burkholderia xenovorans_ LB400 exposed to chlorobenzoate also showed induction of several TCA cycle enzymes (Martinez et al., 2007). In this study _Rlv_ was grown to mid-log phase in the presence of 2,4-D, providing sufficient time to
upregulate pathways that repair initial damage but require increased energy production. Increased levels of PEP and G-6-P also indicate a more active PEP-dependent glucose–phosphotransferase system (PTS), which acts as a center of carbohydrate flux in the cell, playing a significant role during nutrient starvation and stress adaptation in *E. coli* (Gabor et al., 2011). Since the PTS is actively involved in glucose transport and phosphorylation during glucose starvation, its upregulation indicates increased carbon use during 2,4-D adaptation. Upregulation of glycolysis, the TCA cycle and PTS intermediates signifies organized sensory, transport and energy production systems in *Rlv*, all representative of adaptive changes in response to 2,4-D induced stress.

**3.4.7 *Rlv* has the potential to assimilate 2,4-D**

An intriguing observation was the upregulation of the glyoxylate pathway, consistent with the 1.5 fold increase in malate synthase activity (Figure 3.12) in 2,4-D treated *Rlv*. On the contrary, the activity of isocitrate lyase, the other key enzyme in the glyoxylate pathway, was unchanged with 2,4-D treatment (Figure 3.13). These seemingly contradictory results could be explained if 2,4-D increases glyoxylate levels in the cell, thus activating specifically MS and not isocitrate lyase. Several 2,4-D degradation pathways have been discovered in soil bacteria and the intermediates of these pathways commonly enter the TCA cycle through glyoxylate (Balajee and Mahadevan, 1990; Hausinger and Fukumori, 1995; Suwa et al., 1996). 2,4-D degrading genes have been discovered in a closely related species, *Bradyrhizobium sp*. strain HW13 (Kitagawa et al., 2001), however, to the best of our knowledge a 2,4-D degrading wild type *Rlv* strain has never been isolated. It is clear, however, that the glyoxylate cycle is upregulated during
2,4-D exposure to replenish the TCA cycle intermediates necessary for energy generation during 2,4-D adaptation. 2,4-D and related herbicides induce superoxide dismutase, malate dehydrogenase and auxin responsive genes in soybeans (Kelley et al., 2006) consistent with *Rlv* exhibiting 2,4-D-induced oxidative stress, increased malate synthase activity and possible induction of auxin responsive genes as evidenced by the common auxin- and 2,4-D-induced phenotype.

3.4.8 Protein acetylation and myo-inositol, potential bioindicators of stress adaptation in *Rlv*

The 2,4-D treated *Rlv* had increased levels of acetyl lysine, a key post-translational modification related to intermediary metabolism (Guan and Xiong, 2011). A greater number of proteins are lysine-acetylated in the stationary phase compared to the exponential phase (Yu et al., 2008) to regulate cell growth and proliferation (Weinert et al., 2013). It has been proposed that the majority of central metabolic enzymes are acetylated, and that this reversible process is an efficient means of adapting to changing environmental conditions (Bernal et al., 2014). Therefore, higher levels of acetyl lysine in the metabolite pool would be a key biomarker indicative of active adaptation to 2,4-D stress.

The increase in inositol phosphate metabolism in 2,4-D treated *Rlv*, as indicated by the accumulation of myo-inositol, cannot be fully explained. The role of inositol phosphates in bacteria is not completely understood, but in eukaryotes, this pathway has a significant role in signal transduction at the cell surface, regulation of membrane traffic, the cytoskeleton, and permeability and transport functions of membranes (Di Paolo and De
Camilli, 2006). To date, accumulation of free myo-inositol has not been documented in prokaryotes (Roberts, 2006), but this study indicates that it may play a significant role during stress adaptation in rhizobia.

3.4.9 2,4-D upregulates membrane transport and ATP biosynthesis

Greater activity of the ABC transport system, the two component system along with increased oxidative phosphorylation indicates a need for the import of vital macromolecules, signal transduction and the simultaneous need for ATP to fuel such adaptive changes, respectively, which are consistent with upregulated glycolysis and the TCA cycle. The membrane transport system plays a significant role during stress adaptation and this pathway is upregulated in *Corynebacterium glutamicum* during 2,4-D stress (Fanous et al., 2007) and in *E. coli* during nutrient limitation and oxidative stress (Lemuth et al., 2008). Although ATP requirements are higher during active membrane transport and stress adaptation, several studies indicate a reduction in ATP biosynthesis (Benndorf et al., 2006; Durfee et al., 2008), possibly resulting from reduced membrane integrity and the inability to adapt efficiently, especially during short term exposure. Indeed 2,4-D causes membrane defects, affects proton motive force and oxidative phosphorylation in *E. coli* (Bhat et al., 2015b) and *Comamonas testosteroni* (Loffhagen et al., 2003). Aromatic organic compounds in general, such as benzoate, chlorophenol and dinitrophenol, are known to accumulate in the cytoplasm, reduce internal pH, reduce growth and act as ‘uncouplers’ which affect membrane integrity, proton motive force and ATP biosynthesis (Gage and Neidhardt, 1993; Lambert et al., 1997; Lupi et al., 1995).
Upregulation of ABC exporters is an induced adaptation mechanism that exports xenobiotic substrates out of the cell (Cherepenko and Hovorun, 2005) and P-glycoproteins are known to interact with pesticides, playing a major role in toxicity resistance (Buss and Callaghan, 2008). Increased PTS and membrane transport is also required for the export of polysaccharide precursors for EPS production, an important adaptive response during stress resistance in bacteria (Houot and Watnick, 2008) and consistent with the altered surface properties in Rlv. Upregulation of glycolysis, the TCA cycle, oxidative phosphorylation and the membrane transport system provides Rlv with the energy required for adaptation to stress.

3.5 Conclusions

Anthropogenic chemical compounds are known to disturb the legume-rhizobia symbiotic relationship, forcing rhizobia to reprogram integral pathways of cellular metabolism to adapt with consequent changes to their cellular structures. This study shows that 2,4-D alters key pathways in Rlv central metabolism necessary for long term adaptation during 2,4-D stress. Other 2,4-D-induced effects, such as changes to cell morphology similar to those found in bacteroids can be ascribed to its structural relationship with auxin. Some of the specific metabolites showing significant changes in response to 2,4-D exposure, namely acetyl-lysine, glyoxylate, myo-inositol and polyamines, can serve as sensitive biomarkers of adaptation to stress. This study provides valuable insights into the effects of 2,4-D exposure at sub-lethal levels on the beneficial model soil bacterium Rlv.
3.6 Bibliography


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CHAPTER 4 – OXIDATIVE STRESS AND METABOLIC PERTURBATIONS IN

ESCHERICHIA COLI EXPOSED TO SUB-LETHAL LEVELS OF 2,4-
DICHLOROPHENOXYACETIC ACID

This majority of this chapter is part of the open access publication,


GC-MS of the metabolomics samples and part of the statistical analysis were performed by Sean Booth at the University of Calgary.

The *E. coli* BL21 pET21b genotyping was performed by Shirin Afroj from the Yost laboratory at the University of Regina.
4.1 Introduction

Modern agricultural and urban landscaping practices have led to the widespread use of pesticides, some of which persist in and affect the environment to a greater extent than intended. The effects of chronic, sub-lethal exposure to increasing combinations of pesticides and other xenobiotics remain largely understudied, despite the potential risk to human health and the environment. The chlorophenoxy herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been used extensively world-wide since the 1940s to combat residential and agricultural dicotyledonous weeds (Bukowska, 2005). With a half-life of 20–200 days, 2,4-D is able to leach into ground water, or be transported into rivers and lakes, contaminate water reservoirs through precipitation, and can be transported long distances through the atmosphere from the initial area of application (Boivin et al., 2005).

As the first synthetic auxin analogue of indole-acetic acid, 2,4-D, acts as an herbicide disturbing the normal growth and development of target plants, provoking lethal damage that leads to uncontrolled cell division and epinasty (Pazmino et al., 2011). Although, its exact mode of action in plants is unknown, 2,4-D exposure leads to the accumulation of reactive oxygen species (ROS), disrupts calcium channels and cell division, produces cell wall and membrane defects resulting in localized cell death (Pazmino et al., 2014). 2,4-D is known to be toxic and carcinogenic to non-target mammals but reports of toxicity to soil bacteria are conflicting, possibly due to the lack of suitable comparative approaches and methodological platforms (Chinalia et al., 2007). Nonetheless, 2,4-D has shown the potential to cause significant disturbances to soil microbial communities (Balague et al.,
2001; Balague et al., 2002; Fabra et al., 1993; Prado and Airoldi, 2000; Zhang et al., 2010), but the mechanisms of cellular toxicity remain unknown.

The development of robust analytical platforms such as genomics, proteomics and metabolomics has made it possible to assess the detailed mechanisms of how hazardous chemicals affect microbes. The proteomic analysis of Burkholderia sp. (Cho et al., 2000), Pseudomonas putida (Benndorf et al., 2006), Saccharomyces cerevisiae (Teixeira et al., 2004; Viegas et al., 2005), Deftia acidovorans (Benndorf et al., 2004) and Corynebacterium glutamicum (Fanous et al., 2007) in response to 2,4-D exposure showed induction of stress proteins, altered protein expression related to energy metabolism and membrane transport, and inferred oxidative stress. While metabolomics studies cataloguing the effects of xenobiotics on bacteria are limited, they have been effective in elucidating pesticide degradation pathways and underlying mechanisms of cellular toxicity (Lenart et al., 2013; Wang et al., 2013). Metabolomics is ideal for generating intracellular metabolic profiles, while SEM and AFM are best suited to probe cell surface ultrastructure, physical and chemical properties (Kaminskyj and Dahms, 2008). AFM has been widely used to image E. coli as a function of genetic mutation (Alsteens et al., 2013), chemical insult (La Storia et al., 2011) and for nanoindentation to determine the viscoelastic properties as a function of environmental factors (Abu-Lail and Camesano, 2006; Bhat et al., 2012). We used a unique analytical platform that combines metabolite profiling techniques (metabolomics) and complementary surface scanning methods to systematically assess the effects of 2,4-D on E. coli.

E. coli, a Gram negative bacterium with four distinct phylogroups A, B₁, B₂, and D (Gordon et al., 2008), consists of commensal and pathogenic groups that are primarily
associated with intestinal tracks of animals, however they have also been observed to persist in aquatic and soil ecosystems (Chaudhuri and Henderson, 2012). *E. coli* is a well characterized bacterium that has been used as a model organism for studying bacterial physiology and genetics. It is a robust model to study the effects of xenobiotics since its genomes, proteomes and metabolomes are well defined. Clinically isolated strains of *E. coli* exposed to 1 mM 2,4-D exhibited lipoperoxidation, alterations to cell envelope hydrophobicity, diminished growth, total protein content, motility and fimbriation (Balague et al., 2001). Here we explore the effects of a wide range of 2,4-D exposure levels on environmental isolates of *E. coli* including structural, physical, biochemical and metabolic characteristics to further understand the mechanisms underlying its effects on bacterial physiology.

SEM was used to track phenotypic changes, AFM to quantify surface ultrastructural and physical changes at nm and pN resolution and GC-MS based metabolomics to track corresponding physiological changes. Several strains of *E. coli* isolated from areas impacted by pasture and wastewater effluent were examined to determine common biomarkers. This study is the first to investigate the extracellular and intracellular stress responses of *E. coli* to 2,4-D at sub-lethal levels.

4.2 Materials and methods

The 2,4-D amine formulation source, composition and HPLC analysis are described in chapter 2.
4.2.1 *E. coli* growth conditions, genotyping and MIC assays

The lab strain *E. coli* BL21 (DE3) media, growth conditions and MIC assays are described in chapter 2. *E. coli* genotyping for BL21 and the environmental strains were conducted in the laboratory of Dr. Chris Yost by graduate student Shirin Afroj. Environmentally isolated strains of *E. coli*, were obtained from water samples collected from Wascana Lake (NECD 1 and NECD 2), and the Qu’appelle River (NECD 3 and NECD 4) near Lumsden, Regina, SK, Canada. The environmental strains and lab strain *E. coli* BL21 (DE3) were genotyped using the triplex PCR method developed by Clermont and coworkers (2000) and later modified by Higgins and coworkers (2007), and four specific strains (NECD 1-4) were selected to represent the four broad genotypes (A₀, A₁, B₁ and D respectively). DNA was extracted from two isolated colonies for each strain growing on agar plates using the Colony Fast-Screen kit (Epicenter Biotechnologies, Madison, USA) following the manufacturer’s instructions. The extracted DNA was used directly as a template for the PCR amplification. *E. coli* ATCC 25922, genotyped as B₂, was used as a positive control. PCR was conducted under standard conditions in 25 µL reaction volumes, containing 2.5 µL of 10 × PCR buffer (BIO BASIC INC.), 1 µL template, 2 mM MgSO₄ (UBI), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.25 µM each of the *chuA* primers, 1 µM each of the *yjaA* primers, 0.5 µM each of the TSPE4.C2 primers and 0.6 U of *Taq* polymerase (BIO BASIC INC., Canada). For multiplex reactions, PCR amplification conditions included an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 15 s, annealing at 60 °C for 30 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 5 min.
4.2.2 Biofilm assays

*E. coli* biofilms were grown on glass coverslips by immersing them in LB (amp) broth media with 4.2 mM 2,4-D inoculated with *E. coli* BL21 starter culture and incubation at 37 °C for 3 days in a shaker incubator at 10 rpm. Similarly, control biofilms were grown in the absence of the 2,4-D formulation. Coverslips with biofilms were rinsed with phosphate buffer (pH 7, 0.5 M) and lightly stained with crystal violet (0.05 mg/mL) for better contrast, observed by light microscopy (Olympus BX51 with fluorescent capabilities) under oil immersion (1000 ×) and images recorded with a digital camera (Olympus DP70).

For fluorescence imaging, see section 2.6.2 of chapter 2. Coverslips were rinsed with phosphate buffer and incubated with the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole dihydrochloride hydrate) at a concentration of 0.2 µg/mL and allowed to stain for 5 min. The coverslips were rinsed thoroughly with DI water and air dried. The entire staining and imaging procedure was conducted in the dark. Over 20 fluorescent images were collected for each sample with an inverted fluorescent microscope (Carl zeiss, Axiovert 40). SEM images were collected for the 4.2 mM 2,4-D treated and control samples using the procedure described in chapter 2, section 2.6.4.

4.2.3 Intracellular reactive oxygen species assay

Intracellular ROS was measured using the procedure described in chapter 2, section 2.8.

4.2.4 Microscopy

The 2,4-D dose-response relationships at higher structural resolution were based on suitable sub-lethal concentrations chosen by LM. To study the influence of induced
oxidative stress, *E. coli* BL21 (DE3) was grown in the presence of 4.2, 2.1 and 0.21 mM hydrogen peroxide, and cells imaged by LM. To determine the effects of polyamines on the phenotype in the presence of 2,4-D, cells were grown in the presence of 4.2 mM 2,4-D and 1–50 mM of spermine, spermidine and putrescine. LM, SEM and AFM sample preparation and image analysis are described in detail in chapter 2.

### 4.2.5 Metabolite extraction and analysis

Six replicates of *E. coli* BL21 (DE3) treated with formula control, control and 4.2 mM 2,4-D were used for metabolite quenching, extraction and GC-MS analysis. The associated protocols and statistical analysis are described in detail in chapter 2.

### 4.3 Results

Triplex PCR analysis indicated that *E. coli* BL21 (DE3) belongs to the A₁ sub-genotype (Figure 4.1).

The minimum inhibitory concentration (MIC) of 2,4-D after 24 h (OD_{600 nm}) was 6.3 mM for *E. coli* BL21 and 6.0, 7.0, 9.0 and 9.0 mM for the NECD 1, 2, 3 and 4 strains, respectively. Exposure to 4.2 mM 2,4-D provided adequate BL21 cell mass for metabolomic analysis, and corresponded to the highest sub-lethal dose for imaging. Similarly, the highest sub-lethal doses of 2,4-D used for microscopic analysis of the NECD 1, 2, 3 and 4 strains were 5.0, 6.0, 8.0 and 8.0 mM, respectively.
Figure 4.1 Polyacrylamide gel separation of triplex PCR. Lane M - 50 bp markers, lanes 1 and 2 - replicates of the *E. coli* BL21 strain appearing as sub-genotype A₁, P-positive control (*E. coli* ATCC 25922, B2 genotype), N- Negative control. (Taken from Bhat et al., 2015)
4.3.1 2,4-D alters *E. coli* biofilm architecture

LM images were analyzed for individual cell morphology and biofilm distribution. Treated cells were capable of settling, substrate attachment and biofilm formation (Figure 4.2) but the biofilms were not as thick and uniformly distributed as the untreated biofilm. Treated cells formed an irregular biofilm with a greater number of clumped cells as compared to the untreated cells having a thick multilayer mat with fewer clumps. Cells residing in the treated biofilm were less elongated compared to cells that were loosely dispersed.

FM images were similar to those collected by LM (Figure 4.3), but FM is capable of providing images with better contrast and depth. FM showed the control cells generating a much thicker and denser biofilm as compared to treated cells that were incapable of forming complete biofilms. SEM imaging further confirmed the LM and FM results, but better articulated the associated texture and morphological patterns. The control *E. coli* biofilms were multilayered with a thick matrix (Figure 4.4), and a unique pattern of matrix distribution which was lacking in the treated biofilm.

4.3.2 *E. coli* exposed to sub-lethal 2,4-D produce ROS

*E. coli* BL21 treated with 4.2 mM 2,4-D showed three times higher intracellular ROS content compared to the control and formula control, with intracellular ROS directly proportional to 2,4-D levels (Figure 4.5). Positive controls treated with 4.2 and 2.1 mM H₂O₂ also showed ROS accumulation.
Figure 4.2 Light microscopy images showing effects of 2,4-D on *E. coli* biofilm formation. Images show the low (a, b) and high distribution areas (c, d) of control (a, c) and treated (b, d) biofilms stained with crystal violet. The treated cells also show the elongated morphology. As shown in the images, the control biofilms were thick, multilayered and uniform compared to the treated biofilms which formed irregular, clumpy, but thinner biofilms.
Figure 4.3 Epifluorescence microscopy images showing the effects of 2,4-D on *E. coli* biofilm formation. Showing the DAPI stained FM images at low (10 ×; a, b) and high (40 ×; c, d) resolution in control (a, c) and treated (b, d) *E. coli* biofilms. Control biofilms were much thicker and multilayered with shorter cells as compared to the 4.2 mM treated biofilms.
Figure 4.4 SEM images showing *E. coli* biofilm architecture in the presence of 2,4-D.

Representative SEM images show biofilm architecture at low (a, b) and high (c, d) resolution for control (a, c) and treated (b, d) *E. coli* biofilms. The control biofilm is thicker and shows distinct channels unlike those of treated samples. Scale bar, a - 500 µm, b – 200 µm, c, d – 10 µm
Figure 4.5 Plot showing an increase in ROS accumulation as a function of increase in 2,4-D and H$_2$O$_2$ concentrations. ROS was directly measured by the increase in the fluorescence signal of the ROS indicator 2,4-dichlorodihydrofluoresceine diacetate (DCFDA) ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 528$ nm). Opaque bars represent 2,4-D concentrations and clear bars represent H$_2$O$_2$ concentration. (Taken from Bhat et al., 2015)
4.3.3 *E. coli* exhibits a filamentous phenotype under oxidative stress

Exposure to 2,4-D affected all *E. coli* strains by inducing a filamentous phenotype up to 200 μm in length (Figure 4.6, Figure 4.7, Table 4.1). *E. coli* BL21 treated with 4.2 mM 2,4-D gave rise to highly altered morphological features and variable cell length compared to both the formula and standard controls. Filamentous cells were apparent at concentrations 300 times below the MIC, but average cell length was statistically identical (5.4 ± 1 μm) to controls when exposed to ≤0.0021 mM 2,4-D. All the environmental genotypic groups responded similarly, but the NECD 3 and 4 strains had less pronounced elongation, showing subtle differences in response to 2,4-D (Table 4.1). Both average cell length and number of elongated cells increased as a function of 2,4-D concentration (Figure 4.7). Hydrogen peroxide also induced the filamentous phenotype in *E. coli* BL21, with the degree of elongation directly proportional to its concentration (Figure 4.7).

Since the whole metabolome GC-MS analysis indicated significant reduction in polyamine metabolites, *E. coli* media was supplemented with putrescine, spermidine and spermine to determine the effects of polyamines in the presence of 2,4-D. *E. coli* BL21 treated with 4.2 mM 2,4-D and 15 mM of spermidine and putrescine were rod-shaped and statistically similar (n = 100, p > 0.05) in cell length (3.7 ± 1.4, 3.09 ± 1.18 respectively), to that of the control (3.7 ± 1.6) and formula control (3.83 ± 1.06). In contrast, spermine inhibited the growth of *E. coli* BL21 at concentrations > 1 mM in the presence of 4.2 mM 2,4-D, and was unable to rescue the rod-shaped phenotype.
Figure 4.6 SEM images highlighting the filamentous phenotype in *E. coli*. *E. coli* treated with control (a, d, g), formula control (b, e, h) and the highest sub-lethal level of 2,4-D (c, f, i) for strains BL21 (a, b, c), NECD 1 (d, e, f) and NECD 2 (g, h, i) each showing filamentous phenotypes. The highest sub-lethal concentrations used were 4.2, 5 and 6 mM for BL21, NECD 1 and NECD 2 respectively. Magnification: 5000 ×; scale bar: 5 μm. (Taken from Bhat et al., 2015)
Figure 4.7 Histogram of *E. coli* BL21 DE3 cell length dispersion and associated variability as a function of 2,4-D and H₂O₂ concentration. (Taken from Bhat et al., 2015)
Table 4.1 *E. coli* cell length as a function of 2,4-D.† (Taken from Bhat et al., 2015)

<table>
<thead>
<tr>
<th><em>E. coli</em> strains</th>
<th>Average cell length values (n=100)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>Formula</td>
<td>treated</td>
</tr>
<tr>
<td>BL21</td>
<td>5.0 ± 1.0</td>
<td>4.2 ± 1.0</td>
<td>27.9 ± 29.0*</td>
</tr>
<tr>
<td>NECD 1</td>
<td>3.5 ± 0.8</td>
<td>3.7 ± 1.1</td>
<td>13 ± 6.9*</td>
</tr>
<tr>
<td>NECD 2</td>
<td>4.1 ± 1.2</td>
<td>4.3 ± 1.1</td>
<td>15.7 ± 8.8*</td>
</tr>
<tr>
<td>NECD 3</td>
<td>4.3 ± 1.2</td>
<td>3.8 ± 1.0</td>
<td>7.0 ± 2.2*</td>
</tr>
<tr>
<td>NECD 4</td>
<td>3.3 ± 1.0</td>
<td>3.5 ± 0.9</td>
<td>6.6 ± 2.1*</td>
</tr>
</tbody>
</table>

†Highest 2,4-D concentration producing adequate cell mass (see chapter 2).

*Values are statistically significant from the control, p < 0.0001
4.3.4 The cell surface of *E. coli* is remodeled with 2,4-D exposure

AFM images revealed surface subunit features (Figure 4.8) used to calculate roughness, each of which were quantified for all cells, irrespective of cell length. Surface features of both *E. coli* controls were statistically identical (86 ± 13 nm and 87 ± 8 nm) and increased, along with their irregularity, as a function of 2,4-D concentration. At 0.0021 mM 2,4-D, cell surface roughness and feature diameter were statistically identical to controls. At exposure levels higher than 0.0021 mM 2,4-D there were no longer regular surface subunit features (Table 4.2). Surface roughness varied accordingly, with the lowest values identical to the well-ordered surface features of the control samples, and higher levels of 2,4-D leading to increasingly higher (p < 0.001) and more variable roughness (Table 4.2).

The e-f segment on the force curve (Figure 4.9, middle) quantifies adhesion between the *E. coli* surface and hydrophilic AFM tip during the force curve retraction phase. The 2,4-D treated cells showed significantly lower (n = 60, p < 0.0001) adhesion (6.16 ± 0.5 nN) compared to the formula (11.12 ± 2 nN) and sample controls (13.09 ± 4 nN). QI adhesion maps (Figure 4.9) show much lower adhesion at central regions of treated cells compared to that of controls, for which peripheral cell regions represent large height changes and unreliable adhesion values (Valegol and Longan, 2002). The b-c segment of the approach curve (Figure 4.9, middle) was used to calculate Young's moduli, for which cells treated with 4.2 mM 2,4-D had lower (n = 40, p < 0.0001) values (114 ± 55.6 MPa) compared to the statistically identical values of formula (421 ± 256 MPa) and sample controls (405 ± 261 MPa).
Figure 4.8 AFM images illustrate surface remodeling during 2,4-D exposure. *E. coli* BL21 (a, A, i) control; (b, B, ii) formula control; (c, C, iii) and treated with 0.0021 mM; (d, D, iv) 0.021 mM and (e, E, v) 4.2mM 2,4-D. A–E are high resolution images (500 × 500 pixels), a–e are lower resolution (300 × 300 pixels) and i–v show changes in surface ultrastructure and roughness in 3-D topographical representations. Cells treated with > 0.0021 mM 2,4-D show filamentous morphology and an altered surface ultrastructure. Scale bars, a–e: 5 μm; A–E: 1 μm. (Taken from Bhat et al., 2015)
Table 4.2. *E. coli* surface roughness and subunit size as a function of 2,4-D (Taken from Bhat et al., 2015).

<table>
<thead>
<tr>
<th>2,4-D concentration (mM)</th>
<th>Average surface roughness (nm)</th>
<th>Average subunit size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11 ± 8</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>Formula control</td>
<td>13 ± 4</td>
<td>86 ± 13</td>
</tr>
<tr>
<td>0.0021</td>
<td>14 ± 8</td>
<td>91 ± 37</td>
</tr>
<tr>
<td>0.021</td>
<td>29 ± 14*</td>
<td>163† (Range: 101-254)</td>
</tr>
<tr>
<td>0.21</td>
<td>33 ± 12*</td>
<td>-</td>
</tr>
<tr>
<td>2.1</td>
<td>35 ± 27*</td>
<td>-</td>
</tr>
<tr>
<td>4.2</td>
<td>28 ± 18*</td>
<td>-</td>
</tr>
</tbody>
</table>

* Difference in the average cell surface roughness and † subunit size between the control, formula control and treated *E. coli* cells is statistically significant (p < 0.01 and p < 0.0001 respectively; paired student’s t-test), surface subunit sizes could not be quantified for samples treated with ≥ 0.21 mM 2,4-D.
Figure 4.9 AFM QI images showing changes in surface physical properties due to 2,4-D exposure. Deflection images of E. coli BL21 treated with (a) control, (b) formula control and (c) 4.2 mM 2,4-D, including adhesion maps of a 200 × 200 nm surface area. Darker pixels indicate a lower adhesion. Representative force curves (a–c extend, c–g retract) in which the f-e segment of retraction is used to measure the adhesion force and b-c was used to calculate Young’s moduli. Histograms generated from force curves (n = 60) show decreased adhesion force between the hydrophilic AFM tip and cell surface with 2,4-D treatment. Scale bar, 1 μm. (Taken from Bhat et al., 2015)
4.3.5 2,4-D alters the metabolic profile of *E. coli* BL21

Principal component analysis (PCA) showed good separation between the control and treated sample sets as well as formula and treated sample sets, as indicated by clustering on the scores plot. However, there was little difference between the two control sample sets (data not shown). OPLS-DA supervised pairwise models had strong $R^2_Y$ and $Q^2$ values, indicative of good cross-validation (Table 4.3) (Wiklund et al., 2008), with significantly different metabolite profiles for the treated samples compared to the two controls (Figure 4.10). A satisfactory model to separate the all class profiles was precluded by the absence of variation between the two controls.

4.3.6 *E. coli* alters central metabolism in response to 2,4-D stress

The VIP SUS plot indicates a common general trend for significant and non-significant metabolites between control-treated and formula-treated models, demonstrating that metabolic effects can mainly be attributed to 2,4-D exposure. The p(corr) SUS plot showed a similar type of correlation with very few metabolites having a change unique to the formula ingredients (Figure 4.11).

There were a total of 114 metabolites common to all sample types, from which 44 were identified and assigned a unique KEGG ID. We used both *E. coli* BL21 and A1 pathway libraries in MBrole (Chagoyen and Pazos, 2011) to study the affected pathways, each giving identical results. Only pathways containing metabolites with a VIP value > 1 and a p-value of < 0.05 (as determined by MBrole) were considered to represent effects attributable to 2,4-D exposure. Specifically, metabolites associated with oxidative phosphorylation, ABC transporters, peptidoglycan biosynthesis, glutathione metabolism
Table 4.3. OPLS-DA model statistics (Taken from Bhat et al., 2015).

<table>
<thead>
<tr>
<th>Model</th>
<th>$R^2_Y$</th>
<th>$Q^2$</th>
<th>CV-ANOVA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>All class</td>
<td>0.40</td>
<td>0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>Control-treated</td>
<td>0.79</td>
<td>0.57</td>
<td>0.02</td>
</tr>
<tr>
<td>Formula-treated</td>
<td>0.84</td>
<td>0.68</td>
<td>0.005</td>
</tr>
<tr>
<td>Control-formula</td>
<td>0.89</td>
<td>0.35</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Low $R^2_Y$, $Q^2$ and high CV-ANOVA values for all classes and control-formula models indicates lack of statistical significance between sample sets.
Figure 4.10 Score plot of OPLS-DA. Plot shows control-treated (left) and formula-treated (right) models having clustering of sample sets (▲-treated, ●-control and ○-formula) and pairwise models with statistically significant variations. (Taken from Bhat et al., 2015)
**Figure 4.11 SUS plots for VIP and p(corr) values.** SUS (shared and unique structure) plots constructed for VIP (variable influence on projection) and p(corr) values showing the significant metabolites and metabolite correlation, respectively, with shared features. VIP (left) and p(corr) (right) values of formula and control samples were compared against those of the treated sample. The diagonally aligned metabolites (highlighted with an ellipse) in both the plots show the similarity between the control and formula OPLS profiles indicating that the metabolic effects are mainly due to the 2,4-D component of the formulation. VIP indicates the importance of a variable (metabolite) to distinguish model classes, while p(corr) represents a scaled correlation coefficient between the variable (metabolite) and component of variation. Larger VIP values indicate a larger influence, whereas larger p(corr) magnitudes specify a stronger correlation, with negative numbers representing a negative correlation. (Taken from Bhat et al., 2015)
and purine/pyrimidine metabolism were diminished, while metabolites associated with amino acid/protein metabolism and sugar/starch metabolism increased with 2,4-D exposure compared to those of control and formula control. The most obvious change was the significant reduction in several metabolites associated with membrane and cell wall components. Consequently, there was also a significant increase in amino acids such as threonine, glycine, alanine, tyrosine, and sugars such as sucrose, fructose and glucose (Figure 4.12).

4.4 Discussion

To shed light on the effects of xenobiotics on cellular processes, I developed a methodical platform that combines a suite of microscopy techniques with metabolomics to systematically assess the sub-lethal effects of 2,4-D on cell envelope organization, morphology and cellular metabolism for the model bacterium *E. coli*.

4.4.1 2,4-D alters *E. coli* biofilm formation

Biofilm development is a complex process dependent upon environmental factors, genetic influences and quorum sensing, involving a multistep mechanism. Biofilm matrix (Figure 4.4 c), likely consisting of EPS and/or colanic acid (Danese et al., 2000), forms channels within the 3-D biofilm structure known to provide gas exchange, influx and efflux of nutrient and waste materials, respectively, bacterial motility and stability (Francius et al., 2011). Biofilms are known to be highly resistant to antibiotics and disinfectants, making them a serious medical problem (Kania et al., 2010; Paulitsch et al., 2009). Several antibacterial compounds such as Triclosan (Lubarsky et al., 2012),
Figure 4.12 Metabolic pathways affected after overnight incubation with 2,4-D.

Metabolites colored in red and green represent higher and lower metabolites, respectively, in 2,4-D stressed *E. coli* BL21 extracts compared with formula and sample controls. Metabolite enrichment analysis identified metabolites with altered concentrations due to 2,4-D exposure, mainly involved in glutathione metabolism, oxidative phosphorylation, amino acid metabolism, starch and sucrose metabolism, peptidoglycan biosynthesis, purine and pyrimidine metabolism and the ABC transport system. (Taken from Bhat et al., 2015)
chlorine salts (Jones et al., 2011), antibiotics, toxic metals and disinfectants (Arias-Moliz et al., 2012; Behnke and Camper, 2012; Jaramillo et al., 2012; Shen et al., 2012; van der Waal et al., 2012; Wu et al., 2013) are known to affect the dispersion, stability, viscoelastic and rheological properties of biofilms. Exposure to 2,4-D impaired the 3-D architecture of the *E. coli* biofilm, consistent with alterations to metabolites involved in quorum sensing and EPS production.

### 4.4.2 *E. coli* forms a filamentous phenotype in response to 2,4-D stress

The filamentous phenotype was common to all *E. coli* strains upon exposure to sub-lethal levels of 2,4-D (Table 4.1, Figure 4.6), indicating a general physiological underlying mechanism. Similar morphological responses between the domesticated *E. coli* BL21 (DE3), and the recently isolated environmental strains indicate that the filamentous phenotype is not a result of a lab artifact from a long-term cultured model strain.

While the mechanism underlying cell elongation is unclear, it is thought to result from a combination of growth and cell division defects and has been proposed as a stress survival strategy associated with various environmental conditions (Justice et al., 2008). Exposure to both H₂O₂ and 2,4-D led to the accumulation of intracellular ROS and the filamentous phenotype in *E. coli* (Figure 4.5). Dose-dependent ROS accumulation at even very low levels of 2,4-D (0.021 mM) demonstrates significant oxidative stress-induced cellular changes. ROS are known to cause lipid peroxidation, irreversible damage to proteins and nucleic acids (Lushchak, 2011). ROS-induced DNA damage activates the SOS response in *E. coli*, arresting cell division, promoting elongation and initiating the
DNA repair process, thereby preventing the transfer of mutated genes to daughter cells (Janion, 2008).

4.4.3 2,4-D likely induces DNA damage in *E. coli*

Several glutathione and polyamine metabolites were reduced with 2,4-D exposure and the rod-shape wild type phenotype of *E. coli* was rescued when 2,4-D-exposed cells were externally supplemented with spermidine and putrescine. Polyamines protect cells from DNA damage and outer membrane peroxidation by scavenging ROS and inducing antibiotic resistance (Johnson et al., 2012; Tkachenko et al., 2012). The ability of polyamines to reverse the filamentous phenotype indicates that the DNA damage likely resulting from the oxidative stress are the cause of abnormal cell division. The complete growth inhibition by > 1 mM spermine in the presence of 4.2 mM 2,4-D is intriguing, potentially explained by a synergistic effect of spermine with the herbicide. A similar observation has been observed for spermine in the presence of β-lactams and other antibiotics in *S. aureus*, but the specific molecular mechanism is currently unknown (Kwon and Lu, 2007).

4.4.4 2,4-D alters surface ultrastructure and physical properties in *E. coli*

The bacterial cell envelope forms the protective barrier that senses external challenges, such as exposure to xenobiotics, and transmits signals into the cell that lead to a cellular response. The cell surface of *E. coli* exposed to 2,4-D was markedly remodeled, with altered surface ultrastructure, roughness and hydrophobicity (Table 4.2). Exterior macromolecules contribute to the surface ultrastructure, roughness and adhesion in
bacteria and their loss, damage or irregular packing leads to surface remodeling (Paul et al., 2011). Reversible adhesion in Gram negative bacteria is largely influenced by surface residues, particularly lipopolysaccharides and other extracellular polysaccharides contributing to surface adhesion, wettability and hydrophilicity of bacterial cells (Francius et al., 2011). Studies examining the role of outer membrane proteins on adhesion indicate that they also play a significant role in adhesion to hydrophilic surfaces and biofilm formation (Rivas et al., 2008). The surface of E. coli is normally hydrophilic (Rivas et al., 2005), and so the increase in surface roughness and the negative charge (Table 4.2) directly measures the loss of envelope integrity. The cell envelope mechanical properties contribute to cell shape in Gram negative bacteria and originate from intermolecular interactions between phospholipids, proteins, peptidoglycan, and lipopolysaccharides (Vadillo-Rodriguez et al., 2008). The greater envelope compliance (Young’s moduli) for 2,4-D treated cells indicates the loss of such interactions. It is clear from the ROS accumulation that 2,4-D is capable of entering the cell and causing oxidative damage that can lead to surface remodeling, but it is also capable of interacting directly with surface macromolecules.

4.4.5 E. coli cell envelope is remodeled with 2,4-D exposure

The key metabolic pathways altered by 2,4-D exposure are consistent with the observed morphological, ultrastructural and mechanical changes. Reduction in metabolites involved in peptidoglycan biosynthesis, such as uridine monophosphate (UMP), with a simultaneous accumulation of alanine, lysine and glutamate may indicate loss of peptide crosslinking in the cell wall. Corynebacterium glutamicum shows peptidoglycan damage
upon exposure to 2,4-D, which has been related to an increased expression of peptidoglycan biosynthetic enzymes (Fanous et al., 2007). Alteration to the cell envelope is known to have a direct effect on cell signaling mechanisms, leading to global transcriptional changes associated with cell division and shape regulation (Typas et al., 2011). The Cpx response in *E. coli* mediates envelope stress adaptation and is activated by surface macromolecular damage (Ruiz and Silhavy, 2005), consistent with this study. Such a response is conferred through increased expression of chaperones and proteases that degrade or repair the damaged proteins responsible for maintaining cell wall and membrane integrity (Raivio et al., 2013), also explaining the accumulation of amino acids. The observed repression of ABC transport and phosphotransferase systems, which take active roles in the export of cell wall and membrane biosynthesis precursors across the inner membrane (Sobhanifar et al., 2013), may indicate alteration to cell wall biosynthesis. In line with these findings, alteration to peptidoglycan biosynthesis is an indication of 2,4-D induced envelope and membrane damage that we observe as changes to surface roughness and charge.

### 4.4.6 2,4-D alters the membrane transport system in *E. coli*

Passive transport of 2,4-D through the cell envelope has been shown for *Rhizobium* sp. M4 (Arias and Fabra de Peretti, 1993), along with breakdown of the membrane permeability barrier and reorganization of membrane fatty acids in yeast (Viegas et al., 2005). This is consistent with the increased amounts of fatty acids such as octadecanoic acid, pentadecanoic acid, undecanoic acid and sugars with 2,4-D exposure. In response to 2,4-D, several proteins involved in the ABC transport system in *Pseudomonas putida*
were down regulated, (Benndorf et al., 2006) while ABC transport proteins in *Corynebacterium glutamicum* were expressed (Fanous et al., 2007). Taken together with the current study, this indicates that 2,4-D can have a direct impact on the membrane transport system.

**4.4.7 E. coli reduces oxidative phosphorylation during sub-lethal 2,4-D exposure**

The reduction of succinic acid, pyrophosphate and phosphoric acid with 2,4-D exposure suggests a negative impact on oxidative phosphorylation and ATP biosynthesis, likely leading to repression of energy consuming biosynthetic pathways. A reduction in the total ATP content, respiration and growth has been observed for *Pseudomonas putida* exposed to 2,4-D (Benndorf et al., 2006). Negative impacts on the cell envelope and membrane integrity by 2,4-D can affect ATP biosynthesis and growth. Polyamines are crucial for glucose utilization and optimum growth in *E. coli* (Gutierrez-Rios et al., 2007), and exposure to 2,4-D led to reduced polyamine metabolites such as putrescine and cadaverine. Such effects, together with the sharp decrease in nucleotide derivatives, uridine monophosphate, urea and uracil, which are direct indicators of nucleotide metabolism, DNA replication, cell division and growth states, reveal a significant reduction in cell growth and division.

Co-reduction in ATP biosynthesis and nucleotide metabolism, coinciding with a filamentous phenotype, offers a highly effective energy conservation system. The increase in sugar levels along with reduced phosphotransferase system metabolites is a good indication for loss of coupling between glycolysis, the TCA cycle and oxidative phosphorylation, along with the inactivation of membrane transport proteins involved in
sugar transport (Escalante et al., 2012). By down regulating energy intensive pathways, in this case repression of cellular respiration, cell division and nucleotide metabolism, the bacterium is able to adapt to the 2,4-D induced stress.

4.5 Conclusion

The lipophilic nature of 2,4-D and the associated oxidative stress produces a series of primary stress responses involving the alteration of cell envelope integrity and DNA damage. Such changes lead to a series of secondary stress adaptations with reduced cell division as a main survival mechanism. *E. coli* cell shape, physical and biochemical properties, along with vital pathways such as oxidative phosphorylation and the membrane transport system serve as effective indicators of 2,4-D exposure in the environment. Future studies will examine the specific molecular targets of 2,4-D in *E. coli* to better understand the associated toxicity mechanism on microbial communities.

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CHAPTER 5 - LIVE CELL IMAGING OF REAL-TIME CELL DIVISION AND ENVELOPE REMODELING IN \textit{ESCHERICHIA COLI} EXPOSED TO 2,4-DICHLOROPHENOXYACETIC ACID

The majority of this chapter is part of a paper currently under review,

Bhat, S. V.; Kamencic, B.; Kornig, A.; Dahms, T. E. S. Live cell imaging of real-time cell division and envelope remodeling in \textit{Escherichia coli} exposed to 2,4-dichlorophenoxyacetic acid, \textit{App. Env. Micro}. Manuscript # AEM01511-16
5.1 Introduction

*E. coli* is a robust, easily adaptable and culturable bacterium *in vitro*, making it an excellent model in which to study bacterial response mechanisms to xenobiotic exposure. *E. coli* has been used to characterize the impact of several antimicrobial compounds including peptides, antibiotics, pesticides and other xenobiotics (Asghar et al., 2006; Guven et al., 2005; Ruiz and Silhavy, 2005). Survival of this organism in diverse hostile environments comes from its ability to persist and reproduce (Touchon et al., 2009). Morphological change, which provides a survival advantage, is one of the key adaptation mechanisms exhibited by bacteria under stressful conditions. In particular, *E. coli* exhibits a filamentous phenotype during temperature stress (Ricard and Hirota, 1973), oxidative stress (Bhat et al., 2015), host invasion (Henry et al., 2005; Justice et al., 2006) and xenobiotic stress (Bhat et al., 2015; Boberek et al., 2010).

Cell division in *E. coli* is initiated by the assembly of the divisome complex at the mid-cell septa, with FtsZ forming the Z-ring scaffold which recruits other colocalizing partners (Adams and Errington, 2009). FtsZ is a GTP-dependent tubulin homologue, and its action is tightly regulated by a number of intracellular and environmental factors. FtsZ forms the Z-ring along with a group of over twenty colocalizing proteins, many of which are unidentified and uncharacterized. The complete architecture of the Z-ring is not known (Egan and Vollmer, 2013).

FtsA, which colocalizes with FtsZ, is possibly the second most characterized protein after FtsZ. FtsA forms actin-like protofilaments, anchors FtsZ to the inner membrane and helps in recruiting downstream proteins to further stabilize the divisome complex. A short
amphipathic C-terminal sequence helps attach FtsA to the membrane and the FtsZ C-terminal peptide in turn interacts with FtsA, resulting in a cooperative interaction of the three components (Geissler et al., 2007; Pichoff and Lutkenhaus, 2005).

There are two factors known to influence Z-ring formation at the mid-cell, the action of MinCDE proteins and nucleoid occlusion (Sun and Margolin, 2004). The latter is based on cytological evidence that FtsZ does not form a Z-ring over the dividing nucleoid and is mediated by the SlmA complex in *E. coli*. SlmA binds to specific DNA sequences at the periphery of the nucleoid region and to FtsZ filaments tethered to the membrane by FtsA. Upon binding to the tail of the FtsZ filament, SlmA makes additional contact with FtsZ inducing a conformational change which breaks the filament and directly prevents FtsZ polymerization on the nucleoid (Cho et al., 2011; Du and Lutkenhaus, 2014). On the other hand the Min system prevents FtsZ polymerization at the poles (Wu and Errington, 2011). SulA, a product of the SOS response that is induced following DNA damage during oxidative stress, is also known to inhibit FtsZ polymerization by binding to its C-terminus and inhibiting the GTPase activity necessary for polymerization (Cordell et al., 2003). The free radical induced SOS system in bacteria activates RecA, a coprotease that helps autocleave the LexA repressor. In the absence of LexA, the operator sequence allows the expression of more than forty SOS genes, including SulA (Janion, 2008). SulA binds to FtsZ, blocking cell division preventing damaged DNA from passing to daughter cells. SulA is also known to sequester FtsZ monomers, thereby decreasing its critical concentration necessary for assembly (Chen et al., 2012). SulA is known to cause disassembly of the existing Z-rings and prevent assembly of new rings (Adams and Errington, 2009).
Environmental factors such as temperature, oxidative stress, UV radiation, antibiotics and xenobiotics are known to impact bacterial cell division machinery (Adams et al., 2011; Chimerel et al., 2012; Taschner et al., 1988). Since cell division is an attractive target for clinical research, a number of antibiotics and other antimicrobial compounds have been screened for this activity, more specifically molecules targeting FtsZ (Li and Ma, 2015).

In addition to the intentional development of compounds targeting cell division, certain xenobiotics may act in a similar, unintended manner and it is therefore important to understand their mechanism before releasing them into the environment. The commonly used herbicide, 2,4-D is designed to target and eliminate broad leaf plants for agricultural monocot crops. This compound persists with a half-life of 10–200 days in the environment and is known to have undesired effects on diverse species in the food chain, from mammals to soil bacteria (Boivin et al., 2005; Chinalia et al., 2007). Despite the field application of 2,4-D for more than several decades, the specific molecular action of this herbicide in target and non-target biological systems remains unclear.

Visualizing the impact of xenobiotics in live cells at high resolution is highly desirable, but extremely challenging. Traditional AFM imaging to examine biological samples includes contact, non-contact and tapping modes. The latter less invasive mode was initially thought to be optimal for biological samples (Putman et al., 1994), however contact mode with low spring constant cantilevers provides better data and is sufficiently gentle (Le Grimellec et al., 1998). For AFM imaging, living cells must be immobilized firmly enough to withstand the lateral forces exerted by the scanning tip, but without causing macromolecular damage (Lonergan et al., 2014). Tapping mode AFM in liquid initially gave substantial improvement in image quality and stability while producing
minimal damage to the sample over conventional contact mode, however this technique has a number of challenges. In tapping mode, the user has no control over the distance between tip and sample (Z-length), so only the oscillation amplitude can be controlled, which can still cause frictional forces as contours change across the sample. It is highly important to reduce the lateral forces exerted on the sample since even nanoNewton-scale forces can irreversibly damage the macromolecules being imaged on the surface. Tapping mode can only provide height and lateral force images and uses much stiffer cantilevers, requiring tuning each time. The cantilever resonance frequency is often shifted by damping in liquid during imaging and the tip induces a hydrodynamic pressure on the specimen leading to image distortion (Shih and Shih, 2015). Therefore tapping mode is not ideal for very soft, loosely attached samples with steep edges such as bacteria.

Contact mode on the other hand, is the most well established mode used to investigate biological samples. Here the tip stays in contact with the sample surface at all times and scans the surface with a desired low force to develop topographic details at the nm level (Le Grimellec et al., 1998).

Force-distance curves have been used to examine and characterize the surface properties of many biological and non-biological materials (Hinterdorfer and Dufrene, 2006). The traditional setup determines surface physical properties by moving the AFM cantilever with a piezoelectric micro positioner to contact the surface with a known applied force, also called the set point. Retracting the tip in the same manner produces a force-distance curve, simply called a force curve (Dufrêne, 2003). A constant approach and retraction velocity is maintained by the Z piezo, and the amount of force applied is established based on sample properties. Force curves can be obtained at any desired point on the
sample surface of small biological sample such as bacteria and mammalian cells, with a contact mode topography image used to determine the location of interest. Force curves at a single point, multiple points of interest or an organized force map can be obtained using traditional AFMs. Force mapping has been used to probe samples in air, liquid or in air-liquid interfaces (Gan et al., 2015; Kiracofe and Raman, 2012). Quantitative imaging is a new variation of traditional force mapping that offers higher resolution and speed, providing much more data than force mapping alone. QI is a relatively new technique that overcomes the majority of drawbacks associated with tapping mode, since it induces no lateral forces and the user can completely monitor the tip-sample distance at every point on the sample, making imaging in liquid while maintaining sample integrity much easier. QI simultaneously measures height, lateral forces and collects force curves at every pixel on the sample surface. The force curves obtained during QI can be used to quantify physical characteristics and topography, providing complete AFM sample analysis. QI also allows a range of cantilevers appropriate for delicate samples making this technique highly useful for biological specimens (Chopinet et al., 2013).

AFM has been widely used to image living mammalian cells which is less challenging than live bacteria as they are large, non-motile and can strongly attach to flat surfaces. Bacteria pose unique challenges due to their small size and high motility. Several immobilization techniques have been tested (Lonergan et al., 2014; Zeng et al., 2014), each with their individual drawbacks for imaging in liquid media. Cell-Tak™, a commercially available polyphenolic protein adhesive isolated from a marine mussel has demonstrated robust immobilization of bacterial cells on hard surfaces with the least disturbance to the bacteria surface (Louise Meyer et al., 2010).
AFM and LSCM are both powerful tools for probing different aspects of a cellular system, and when integrated for simultaneous data collection they provide an efficient means for examining bacterial response mechanisms. AFM provides exceptional spatial resolution that is not diffraction limited since it uses an atomically sharp tip on a cantilever to raster scan the surface of the sample, but the latter limits its temporal resolution. AFM generates high content data describing not only sample surface ultrastructure at the nm scale but also cell physical properties (Muller and Dufrene, 2011; Yip, 2001). On the other hand, LSCM is light based and so diffraction limited, but produces images with excellent temporal resolution (Kondra et al., 2009). Integrating AFM with LSCM offers nanoscale spatial resolution of surfaces and real-time imaging of intracellular physiology of live cells in situ. Live quantitative imaging (QI) by AFM is the least invasive of all surface imaging methods, based on its superior control over vertical forces and minimal lateral forces (Chopinet et al., 2013). QI provides data rich images with force curves of pN sensitivity at each pixel that reflect cell surface adhesion and elastic properties. I have physically integrated AFM-QI-LSCM for simultaneous correlative imaging to examine the specific response mechanisms of E. coli during exposure to 2,4-D.

A typical commercially available AFM-CM or AFM-epifluorescence instrument provides the ability to view/image the sample using the light microscope while simultaneously obtaining high resolution topographical data using AFM. Such a modality has been used for various applications in mammalian cells such as obtaining high resolution topography while localizing fluorescently tagged molecules to map molecules of interest (Park et al., 2010). This technique has also been used to characterise biomaterial interfaces, determine
the mechanical properties of the mammalian cell cytoskeleton (Deng et al., 2009; Geisse et al., 2009) and nano-manipulation studies (Chen et al., 2007; Silberberg and Pelling, 2013; Trache and Lim, 2009). However, only a few studies have used this technique on microbial cells and to a much lower capacity. For example, a study by Kuyukina et al. (2014) used integrated AFM-LSCM to determine the viability and changes in morphology of solvent exposed bacteria. El-Kirat-Chatel and Dufrêne (2012) used correlated AFM-epifluorescence to examine the interaction of Candida albicans with mammalian macrophages, however the correlated imaging was conducted on fixed cells in air. This technique has not been previously used for high content data of live bacteria in liquid due to the lack of suitable sample immobilization strategies and an imaging technique gentle enough to keep bacteria attached while the AFM probe scans their surface.

We previously showed that sub-lethal levels of 2,4-D induced a filamentous phenotype in E. coli (Bhat et al., 2015; see chapter 4) leading to the hypothesis that 2,4-D affects components of cell division. Here I demonstrate the AFM-QI-LSCM technique on live bacteria under native conditions and use the technique to provide evidence for the impact of 2,4-D on cell division proteins and simultaneous temporal changes to surface ultrastructure and physical properties. Based on cytological and biochemical evidence I propose that 2,4-D induces oxidative stress leading to DNA damage, immediately impacting FtsA, FtsZ and SulA localization which disrupts cell division and ultimately leads to the filamentous phenotype at longer exposure times.

5.2 Materials and methods
5.2.1 Strains and growth conditions

The *E. coli* wild type parent strain WM1074 and its mutants WM2026 (WM1074+stable chromosomal fusion FtsZ-GFP), WM2760 (WM1074 containing pWM2760) and WM2739 (WM1074+stable chromosomal fusion SulA-GFP) were a kind gift from Dr. William Margolin (Geissler et al., 2007). Growth media, conditions and MIC determination are described in chapter 2.

5.2.2 DNA damage assay

The extent of 2,4-D induced DNA fragmentation was tested using the agar diffusion method (Fernandez et al., 2008). *E. coli* WM1074 cells were exposed to 0–4 mM 2,4-D (5 s, 10 s, 30 s, 60 s, 3 h, overnight), H$_2$O$_2$ (6 h, overnight) or 50 °C (2 h). The culture was diluted to an OD$_{600}$ of approximately 0.1 and 25 µL was mixed with 60 µL of 0.1 % molten agarose at 37 °C and vortexed thoroughly. A 20 µL aliquot of this mixture was then spotted onto slides pre-coated with agarose and a coverslip was carefully placed on the sample to prevent air bubbles in the gel. The slide was incubated at 4 °C for 10 min allowing the gel to solidify. The coverslip was removed carefully and the slides were incubated in lysis buffer (2% sodium dodecyl sulfate, 0.05 M EDTA, and 0.1 M dithiothreitol, pH 11.5.) at 37 °C for 5 min. All samples were submerged in lysis solution in the same tray to avoid bias in treatment. The lysis buffer was removed and the slides carefully washed, without tilting, in deionized water (5 ×) and dried in ethanol (70, 95 and 100 %, 3 min each at -20 °C). The slides were dried in an oven under vacuum overnight, stained with SYBR gold (Life technologies) for 5 min, washed, mounted in
TBE buffer with a 18 × 18 Zeiss precision coverslip and imaged by epifluorescence microscopy (Ex: 497 nm, Em: 537 nm).

The degree of DNA damage was determined by measuring the DNA spreading around each cell. Images were processed to maximize contrast and remove background noise (Zeiss Zen software) and the diameter of DNA halos surrounding 100 cells for each sample were measured using ImageJ.

5.2.3 Epifluorescence microscopy

Samples from the assay (5.2.2) and the E. coli WM2026 strain (5.2.1) exposed to 4 mM 2,4-D after 3 h and overnight exposure were imaged (Ex: 488 nm, Em: 509 nm) on a Zeiss Axio Observer Z1 inverted wide-field fluorescence microscope to determine DNA damage, and the Z-ring and nucleoid positioning, respectively. Cells were stained with DAPI (100 µg/mL, Ex: 358 nm, Em: 461 nm) and mounted in 0.01 M PBS and imaged. Similarly, E. coli WM2739 was also imaged to determine the oxidative stress induced SOS response.

5.2.4 Sample preparation

A 30 µL aliquot of freshly prepared Cell-Tak™ solution (145 µL of pH 8 NaHCO₃ buffer, 5 µL of 1 mM NaOH and 5 µL of Cell-Tak™) was spread (1 sq. cm) onto plasma or piranha-cleaned (by sequentially immersing in 1 M HCl for 1 min, piranha solution - 3:1 v/v of H₂SO₄:H₂O₂, for 1 h, rinsed with deionized water, immersed in methanol and acetone for 2 min and air dried) coverslips, incubated (RT, 30 min), rinsed gently with ultrapure water, air dried and stored for up to 2 weeks at room temperature. The coverslip
was mounted onto the JPK Biocell coverslip holder which was placed on the AFM petridish heater.

A 500 µL overnight culture of *E. coli* was inoculated into 5 mL of fresh LB media and allowed to grow for 3 h to obtain culture at the mid-log phase. Approximately 500 µL of the culture was added to the coverslip and incubated (32 °C, minimum of 30 min) in the dark. The samples were imaged under PBS (0.01 M, pH 7), 1:1 diluted LB in PBS and plain LB to observe the influence of imaging media. In all the cases the sample was rinsed thoroughly with the imaging media and mounted with 500 µL of the same solution onto the AFM stage. *E. coli* WM2026 imaging media contained 40 µg/mL of IPTG during imaging. The PBS and media were filtered (0.2 µm sterile filters) and maintained at 32 °C prior to use.

The coverslips were mounted either onto a JPK Biocell coverslip holder or were glued to the bottom of a petridish and placed in the JPK petridish heater. During the experiments the sample was maintained at a temperature of 32 °C.

### 5.2.5 Live-AFM-LSCM

The essential elements of the AFM-LSCM setup are shown in Figure 5.1. The entire system was placed on a vibration isolation platform in a home-built acoustic enclosure.

Our AFM-LSCM setup consists of the Nano Wizard AFM (JPK Germany) placed on an inverted LSCM 710 equipped with 34 channels of highly sensitive GaAsP detectors, five steady state excitation lasers (458, 488, 514, 543, 594 nm) and a Ti:Sapphire tunable
**Figure 5.1.** Cartoon (not to scale) of the integrated AFM-LSCM instrument used for simultaneous imaging. The schematic view of the setup shows the cantilever imaging the surface of cells immobilized on the coverslip with the inverted LSCM objective focused at the bottom. The coverslip was glued against the bottom edge of a circular hole cut into a petridish. Liquid media was added from the top, with cells immobilised firmly using Cell-Tak™ on the top side of the coverslip. The whole setup was placed inside the AFM petridish heater maintained at a desired temperature. The middle image shows an optical image taken using the front port camera of the LSCM to show the AFM cantilever suspended in liquid with the tip projecting towards the fast dividing *E. coli*. This optical image was also used for calibration of the tip position to overlay AFM and optical images. On the right are LSCM images showing the Z-ring as a function of FtsZ-GFP localization (top) and the simultaneously collected AFM-QI height image (bottom).
femtosecond pulsed IR laser. The LSCM stage was replaced with the custom designed AFM stage (JPK) and the sample mounted in a manner similar to that described above. A camera was mounted onto the front port of the confocal inverted microscope base and connected to the AFM-ECU to produce LM images of the sample and to allow alignment of the laser on the AFM tip. The sample was initially brought into focus using the low resolution 20 × objective to allow laser alignment and to assess the distance between AFM tip and sample. A drop of the sterile imaging media was placed slowly onto the cantilever and allowed to slide onto the tip to avoid sudden jump into contact with the liquid and possible tip damage due to change in the surface tension. The tip was lowered into the liquid and focused using the 20 × objective and positioned on a suitable point on the sample using the x and y control screws on the AFM head. Once the sample and the AFM tip could be viewed using the low resolution objective, the laser was aligned onto the cantilever to obtain the best signal and the laser was focused onto the center of the photodiode.

The sample was subsequently viewed using a 63 × oil immersion objective after placing a drop of oil on the lower side of the coverslip. The sample was focused and a suitable single cell, which appeared immobile by DIC and confocal but actively dividing (FtsZ-GFP ring at the mid cell) was chosen for imaging. The optical calibration was used to precisely position the AFM tip in relation to the cell for generating optical overlay of the AFM-LSCM images. A force constant calibration of the AFM cantilevers (Nanoscience, model no: HYDRA4V-100N), having a low nominal spring constant \(k = 0.08\) N/m and calibrated \(k = 0.05 \pm 0.03\) N/m in imaging media), was followed by a low resolution force map to evaluate the integrity of the chosen cell. The spring constant of the AFM tip
was calibrated using the thermal noise method (Lubbe et al., 2013) each time prior to data collection. The height image from the force map was used to choose an appropriate area for collecting the QI image. Parameters such as set point, Z-length, and approach/retract speed were adjusted for live QI to minimise noise. The *E. coli* WM1074 was imaged by AFM-QI alone, while WM2026 containing the FP construct was imaged by integrated AFM QI-LSCM.

5.2.6 AFM-LSCM data processing

The AFM and LSCM images taken approximately at the same time were overlaid using Photoshop 11. LSCM images were processed for contrast (Zen image processing software) and digitally enlarged to fit the AFM height image.

QI force curves obtained at each pixel on a 128 × 128 image were corrected (JPK software) with the calibrated cantilever force constant for baseline and tip-sample separation. The adhesion was determined using the distance between the lowest point and baseline of the retract curve. Young’s modulus was determined using a Hertzian fit which is an estimate of cell envelope elasticity (JPK software). Surface roughness was measured at the mid-point of the cell from QI height images as described previously in chapter 4 (Bhat et al., 2015). All the force curves in the image were batch processed using a 200 × 200 nm square in the center of the cell, where curvature was minimized. Histogram data was exported from the JPK software for statistical analysis and plotting.

All data were statistically analysed using unpaired student’s t-test and one-way ANOVA (Graph Pad Prism 5.01).
5.3 Results

The minimum inhibitory concentrations (MIC) of 2,4-D for all the *E. coli* strains were determined to be 6 mM, and 4 mM was used as the highest sub-lethal concentration that produced consistent and sufficient cell growth for microscopy and biochemical assays.

5.3.1 Correlative AFM-LSCM simultaneously probes intra and extracellular changes in response to 2,4-D

A suitable actively dividing cell as indicated by LSCM with a distinct Z-ring at the center of the cell was chosen for live AFM-LSCM. Consequently, a force map was taken to further locate the precise position of the cell with respect to the cantilever at low resolution (20 × 20 pixels, 15 µm square). The presence of the cell and its specific location can be confirmed by the presence of very bright pixels in the height image. The area with the brightest pixels was further enlarged and imaged at high resolution QI (126 × 126) to obtain a complete map of Young’s moduli and adhesion. A high resolution QI image of the cell was taken while adjusting the Z-length, set point and imaging speed. A Z-length of ~3000 nm, set point ~0.5 nN and an extend/retract speed of 100 µ/s worked well and produced the least noise in most cases. Imaging time associated with the above mentioned parameters was approximately 17 min. Confocal time lapse images were collected directly before and after the QI images. We were able to simultaneously collect QI and confocal images but they often resulted in AFM tip crashes or large noise in the QI image. The QI imaging conditions that provided the best results are outlined in the table 5.1.

Thorough sample rinses ensured that the majority of planktonic cells were removed, but
Table 5.1 Optimal imaging parameters and conditions for live *E. coli* in half diluted LB media *.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-length</td>
<td>3000 µm</td>
</tr>
<tr>
<td>Extend speed</td>
<td>100 µm/s</td>
</tr>
<tr>
<td>Extend time</td>
<td>30 s</td>
</tr>
<tr>
<td>Retract speed</td>
<td>100 µm/s</td>
</tr>
<tr>
<td>Retract time</td>
<td>30 s</td>
</tr>
<tr>
<td>Sample rate</td>
<td>100 kHz/s</td>
</tr>
<tr>
<td>Set point</td>
<td>0.4 – 0.7 nN</td>
</tr>
<tr>
<td>Time taken to complete each image</td>
<td>15.6 min</td>
</tr>
<tr>
<td>Resolution</td>
<td>125 × 125 pixels</td>
</tr>
<tr>
<td>Temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Cantilever spring constant (measured using thermal noise method)</td>
<td>0.04 ± 0.02 N/m</td>
</tr>
</tbody>
</table>

* LB media was diluted in 0.05 M PBS 1:1 and filtered using a 0.2 µm nylon filter.
with an average *E. coli* doubling time of 20 min the imaging environment became rapidly over crowded. The same cell was repeatedly imaged simultaneously by AFM and LSCM for 2–3 hours until image quality was drastically reduced. Increased division led to microcolony formation, more sticky film around the cells and many planktonic cells due to loss of immobilization, which obstructed cantilever movement and laser alignment. At this point, the AFM head had to be removed and the sample washed gently several times, replacing the media, and imaging was resumed as described previously. The AFM head was replaced directly onto the LSCM stage and the optical overlay ensured that the cell of the same progeny, which after generations of division (2–3 h) often appeared as a mini colony, was selected for further imaging. Since a single AFM image takes ~ 20 min for collection, AFM images of cells showed dramatic changes from one image to the next. The addition of fresh media every few hours ensured that cells were provided with adequate nutrients, and that waste products were removed to prevent toxicity. AFM time lapse images show every step of cell division, including cell elongation, the beginning of constriction at the mid cell, extension of constriction and the separation of daughter cells (Figure 5.2).

The central challenge with the integrated imaging was sample immobilization, and although Cell-Tak™ (Zeng et al., 2014) appeared to work well consistently, finding an immobilized and actively dividing cell for imaging was a challenging task. Also, after several hours of imaging Cell-Tak™ appeared to lose its efficacy so that the majority of cells detach from the surface.
Figure 5.2 AFM-QI time lapse images showing various stages *E. coli* cell division.

AFM-QI images were taken every 15.6 min in 0.05 M PBS. In PBS, cells showed a much slower division compared to pure and half diluted LB in PBS, taking approximately 2.5 h. Shown are cell elongation, constriction at the mid-cell, and separation of daughter cells. Arrows show a cell slowly detaching from the surface immediately after division which eventually becomes fully planktonic. Scale bar, 1 µm
5.3.2 Surface elasticity and adhesion in *E. coli* during active division in PBS, diluted LB and LB media

During AFM-QI-LSCM imaging we observed typical FtsZ localization and Z-ring formation patterns and real-time changes in surface physical properties in actively dividing *E. coli*. Cell division was slower (~2.5 h) in PBS (Figure 5.2) as compared to LB/PBS (1:1) and in LB media (20 min). Diluting the LB media 1:1 with PBS did not appear to have an effect on the speed of cell division. Cells became planktonic after several divisions indicating their loss of adhesion to Cell-Tak™, or detachment of Cell-Tak™ from the coverslip. Cells that were still immobilized formed a microcolony, due to continuous division, however their surface properties remained the same.

As a first demonstration of integrated AFM-LSCM, we investigated the wall elasticity (Young’s moduli) and surface adhesion in *E. coli* from force curves (Chapter 2, section 2.6.5) during active cell division. We first selected a single cell that was strongly immobilised to show active cell division with the presence of a distinct Z-ring at the mid-cell. A series of time lapse images by LSCM confirmed a stable FtsZ-GFP with the typical localization patterns of FtsZ during cell division.

An actively dividing cell showed a spectrum of surface elasticity (Figure 5.3) changes with a range of 0.1–2 MPa. The center of the cell had a much higher elasticity (1–2 MPa) when compared to the edges (100–300 KPa) for all the samples, regardless of the imaging media. Values only at the center of the cell were considered for analysis and there was no significant change in the elasticity between different imaging media. On the other hand, the surface adhesion varied slightly with the imaging medium. *E. coli*
Figure 5.3 QI map of *E. coli* cell surface taken during cell division. QI map of *E. coli* WM1074 surface taken at 0 min and after many cell divisions at 156 min showing no changes in the Young’s modulus on the *E. coli* cell surface. Cells showed much higher Young’s modulus at the center (1–2 MPa) when compared to the peripheral region (100–300 KPa) due to artifacts resulting from increased curvature.
when imaged in 0.01 M PBS showed the highest adhesion (38.1 ± 2.29 nN), followed by ½ diluted LB (28.8 ± 1.5 nN) in PBS, and pure LB (17.3 ± 1.06 nN) showed the least adhesion (n ≥ 180, p < 0.0001) to the silicon tip. Adhesion did not vary significantly with the different stages of cell division.

5.3.3 Sub-lethal 2,4-D alters *E. coli* cell division at longer exposure times

In cells treated for 3 h, there was no significant elongation, however most cells did not show a typical Z-ring, but rather diffuse green fluorescence distributed throughout the cell but occluded from nucleoid regions (Figure 5.4). Cells treated overnight showed irregular and multiple partially formed Z-rings arranged side by side (Figure 5.5 b). Simultaneous imaging of the two channels (DAPI and GFP) following overnight exposure showed an altered localization of both the Z-ring and cell nucleoid. Images showed irregular clumps of undivided DNA with some Z-rings localized between, and others above the nucleoid masses (Figure 5.5 a). SulA-GFP after 3 h and an overnight treatment, produced brighter signals overall (Figure 5.6), and more elongated cells had a higher signal intensity indicating an increased expression of SulA.

5.3.4 2,4-D rapidly alters FtsZ, FtsA and SulA localization in live *E. coli*

Since *E. coli* are highly motile, immobilizing them for confocal and AFM scanning was a daunting task. *E. coli W* is a robust and fast growing strain (Archer et al., 2011), forming short rods visible during integrated imaging. Live *E. coli* WM2026, WM2760 and *E. coli* WM2739 were imaged with LSCM to examine FtsA,
Figure 5.4 Changes to FtsZ-GFP and DAPI-labelled nucleoid after 3 h exposure to 4 mM 2,4-D imaged by epifluorescence microscopy (GFP 488/509 nm, DAPI 358/461 nm). Formally exposed cells (A–C) show a distinct Z-ring at the mid-cell and a dividing nucleoid. Upon 4 mM 2,4-D exposure for 3 h (a–c), the majority of cells lacked a Z-ring and instead FtsZ appeared to be localized away from nucleoid regions. Although the majority of the cells lacked a Z-ring, some cells were able to retain the Z-ring (arrow in b) and continued dividing, showing population heterogeneity. Scale bar, 2 μm
Figure 5.5 Changes to the GFP-FtsZ and DAPI stained nucleoid localization after an overnight 2,4-D exposure imaged by epifluorescence microscopy (GFP 488/509 nm, DAPI 358/461 nm). GFP and DAPI are rendered in green and blue respectively. *E. coli* exposed to 4 mM 2,4-D (a, b, c) showed multiple Z-rings (arrow in b) and what appeared to be improperly divided nucleoids (arrow in c). Cells treated with formula (A, B, C) and unexposed cells show divided nucleoids (arrow in C) with a single Z-ring (B) located between. A, a) show DAPI and GFP channels merged, whereas B, b) and C, c) show only GFP and DAPI respectively after separating the two channels. Scale bar, 2 μm
Figure 5.6 Changes to SulA-GFP localization after 3 h and overnight treatment to 4 mM 2,4-D imaged using epifluorescence microscopy (GFP 488/509 nm). Formula treated *E. coli* (A) show a uniformly low signal from SulA-GFP, however after 3 h exposure to 4 mM 2,4-D (B) cells have an overall brighter signal (C), with elongated cells showing greater signal compared to the shorter cells. Scale bar, 2 μm
FtsZ and SulA signal in real-time. Time-lapse images taken using live-LSCM of FtsZ before, during and after the addition of 2,4-D indicate changes to FtsZ localization within 5 s after the addition of 1 mM 2,4-D (Figure 5.7). In the absence of 2,4-D, with < 1 mM 2,4-D or formula treatment, FtsZ-GFP shows a distinct localization pattern associated with various stages of its assembly, involving formation of bright spots near the cell-periphery at the polar and sub-polar regions, which move towards the mid-cell to form a band which eventually gets brighter and begins to constrict (Figure 5.7). Immediately following the addition of 1 mM 2,4-D, the Z-ring disappeared within seconds for all the cells (~ 4–5 s, time frame of confocal scan) to form bright fluorescence spots in a randomly distributed punctate pattern (Figure 5.7 a–f). In some cells FtsZ was more concentrated near the mid-cell and poles but in others a random distribution was observed. In each case, the signal was stagnant and its fluorescence signal decayed rapidly over a period of 2–3 min.

In the absence of 2,4-D, with < 1 mM 2,4-D or formula treatment, FtsA-GFP showed a similar localization to FtsZ-GFP, with a distinct Z-ring at the mid-cell (Figure 5.8 A). Immediately (< 5 s) after the addition of 1 mM 2,4-D, FtsA-GFP relocalized and the Z-ring disappeared. FtsA-GFP formed large bright masses (Figure 5.8 B-D) in a randomly distributed punctate and banded pattern throughout the cell. SulA-GFP produced a significantly weaker, uniform and delocalized fluorescent signal across the cell in the absence of 2,4-D and in the presence of formula control (Figure 5.8 a). However ~ 4–5 s after the addition of 1 mM 2,4-D, SulA appeared occluded from nucleoid regions forming a figure eight pattern (Figure 5.8 b–d) and localization was irreversible as SulA-GFP was never again observed in a uniform distribution and its signal decayed over time.
Figure 5.7 Time lapse images showing the change in FtsZ-GFP localization imaged by LSCM (GFP 488/509 nm). *E. coli* exposed to only formula (A–F) show FtsZ as bright fluorescent signals, with a distinct Z-ring at the center. Within 5 s of treatment with 1 mM 2,4-D (a–f) the Z-ring disappeared, and FtsZ formed stagnant bright spots throughout the cytoplasm. In some cells FtsZ appeared to be more near the cell center and periphery. Scale bar, 1 μm
Figure 5.8 Time lapse images showing changes to FtsA-GFP and SulA-GFP localization imaged by LSCM (GFP 488/509 nm). *E. coli* exposed to formula only (A) showed FtsA-GFP distinctly localized to the Z-ring, but upon exposure to 1 mM 2,4-D FtsA dissociated from the Z-ring within 5 s forming bright bands throughout the cell (B-D). SulA-GFP in formula exposed cells showed a uniform distribution throughout the cell (a). Within ~5 s following 1 mM 2,4-D treatment SulA formed figure eight patterns around dark regions corresponding to nucleoid regions (b-d). Scale bar, 1 μm
5.3.5 2,4-D causes DNA damage during short and long time exposures

The extent of DNA damage was examined during very short time periods to characterise its possible role in changes to the FtsZ, FtsA and SulA localization in the presence of 2,4-D. After 5 s exposure to 1 mM 2,4-D, there was a statistically significant increase in DNA damage, which further increased after 10, 30 and 60 s exposures as compared to controls (Figure 5.9 and 5.10). The DNA damage assay after 3 h and overnight exposure to 4 mM 2,4-D showed significantly increased DNA fragmentation (p < 0.0001, n = 100) for treated cells compared to formula and sample controls (Figure 5.11 a–c and d–f, and Figure 5.12). Positive controls, in which oxidative stress had been induced for 6 h and overnight using 4 mM H₂O₂ (Figure 5.11 g–i and j–l, and Figure 5.12) or from temperature stress at 37 °C overnight (p < 0.03, n = 100) or 50 °C for 2 h (p < 0.0001, n = 100), had increased DNA fragmentation (Figure 5.11 m–o and Figure 5.12).

5.3.6 2,4-D rapidly arrests E. coli cell division with simultaneous changes to surface roughness and elasticity

After the addition of 4 mM 2,4-D, cell division arrested abruptly, regardless of the stage of cell division, and remained stagnant (Figure 5.13 E–H). Although imaging was continued for several hours in the presence of 2,4-D, cell division did not resume nor did it with the removal of 2,4-D and the addition of fresh media. QI height images showed a significant change (p < 0.0001, n = 30) in surface roughness after 20 min exposure to 4 mM 2,4-D, from 5.24 ± 3.23 nm (control) and 7.67 ± 2.23 (formula control) to 22.07 ± 12.2 nm following 2,4-D exposure. The high standard deviation of roughness values following 2,4-D exposure indicates the variability of surface roughness, which did not
Figure 5.9 DNA damage assay showing the DNA halos stained with SYBR-gold over short exposure periods imaged by epifluorescence microscopy (SYBR gold, Ex: 497 Em: 537 nm). Unexposed and formula exposed *E. coli* after 60 s had no DNA damage as indicated by a lack of halos around cells (A). There was a significant increase in DNA damage after 5 (B) (*p* < 0.03, *n* = 100), 10 (C) (*p* < 0.0001, *n* = 100), 30 (D) (*p* < 0.0001, *n* = 100) and 60 s (E) (*p* < 0.0001, *n* = 100) exposure to 1 mM 2,4-D. Scale bar, 5 μm
Figure 5.10 Plots of DNA damage as a function of time after short exposure period.

_E. coli_ showed significant DNA damage after 5 (\(p < 0.03, n = 100\)), 10 (\(p < 0.0001, n = 100\)), 30 (\(p < 0.0001, n = 100\)) and 60 s (\(p < 0.0001, n = 100\)) exposure to 1 mM 2,4-D. Error bars indicate standard deviation.
Figure 5.11 DNA damage assay showing DNA halos stained with SYBR gold and imaged by epifluorescence microscopy (SYBR gold-495/537 nm) after a long exposure. Images show *E. coli* exposed to 4 mM (a–c, d–f) 2,4-D and 2 mM (g–i), 4 mM
$\text{H}_2\text{O}_2$ (j–l) and high temperature (m–o). *E. coli* cells had increased DNA damage as shown by larger halos around cells after exposure to 4 mM 2,4-D for 3 h (c) ($p < 0.0001$, $n = 100$) and overnight (f) ($p < 0.0001$, $n = 100$) compared to the formula exposed (b, e) and control (a, d) cells. Similarly, cells exposed to 2 mM $\text{H}_2\text{O}_2$ for 6 h (h) ($p < 0.0001$, $n = 100$) and overnight (k) ($p < 0.0002$, $n = 100$) and 4 mM $\text{H}_2\text{O}_2$ for 6 h (i) ($p < 0.0001$, $n = 100$) and overnight (l) ($p < 0.0001$, $n = 100$) also showed increased DNA damage, however, there was no statistically significant difference between the two $\text{H}_2\text{O}_2$ concentrations ($p > 0.05$). *E. coli* exposed to 37 °C overnight (n) and 50 °C for 2 h (o) also showed a significantly increased DNA damage ($p < 0.0001$, $n = 100$) compared to the representative control (m). Scale bar, 5 μm
Figure 5.12 Plots of DNA damage as a function of time after long exposure periods.

* E. coli showed significantly increased DNA damage after longer period exposures to 4 mM 2,4-D (3 h and overnight, p < 0.0001, n = 100) (B). H₂O₂ and high temperature at various exposure times were used as positive controls. Error bars indicate standard deviation.
Figure 5.13 Overlaid time lapse images of simultaneously collected QI topography (gold scale) and LSCM (white on black) images. Images A–D of formula treated cells show LSCM FtsZ-GFP fluorescence and QI topography of cells undergoing binary fission at the mid-cell upon formation of the distinct Z-ring at their center. Even before the constriction and separation of daughter cells is complete, there is formation of new Z-rings at the mid-cell of the soon to be daughter cells. Exposure to 4 mM 2,4-D (a–b) immediately halted cell constriction and caused disassembly of the Z-ring, with bright FtsZ-GFP signal dispersing from the midpoint throughout the cytoplasm. Scale bar, 0.5 μm
Figure 5.14 QI height (gold on black) and elasticity maps (green on black) showing changes to Young’s moduli following exposure to 4 mM 2,4-D for 16.5 and 120 min. Formula exposed cells (A, a, B, b) showed a relatively uniform elasticity, values ranging from 1–4 MPa. Exposure to 2,4-D for 16.5 and 120 min significantly reduced the Young’s modulus to a range of 80–400 KPa. I and II are representative force curves which were generated at every pixel of the QI image. Scale bars, 0.5 μm
Figure 5.15 Plot showing the time dependent decrease in Young’s modulus for *E. coli* exposed to 4 mM 2,4-D and imaged by QI-AFM. *E. coli* showed a significant decrease (p < 0.0001, n > 2000) in elasticity after 20 min exposure to 4 mM 2,4-D which further decreased with time. Large error bars show a high variability in surface elasticity. Error bars indicate standard deviation.
Figure 5.16 Plot showing the time dependent decrease in adhesion for *E. coli* exposed to 4 mM 2,4-D and imaged by QI-AFM. *E. coli* showed a significant increase in adhesion after ~20 min of 4 mM 2,4-D exposure (p < 0.0001, n > 2000), however, there was no statistically significant change after 20 min. Error bars indicate standard deviation.
change significantly after 20 min (p > 0.05). Young’s modulus, an estimate of cell envelope elasticity, was determined from QI force curves at the center of the cell. Values were in the range of 1–4 MPa (Figure 5.14) for cells exposed to formula only and prior to 2,4-D treatment, and did not change significantly during the course of cell division. Elasticity was significantly altered (p < 0.0001, n > 2000) with the addition of 4 mM 2,4-D, with the average elasticity reduced over a hundred fold after 20 min (Figure 5.14, 5.15) and highly variable. In general, elasticity decreased as a function of exposure time, but to different degrees in each cell. Conversely, surface adhesion increased after 20 min 2,4-D exposure, but with no statistically significant increase thereafter (Figure 5.16).

5.4 Discussion

*E. coli* WM1074 (Geissler et al., 2007) is a robust and a fast growing environmental strain that offers a good model to study the impact of xenobiotics. Here we use integrated AFM-LSCM to reveal stress-induced mechanisms associated with the divisome and changes at the surface of live cells in real time. Our results show that 2,4-D directly alters FtsA, FtsZ and SulA localization within seconds, arresting cell division, concomitant with changes to surface elasticity and adhesion and DNA damage during short exposure times. Perturbation of the Z-ring and nucleoid division is also accompanied by DNA damage in cells exposed to 2,4-D overnight. This method offers a molecular picture of *E. coli* cellular division and cell surface characteristics in live cells, providing mechanistic insights into the bacterial xenobiotic stress response mechanisms in real time.
5.4.1 Correlative AFM-QI-LSCM simultaneously examines localization of fluorecently tagged proteins and surface ultrastructure

We used correlated AFM-QI-LSCM to examine the changes in topography, elasticity, and adhesion while tracking the localization of FtsZ-GFP in real-time during *E. coli* cell division. These parameters were measured in different imaging media to optimise imaging conditions. *E. coli* walls had significantly lower and more irregular elasticity near the edges than at the center as indicated by the dark area near the cell periphery. We also observe dark regions with extremely low elasticity immediately surrounding the bacteria and this was observed consistently for all cells in every imaging media. *E. coli* is a rod-shaped organism with a height of ~1–2 µm and a large surface curvature near the cell periphery producing artificially soft surfaces with low elasticity, not suitable for analysis (Butt et al., 2005). Although a 3 µm Z-length was chosen for QI imaging, the piezo was not able to entirely overcome the lateral forces in those regions and therefore, data only at the center was taken as an estimate of the Young’s moduli. This issue can be overcome to a certain extent by altering tip shape, using longer and sharper tips, while increasing the Z-length (Chopinet et al., 2013). However, increasing the Z-length reduces the imaging speed significantly. With the data analysed at the center region of *E. coli* we observed no significant change in surface elasticity during various stages of cell division or in different media. The Hertzian model is imperfect as it considers the surface to be flat and homogenous and often does not take the shape of the cell into account, leading to more inaccurate values near the cell periphery (Kirmizis and Logothetidis, 2010). The model also considers the cells to be firmly attached, therefore, cells must be immobilised well to prevent artificially low elasticity measurements (Dokukin et al., 2013). The
surface spring constant of *E. coli* has been previously determined in the range of 0.5–1 Mpa when collected in liquid (Francius et al., 2011) which is consistent with the current study, however the values can vary significantly depending on tip shape, whether or not the cells are alive and imaged in liquid or air (Vadillo-Rodriguez., et al 2008).

The retract curve can be analysed for nano-adhesive properties, offering the possibility to visualise tip-sample interactive forces, for example, the hydrophobic properties of the surface proteins, and simultaneous height changes at high resolution (Butt et al., 2005). We observed no change in surface adhesion during cell division, however, there was a change in adhesion when imaged in different media, with PBS showing the highest adhesion to the AFM tip. The presence of exposed LPS on *E. coli* gives the surface a net negative charge and facilitates repulsion from the silicon nitride (Abu-Lail et al., 2003). Although, the entire mechanism is not clearly understood, it has been shown that increase in the ionic strength of the media increases the adhesion between the AFM tip and *E. coli*, due to changes to the steric factors as the buffer alters the length of LPS exposing charged groups (Burks et al., 2003).

LSCM images show a simultaneous change in localization of FtsZ, forming partial or complete rings at various locations along the cell during cell division, as shown with time lapse imaging. A clear groove can be seen in the AFM height images in the position of the Z-ring at the mid-cell, however the Young’s moduli in those regions are difficult to decovolute due to large changes in the surface curvature.

### 5.4.2 2,4-D ultimately inhibits nucleoid division
In chapter 4 I demonstrated that 2,4-D induces a filamentous phenotype in *E. coli* BL21 DE3 and several genotypically diverse environmental strains, indicating an impact on cell division (Bhat et al., 2015). In that study examining the long term effects, we used 4 mM 2,4-D which is the highest sub-lethal level at which cells can still divide and produce sufficient cell density after overnight exposure. Most cells showed no Z-ring after a 3 h exposure to 4 mM 2,4-D (Figure 5.4), although some cells were able to retain the Z-ring structure and continue dividing, producing sufficient cell density. Therefore we imaged *E. coli* after an overnight treatment, to determine the influence of 2,4-D on cell division over longer periods of time. After overnight exposure to 4 mM 2,4-D, cells showed significant elongation as expected, with multiple partially formed Z-rings (Figure 5.5), some of which colocalized to the nucleoid, indicating dysfunction of both the MinCDE and SlmA systems at high 2,4-D concentrations over longer exposure times. The MinCDE and SlmA systems inhibit Z-ring formation at the cell poles and over the dividing nucleoid, respectively (Cho et al., 2011; Sun and Margolin, 2004). We observed extensive DNA damage after an overnight exposure to 2,4-D (see below) which likely surpasses the cell’s ability to recover from this stress, thereby affecting cellular adaptation mechanisms. Multiple system failures likely lead to abnormal *E. coli* cell division as evidenced by multiple partially formed Z-rings located throughout the filamentous cells.

### 5.4.3 2,4-D causes DNA damage after several hours of exposure

To observe the long term adaptive effects of 2,4-D, DNA damage was examined after long term exposure. A 3 h exposure to 2,4-D caused DNA damage in *E. coli* that
increased significantly after an overnight exposure (Figure 5.12). Interestingly, filamentous cells had statistically greater DNA damage than rod-shaped cells (Figure 5.11 C), indicating population heterogeneity for which the latter had greater resistance to DNA damage.

DNA damage can be a direct consequence of reactive oxygen species (ROS) in the cell. In chapter 4 I demonstrated that 2,4-D causes a three-fold increase in ROS following exposure to 2,4-D at sub-lethal levels (Bhat et al., 2015), enough to cause the DNA damage observed in this study. Both the generation of ROS and DNA damage are consistent with an increase in SulA expression as indicated by an increased SulA-GFP signal after 3 h and overnight exposure (Figure 5.6). SulA is a main reporter of the SOS system, an adaptive response that enables bacterial survival following a sudden increase in DNA damage due to oxidative stress (Justice et al., 2006). We observed a similar increase in DNA damage with exposure to H$_2$O$_2$ and high temperature, both known to induce the filamentous phenotype (Figure 5.12). H$_2$O$_2$ is known to produce reactive hydroxyl radicals that damage membrane lipids, proteins and DNA for which we expect an upregulation of the SOS response system. SulA is known to interact with FtsZ to disassemble the existing Z-ring and also sequester FtsZ monomers to prevent its further assembly (Chen et al., 2012; Huang et al., 1996), all consistent with the loss of the Z-ring we observed in live E. coli exposed to 2,4-D.

It is however not known whether SulA remains bound to FtsZ following its disassembly to prevent further FtsZ localization until which time the DNA has been fully repaired. In this study, there is similarity in the distribution between FtsZ and SulA for the majority of cells after 2,4-D treatment, possibly indicating sequestration of FtsZ by SulA. However,
it is difficult to interpret this observation in biological terms since the expression, dynamics and localization patterns of SulA in *E. coli* are not entirely known.

5.4.4 FtsZ localization is immediately perturbed with 2,4-D exposure

Cell division in *E. coli* is initiated by septum formation, facilitated by the accumulation of FtsZ and its colocalizing partners which form a membrane associated complex called the divisome (Natale et al., 2013). FtsZ undergoes GTP-dependent self-polymerization to form a highly dynamic scaffold called the Z-ring for which division proteins are in constant flux between the cytosol and the divisome (Anderson et al., 2004). FtsZ-GFP forms bright fluorescent spots that move along the cell’s longitudinal axis and forms mid-cell bands that eventually constrict, all the while forming a second ring at the middle of daughter cells preparing for the next division (Adams and Errington, 2009) (Figure 5.7). The former was clearly visible in the AFM images, in which constriction began approximately 3–4 min following the complete formation of the Z-ring observed by LSCM.

Within 5 s after the addition of \( \geq 1 \text{mM} \) 2,4-D, the Z-ring disappeared, FtsZ became completely static with a punctate distribution throughout the cytoplasm or near to the cell mid-point and poles (Figure 5.7). The randomly distributed static signals bleached irreversibly, indicating no accumulation of newly synthesised FtsZ. Changes to FtsZ assembly have been previously observed in *Bacillus subtilis* during exposure to benzamide (Adams et al., 2011) for which FtsZ post exposure shows randomly distributed dynamic foci, having no specific localization to the mid-cell and poles.
Immediate blocking of the on-going cell constriction, observed with simultaneous QI imaging (Figure 5.13), implies that the accumulation of newly formed peptidoglycan precursors at the site of the Z-ring is also impaired on the same time scale. Consistently, our previous metabolomics study showed alteration of peptidoglycan biosynthesis during sub-lethal 2,4-D exposure in *E. coli* BL21 (Bhat et al., 2015), described in chapter 4. The presence of FtsZ-GFP spots near the center and poles indicate insensitivity to the negative spatial regulators MinCDE/SlmA, which normally prevent FtsZ localization in those regions (Rothfield et al., 2005). Our previous work showed that 2,4-D inhibits oxidative phosphorylation in *E. coli*, mostly likely as a result of 2,4-D altering membrane potential (Benndorf et al., 2006; Fabra de Peretti et al., 1992), and since FtsZ is GTP dependent, loss of FtsZ assembly may be a direct consequence of changes in cellular respiration. Membrane potential is also crucial for the stability of the divisome complex (Strahl and Hamoen, 2010), which could directly result in Z-ring collapse on the time scales observed in this study. 2,4-D is also known to bind and directly affect polymerization of purified neuronal tubulin *in vitro* (Rosso et al., 2000), and it may have a similar impact on its structural homolog, FtsZ, in *E. coli*. Therefore, changes in FtsZ-GFP localization could be the result of a completely non-specific mechanism wherein the membrane potential is altered by 2,4-D that directly reacts with membrane components, leading to the disassembly of the divisome complex.

**5.4.5 2,4-D immediately causes DNA damage and alters SulA localization in *E. coli***

The random distribution of FtsZ-GFP after 2,4-D exposure likely indicates partially disassembled FtsZ polymers, and the loss of Z-ring structure during 2,4-D exposure could
result from SulA-mediated disassembly of FtsZ. Previous studies show that SulA is induced by the SOS response caused by DNA damage (Janion, 2008), and indeed we observe DNA damage at very short 2,4-D exposure times (Figure 5.9 and 5.10) consistent with changes to SulA localization (Figure 5.8). SulA inhibits FtsZ polymerisation by binding and sequestering FtsZ monomers, thereby decreasing its critical concentration necessary for assembly (Chen et al., 2012). SulA is known to bind to the C-terminal end of FtsZ which inhibits the GTPase activity necessary for polymerization (Cordell et al., 2003). SulA produced a uniformly dim signal in the absence of 2,4-D, indicating low constitutive SulA expression, but apparently not at sufficient levels to block FtsZ. It has been previously shown that slightly elevated levels of SulA are sufficient to inhibit FtsZ, blocking cell division (Huisman et al., 1984). Due to signal decay in the LSCM time-lapse images, it is not clear whether the signal is increased immediately after the addition of 2,4-D. Images collected after longer exposure periods however, clearly show higher SulA-GFP signals compared to controls which can be attributed to increased SulA expression and activation of the SOS response.

Upon exposure to 1 mM 2,4-D, within seconds SulA-GFP formed a figure eight pattern throughout the cell (Figure 5.8 b), in the same time frame required for FtsZ to relocate, consistent with SulA mediated disassembly of FtsZ. The redistribution of SulA is within the same time frame as DNA damage, which may indicate activation of the SOS response, altering the SulA/FtsZ ratio. Indeed, there was a statistically significant increase in DNA damage after 5 s exposure to 1 mM 2,4-D, suggesting a role for SOS and SulA mediated disassembly of FtsZ. The apparent clustering of FtsZ following exposure to 1 mM 2,4-D may represent FtsZ sequestered by SulA. Our observations
indicate that this process is complete within a matter of seconds. The DNA repair feedback loop after DNA damage by UV radiation has been predicted to take ~27 min (Ni et al., 2007) but here we show direct evidence of cell division arrest following DNA damage within a matter of seconds, possibly mediated by a more direct response.

5.4.6 2,4-D causes immediate changes to FtsA localization in *E. coli*

FtsA showed similar localization to FtsZ, as membrane-associated rings at the mid-cell for control and formula-treated cells (Figure 5.8 A). Following exposure to 1 mM 2,4-D, the FtsA relocalized away from the ring with large, bright signals that were similar to those observed for FtsZ. These observations indicate that 2,4-D possibly alters assembly of the entire divisome, impacting other cell division proteins.

5.4.7 2,4-D remodels the cell envelope in live *E. coli* during 2,4-D exposure

QI height images of live *E. coli* show a fairly smooth surface under growth-promoting conditions (Figure 5.13) that became significantly rougher after 20 min exposure to 4 mM 2,4-D. The swift change in *E. coli* surface roughness indicates a reorganization of surface molecules, underlying membrane and/or peptidoglycan, leading to uneven deformations in the cell envelope. The bacterial envelope plays an important role in its survival, protecting it from osmotic pressure, environmental stress, providing shape and resilience (Ruiz and Silhavy, 2005). 2,4-D is able to pass through the envelope, allowing entry into the membrane and cell, leading to further intracellular changes associated with cell division and DNA damage. Consistent with our observations, the majority of antimicrobial compounds including toxins, antibiotics and antimicrobial peptides also
alter surface roughness (Fantner et al., 2010; Formosa et al., 2012; La Storia et al., 2011), one of the primary responses to chemical stressors. Since 2,4-D embeds in and alters the physical properties of membranes (Benndorf et al., 2006; Fabra de Peretti et al., 1992), the altered surface roughness may represent disruption of the outer membrane that disorders the lateral packing of LPS as well as CPS.

The Young’s modulus of live cells decreased dramatically after 20 min exposure to 4 mM 2,4-D (Fig 5.14 and Figure 5.15), concomitant with changes to cell division and oxidative stress proteins. The Young’s moduli of exposed cells were highly variable, with some surface areas softer than others (Figure 5.14). Although no prior study has characterized the effects of aromatic compounds on the bacterial surface in live cells in real time, there are extensive studies on fixed cells imaged in air having been exposed to antimicrobial peptides (Fantner et al., 2010), essential oils (La Storia et al., 2011) and antibiotics (Yang et al., 2006). Our results are consistent with the impact of other antimicrobial compounds, and in this case we resolve the temporal changes to the surface. *E. coli* exposed to sub-lethal levels of 2,4-D had a hundred-fold lower surface elasticity within 30 min, further reduced over a period of few hours. Removal of 2,4-D from the imaging medium did not reverse this effect, implying that 2,4-D irreversibly compromises membranes during its entry into the cell. These observations indicate that 2,4-D perturbs a select group of cell surface molecules, causing an irreversible alteration in their chemical and physical properties. The outer membrane of *E. coli* is highly complex and asymmetric, made up lipids, long chain LPS and membrane proteins (Kleanthous and Armitage, 2015). 2,4-D is known to be lipophilic (Benndorf et al., 2006) allowing 2,4-D
to directly interact with LPS leading to uneven changes in surface elasticity.

Perturbations to proteins by 2,4-D may also play a role in selective surface deformations.

The *E. coli* cell surface contains ~ 90% LPS, making its surface negatively charged, consistent with QI images that show less adhesion between the negatively charged silicon tip and bacteria compared to the Cell-Tak™ covered glass surface (Figure 5.1). An increase in adhesion after exposure to 2,4-D is also a direct indication of LPS reorganization, a reduction in negative charge, and is consistent with softer and rougher cells.

### 5.5 Conclusion

I anticipate that integrated AFM-LSCM will find wide use for future studies, in particular to obtain a more comprehensive view of protein dynamics and cell signaling along with cell wall remodelling and phenotypic changes at high resolution in live cells under external stress. This technique can be easily adapted to mammalian cells and yeast under a variety of environmental conditions.

I demonstrate the successful use of simultaneous correlated AFM QI-LSCM imaging of live cells in real-time to obtain insights into the molecular targets of a commonly used herbicide 2,4-D. This herbicide caused significant DNA damage attributed to oxidative stress, resulting in irregular cell division and cell elongation after long exposure periods. We show that exposure to 1 mM 2,4-D is sufficient to completely arrest cell division in *E. coli* by altering FtsZ, FtsA and SulA localization within seconds, potentially triggered by DNA damage or direct impact of 2,4-D. Simultaneous QI imaging showed time-dependent changes to surface roughness, adhesion and elasticity, indicating a direct
interaction between 2,4-D and the envelope surface. This is the first study attempting to
determine the molecular mechanism underscoring the impact of 2,4-D on *E. coli*. The
incomplete molecular description of *E. coli* cell division makes several observations in
this study difficult to explain, but they nonetheless provide valuable insight into how 2,4-
D affects microbes, with implications for other xenobiotics.

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PORTIONS OF THIS CHAPTER ARE PUBLISHED IN THE ONLINE SCIENCE MAGAZINE,

6.1 Discussion

The extensive use of pesticides in agriculture is compromising soil and aquatic ecosystems and causing major concerns linked to human health. As key players that recycle environmental elements, microorganisms can mineralize, detoxify or immobilize xenobiotic compounds. The application of pesticides and chemicals can adversely affect natural biochemical processes crucial for crop production, agricultural sustainability and environment quality (Binetti et al., 2008). Natural processes such as cellulose breakdown, nitrification and turnover of organic matter driven by microbial and enzymatic activities are adversely affected by pesticides and toxic chemicals (Kinney et al., 2005). Pollutants over a long period of time completely change microorganism communities, replacing them with mutants having altered physiology, causing imbalances in natural processes (Hussain et al., 2009). There is evidence that toxic chemical exposure can induce virulence in bacteria, increasing human pathogen resistance to host immune responses (Allen and Griffiths, 2012).

The structure, function and diversification of microbial communities are a reflection of their habitat, making bacteria excellent indicators of toxic chemicals. Leading reviews have addressed the effects of pollutants on soil microbial communities and the environment (Imfeld and Vuilleumier, 2012; Lo, 2010). However, the literature only addresses minor and transient effects when chemicals are applied at recommended doses, and for most pollutants their mode-of-action is not well understood. Initial research used microbes as toxicity indicators at very high chemical doses, having little predictive value for chronic exposure at low levels. Further, it is almost impossible to predict the ultimate bioavailability of most pollutants under environmental conditions, as it depends on
complex environmental factors such as solubility, adsorption, pH, temperature and other factors (Bierkens, 2000; Danese and Silhavy, 1997). Therefore, alternative methods have been developed to detect morphological, biochemical and genetic alterations at the molecular level for non-lethal toxic exposures. As more and more new chemicals are being manufactured, there is a need for new approaches to understand the magnitude and diversity of responses by natural microflora to chemical exposure.

The herbicide 2,4-D is one of the most extensively used pesticides world-wide, which makes understanding its mechanism-of-action in living organisms very important. The primary objective of this research was to determine the impact of this herbicide at low exposure on the commonly occurring beneficial soil rhizobacterium, *R. leguminosarum* and diversely occurring *E. coli*. By working with two phylogenetically different soil bacteria we expected a more comprehensive view of the impact of 2,4-D on microbes.

The secondary objective was to determine the mechanistic details of 2,4-D and its targets in *E. coli*, based on the primary observations, using a novel microscopy assay. *E. coli* is extremely well-studied, making mechanistic interpretation of the data more feasible. This research demonstrates the activation of distinct stress induction mechanisms in *Rlv* and *E. coli* during sub-lethal 2,4-D exposure, and the newly developed AFM-LSCM assay was successfully used to examine the mechanistic details of adaptations in *E. coli*.

There were three major accomplishments of this research. Firstly, advanced microscopy and metabolomics were used to examine primary phenotypic and metabolic stress responses to pesticides. Two significantly different organisms studied in parallel showed different responses to 2,4-D, including a novel 2,4-D and IAA induced phenotype *in vitro* in rhizobia and a 2,4-D induced filamentous phenotype in *E. coli*. These initial
observations opened multiple avenues to advance our understanding of how 2,4-D impacts microbes. Next I developed a novel correlative AFM-LSCM assay to image live *E. coli* in real-time and determine the mechanistic details of 2,4-D response mechanisms. This assay has broad applications to other microbes and cell types in response to an array of xenobiotics. Finally I used this assay to determine the mechanistic details of sub-lethal 2,4-D targeted cell division in fluorescently tagged live *E. coli* in real time.

**6.2 *E. coli* and *R. leguminosarum* general stress responses**

Microscopy combined with metabolomics was used to assess the sub-lethal effects of 2,4-D on the morphology, cell envelope organization, and cellular metabolism of *E. coli* and *Rlv*. Although the majority of the 2,4-D associated cellular responses were unique (discussed in the next section), many responses were common to both model bacteria (Table 6.1).

The cell envelopes of each were reorganized with an accompanying change in surface roughness, sub-unit characteristics, elasticity and adhesion, indicating loss of envelope integrity. *Rlv* and *E. coli* both showed a large increase in surface roughness, whereas adhesion and elasticity increased in *Rlv* but decreased in *E. coli* exposed to 2,4-D. Differences in adhesion and elasticity may reflect distinct physiological responses to 2,4-D, consistent with diverse metabolomic responses (Table 6.2). The two bacteria are phylogenetically distinct with different life styles, where *Rlv* are symbiotic organisms having a larger genome compared to the free living *E. coli*. Nonetheless, 2,4-D alters surface and envelope macromolecular arrangement in each bacteria. This is consistent with 2,4-D directly interacting with membrane lipids (Viegas et al., 2005), potentially
Table 6.1 Summary of *E. coli* and *Rlv* AFM-QI analysis in response to 2,4-D (Taken from Dahms and Bhat, 2016)

<table>
<thead>
<tr>
<th>[2,4-D] exposure</th>
<th>Roughness trend for <em>E. coli/Rlv</em></th>
<th>Adhesion trend for <em>E. coli/Rlv</em></th>
<th>Elasticity trend for <em>E. coli/Rlv</em></th>
<th>[2,4-D] exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (mM)</td>
<td></td>
<td></td>
<td></td>
<td><em>Rlv</em> (mM)</td>
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<tr>
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</tbody>
</table>

* Arrows (unfilled for *E. coli*, gradient filled for *Rlv*) indicate trends in values as a function of 2,4-D concentration, with the arrow head corresponding to the highest value.
Table 6.2 Summary of *E. coli* and *Rlv* metabolome analysis in response to 2,4-D (Taken from Dahms and Bhat, 2016).

<table>
<thead>
<tr>
<th>Pathway/Metabolite Class</th>
<th><em>E. coli</em></th>
<th><em>Rlv</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricarboxylic acid cycle</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>Purine/pyrimidine metabolism</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>-</td>
<td>NC</td>
</tr>
<tr>
<td>Peptidoglycan biosynthesis</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*"+" is an increase, "-" a decrease and NC is no change in metabolites with 2,4-D exposure.*
affecting cellular respiration, leading to the production of ROS which in turn can react with macromolecules non-specifically causing their partial or complete oxidative stress-induced denaturation.

Both bacteria show a dose-dependent increase in ROS during 2,4-D exposure, which could explain cell envelope remodeling, cell division defects and altered metabolism (Bhat et al., 2015a; Bhat et al., 2015b). Certain metabolic alterations were common to both while some were distinct (Table 6.2). The majority of Rlv metabolic pathways were upregulated contrary to those in E. coli that were downregulated, reflecting Rlv’s adaptive capabilities through a robust and versatile metabolism, well suited to surviving external stress factors and supporting their symbiotic and free living life style (Young et al., 2006a).

6.3 2,4-D and IAA possibly alter cell division in Rhizobia

The combination of microscopy, a detailed metabolomics analysis and additional biochemical assays in Rlv provided a comprehensive view of 2,4-D effects at sub-lethal levels. Although LM indicated subtle changes in the phenotype, SEM provided evidence for a distinct change in the morphology of cells into a ‘Y’-shaped phenotype in vitro. A higher frequency of this phenotype, when directly treated with IAA, indicated a possible link to the Rlv bacteroid phenotype observed when isolated from root nodules. However, I have no evidence that Rlv has the capability to fix nitrogen in vitro, and there is a distinct possibility that 2,4-D simply alters the phenotype by affecting cell division, as is observed for E. coli. Interestingly, a preliminary study conducted by honours student
Alexa Pierce in our lab showed that 2,4-D and IAA alter the localization patterns of FtsZ-GFP in rhizobia, providing some further evidence for this hypothesis.

In Rhizobiales cell division occurs by sub-polar division after the formation of a distinct divisome complex, but unlike *E. coli*, peptidoglycan precursors are added at a single pole resulting in unipolar growth (Brown et al., 2012). Cell division in *Rlv* is not well-studied, especially in free-living cells, and further research is required for proper interpretation of how 2,4-D effects *Rlv* cell division. On the other hand, cell differentiation into bacteroids inside root nodules during symbiosis and the role of plant signal molecules, such as Nod factors in this process have been relatively well studied (Maroti and Kondorosi, 2014).

The environment inside the root nodules is complex and influenced by a number of host factors including a complex interplay between rhizobia and plant derived auxin (Oldroyd and Downie, 2008; Takanashi et al., 2011), making interpretation of our data even more challenging.

Proteins involved in *Rlv* cell division such as FtsZ can be linked with FP tags by transformation. These novel mutants with fluorescent markers can be used for live imaging in the presence of 2,4-D and IAA, which will help determine the effects of these compounds on *Rlv* cell division.

**6.4 The 2,4-D and IAA induced phenotype may be related to stress**

The herbicide 2,4-D is a structural analogue of IAA and this research has demonstrated a similarity in their phenotypic impact on *Rlv*. Numerous reports document IAA production by the majority of rhizobia however, only a few reports discuss the regulation of IAA biosynthesis (Spaepen and Vanderleyden, 2011). The role of environmental factors on
IAA production has been best studied in *Azospirillum brasilense*, a plant growth promoting rhizobacterium. The IAA producing gene in this bacterium *ipdC* is positively regulated and its expression is enhanced by other auxins such as phenylacetic acid, the IAA precursor tryptophan and naphthalene acetic acid (Somers et al., 2005). IAA also regulates gene expression in *A. brasilense*, alters adhesion and induces filamentation in *Saccharomyces cerevisiae* (Prusty et al., 2004). The *ipdC* gene in *Pseudomonas putida* and *Pseudomonas agglomerans* are known to be regulated by the stress response transcription regulator RpoS (Patten and Glick, 2002). All the above observations indicate that 2,4-D, being a structural analogue of IAA, may impact cellular IAA production, which is directly linked to the cell stress response. As discussed in Chapter 3, IAA produced by rhizobia plays a crucial role in symbiosis and likely bacteroid differentiation inside the root nodules necessary for nitrogen fixation. All the evidence therefore points towards 2,4-D being capable of causing cellular changes related to cell division.

Future studies will focus on testing the role of IAA and 2,4-D on *Rlv* cell division and nitrogen fixation *in vitro*. Preliminary experiments attempting to grow *Rlv* in nitrogen free media in the presence of 2,4-D to test nitrogen fixation failed, possibly due to nitrogen contamination in the media. This assay can be further optimized using ultrapure water for media preparation. The acetylene reduction assay would confirm whether *Rlv* can fix gaseous nitrogen in the presence of 2,4-D and IAA *in vitro*. A gap in knowledge related to the role of IAA in bacterial differentiation, gene expression and cell signalling during symbiosis needs to be filled using novel rhizobia mutants. Mutants lacking one or more genes for IAA production can be tested for nodulation and the differentiated
phenotype inside the nodules monitored using LM and AFM. Omics technologies will shed light on the physiology, gene expression and genetic changes occurring during and post bacteroid differentiation inside the root nodules. A complete elucidation of the cell division mechanism in rhizobia will help to examine the biochemical basis of the altered phenotype observed during 2,4-D and IAA exposure.

6.5 Potential 2,4-D degradation by *Rlv*

The metabolomics data showed an enrichment in the majority of metabolic pathways, especially the TCA cycle and glyoxylate pathway, during sub-lethal 2,4-D exposure. Such a response indicates the potential for *Rlv* to catabolize 2,4-D as a carbon source. Several species of soil bacteria such as *Pseudomonas*, *Bacillus*, *Alcaligenes*, several species of fungi and actinomycetes are known to possess genes involved in 2,4-D degradation. Kitagawa et al., 2001 discovered novel 2,4-D degrading genes from *Bradyrhizobium* sp. HW13 isolated from pristine soil. *Rhizobium* and *Bradyrhizobium* both belong to the Rhizobiales class in *Alphaproteobacteria*, indicating that the two bacteria are phylogenetically related.

The 2,4-D degradation pathway from *Bradyrhizobium* shows that 2,4-D degradation byproducts enter the TCA cycle through glyoxylate. Metabolomics data in *Rlv* during sub-lethal exposure to 2,4-D also showed an increase in glyoxylate levels, confirmed by enzyme assays showing a selective increase in malate synthase activity without changes to isocitrate lyase activity. These data demonstrate a selective accumulation of glyoxylate, providing evidence for 2,4-D degradation possibly through a mechanism similar to *Bradyrhizobium*. 
Furthermore, the tfdB gene encoding dichlorophenol hydroxylase, an enzyme crucial in 2,4-D catabolism, and the cadABC gene, transcription regulator for 2,4-D degrading operon from Bradyrhizobium sp. HW13 show some similarity to sequences from the Rlv genome, providing further evidence for possible 2,4-D biotransformation. Initial attempts in our lab to isolate the 2,4-D degradation byproduct 2,4-DCP by HPLC and to grow Rlv in 2,4-D minimal media with 2,4-D as a sole carbon source were unsuccessful due to technical difficulties. In future, more optimized HPLC and growth assays will be used to identify any degradation products and 2,4-D assimilation in the media.

6.6 2,4-D induced phenotypic changes in E. coli

E. coli BL21 (pET21b) was used as a model strain to determine the sub-lethal effects of 2,4-D. This lab strain, likely with a series of environmentally triggered mutations, was genotyped and determined to be an A1 sub-strain. At 1000-fold below field application levels, a concentration of 0.0021 mM 2,4-D was sufficient to disrupt cell division leading to the filamentous phenotype. A series of representative environmental strains from four genotypes A, B1, B2, and D, NECD 1–4 respectively, were also tested for a similar phenotype to demonstrate that the effects were not a result of lab artifacts in a long-time cultured lab strain. E. coli formed irregular biofilms with large clumps in the presence of 2,4-D, indicating possible defects in EPS formation. The majority of these phenotypic effects can be explained by physiological changes during 2,4-D exposure.

In E. coli, the onset of the filamentous phenotype has been found to be a common response to chemical and physical stresses (Justice et al., 2008), however, the associated mechanism has not been explored. Moreover, it is not understood whether the mechanism
behind filamentation is common for all stress stimuli or if there is an overlap in the associated mechanisms. Filamentation has an evolutionary advantage - pathogenic *E. coli* and other bacteria filament to increase their survival rate and avoid consumption by the host immune cells (Justice et al., 2006). It has been predicted that the filamentous fungi and actinomycetes evolved from such behaviour. Therefore, it can be safely predicted that filamentation in *E. coli* is an adaptive response to external stress factors. *E. coli* filaments under stress conditions, despite having strict control over its shape and size under normal conditions (Harry et al., 2006; Young, 2006). Therefore, it is important to explore this behaviour to determine whether the mechanism behind this phenotype is universal or unique to each stressor. I attempted to determine the mechanism as described in chapter 5.

### 6.7 2,4-D induced metabolic changes in *E. coli*

Metabolomic evidence indicated reduced oxidative phosphorylation, amino acid metabolism, peptidoglycan biosynthesis, nucleotide metabolism and membrane transport. These observations indicate the strategy of stress and energy conservation to cope with 2,4-D induced damage, also explaining cell division arrest and the resultant filamentation. The 2,4-D stress response pathways of *E. coli* elucidated in this research enrich what was already known about the mechanism of 2,4-D in bacteria (Figure 6.1). Overall, 2,4-D impacts *E. coli* cell division as indicated by the filamentous phenotype and leads to cell envelope remodelling and ROS, which likely lead to DNA damage and changes to the majority of metabolic pathways.
Figure 6.1 Schematic cartoon of *E. coli* showing our present state of knowledge of 2,4-D mode-of-action on various parts of the bacterial cell. This herbicide is known to pass through the cell envelope by passive diffusion (Arias and Fabra de Peretti, 1993), alter membrane fluidity (Fabra de Peretti et al., 1992), bind reversibly/irreversibly to macromolecules (Balague et al., 2001; Chinalia et al., 2007; Fabra de Peretti et al., 1992; Viegas et al., 2005) and affect protein and nucleic acid biosynthesis (Rivarola et al., 1992). My research shows that 2,4-D also alters the morphology, cell division and cell envelope properties. Metabolomics data indicates that 2,4-D affects the ABC transport system, peptidoglycan biosynthesis, oxidative phosphorylation, amino acid, nucleotide and sugar metabolism. The majority of the effects could be attributed to 2,4-D induced ROS and DNA damage (Bhat et al., 2015a; Bhat et al., 2015b).
The microscopy, metabolomics and biochemical assays offered clues as to how 2,4-D was impacting microbes, but the mechanistic details of how cell division was being disrupted required a more rigorous approach.

6.8 Determining the 2,4-D mechanism of action in *E. coli* using correlative AFM-LSCM

6.8.1 Correlative AFM-LSCM method needs improvement

A method was required to further elucidate the targets of 2,4-D impact in *E. coli*. Studies on *E. coli* BL21 and environmentally isolated strains indicated that 2,4-D disrupts cell division, however, to determine the molecular mechanism in greater detail, I developed a live cell imaging assay. Previously, I used AFM to examine fixed bacterial cells which only showed the long term, adaptive effects of 2,4-D, and which had to be cautiously interpreted since fixation can alter quantitative measurements of the cell envelope. AFM live imaging allows surface characterisation in real-time on single cells at ultra-high resolution *in situ*. The AFM QI mode simultaneously quantifies changes in topography, adhesion and elasticity in live cell envelopes. Since AFM only measures surface changes, it was integrated with LSCM, which can simultaneously measure temporal changes in the localization of fluorescent markers inside the cell. This technique had not previously been established for bacteria and hence required significant optimization. I developed a sample preparation protocol and instrumentation setup for live integrated imaging.

Cell-Tak™ facilitated sample immobilization on coverslips and the integrated instrument setup worked well for correlative AFM-LSCM. However, there are aspects of the sample preparation and integrated instrument operation that could be improved upon.
to obtain high quality noise-free data. *E. coli* becoming planktonic and motile during imaging, and overcrowding of the imaging area after 2–4 h posed serious challenges. To partially overcome these obstacles, the sample could be rinsed and remounted in fresh media, however this does not remove sticky extracellular material observed around the microcolonies after several cell divisions, which reduces the image quality.

Initial sample mounting and focusing of the optical image to find a suitable immobile cell for imaging is still a challenging and time consuming task. For simultaneous imaging, the confocal optical path needs to be perfectly aligned with the AFM cantilever. This can be improved by building a better sample holder for glass coverslips that can be quickly mounted. A confocal objective with a longer working distance will eliminate having to initially align the cantilever and laser using the low magnification objective. The current optical overlay tool on the AFM software does not allow proper alignment for large fields of view and the manual process is time consuming. The optical overlay system on the AFM can be improved by including options to choose different scan sizes. Imaging would also be faster if the LCD camera for optical overlay can be directly operated from the computer associated with the confocal microscope.

Confocal scanning can be noisy, giving rise to acoustic noise during the simultaneous AFM-LSCM scanning. Therefore, the noisy mechanical components could be externalized from the integrated setup to obtain noise-free data collected simultaneously for better overlay. Recently a sound isolation hood was installed which minimises acoustic noise.

I used the AFM-QI-LSCM technique to simultaneously image *E. coli* cell division continuously for multiple generations in real-time while imaging the localization of FtsZ.
surface ultrastructure and physical properties. I quantified these parameters in different media to determine the influence of media on live cell imaging, while optimizing the imaging parameters. Imaging in half diluted LB provided sufficient nutrients for *E. coli* growth, reduced fluorescence background to a certain extent and kept bacteria fairly immobile. The second most favorable imaging medium was pure LB, while imaging in PBS appeared to have a slight negative impact on immobilization and slowed cell division over time due to the lack of nutrients.

Significant improvement is required from the software developers for downstream image processing, especially for QI data processing. The current JPK processing software only allows partial QI analysis and a third party software is required for final data organization and statistical analysis, making data processing inefficient. The tools for properly overlaying high-content QI and LSCM images have yet to be developed. AFM and CM contain very different information with varied dimensionality. Every pixel of an AFM image contains information about topography, physical and mechanical properties whereas LSCM images have high temporal resolution and contain fluorescence emission signal. A point based registration method based on intensity and height profiling, to accurately match the corresponding structures especially near corners and edges, is required for proper overlay. All these developments would make the use of AFM-LSCM live cell real-time imaging much more practical for prokaryotes and eukaryotes, and the method would find much broader application.

6.8.2 2,4-D causes DNA damage and targets cell division to induce the filamentous phenotype in *E. coli*
The phenotypic and metabolic changes observed during 2,4-D exposure were further examined to identify the associated mechanistic details. Since the filamentous phenotype was induced in the presence of 2,4-D, indicating abnormal cell division, *E. coli* strains tagged with GFP cell division protein fusions were tracked in the presence of 2,4-D in real-time while simultaneously quantifying the surface topographical and physical changes in live cells. The newly developed high-content AFM-LSCM assay was demonstrated as proof of principle through examining the intracellular and surface changes simultaneously in live cells.

Long exposure to sub-lethal levels of 2,4-D caused DNA damage and inhibition of proper Z-ring formation, likely through nucleoid occlusion as evidenced by improperly divided nucleoids coinciding with inhibition of cell division and cell elongation. Exposure to 1 mM 2,4-D immediately altered FtsZ and FtsA localization, and resulted in the immediate arrest of cell division. SulA localization was also altered, from a uniform distribution prior to 2,4-D exposure to a figure eight pattern excluded from nucleoid regions. There were concomitant changes to the surface roughness, elasticity and adhesion in a time-dependent manner. The ability of to block cell division in *E. coli* within seconds can be explained in one of the following ways. 2,4-D, by altering membrane potential in a non-specific manner, may affect FtsZ polymerization indirectly causing disassembly of the existing Z-ring, as it has been previously shown that membrane potential is crucial for the stability of the membrane associated divisome complex (Strahl and Hamoen, 2010). Disassembly of the existing Z-ring and inhibition of new Z-ring formation can be mediated by SulA. The latter idea is supported by the observed DNA damage, even at a 5...
s exposure time, as DNA damage is known to induce SulA in *E. coli* as a result of the SOS response.

Many of the explanations for the rapid disruption of *E. coli* cell division in this study are speculative, and it is especially difficult to explain changes to SulA localization within seconds of 2,4-D exposure due to the incomplete molecular description of the SOS response. Loss of the Z-ring, and a FtsZ relocalization pattern similar to that of SulA following 2,4-D treatment indicates the possibility of SulA directly inhibiting FtsZ assembly, however we lack the associated direct evidence for this idea. Prior studies have calculated that it would take ~27 min for SulA expression as a function of the SOS response following DNA damage, however my data demonstrates SulA relocalization and loss of the Z-ring within 5 s. This result is difficult to explain. There is a lack of studies on SulA expression, but the majority of the literature indicates that SulA is expressed only after its induction by ROS, which may explain low, constitutive expression of SulA resulting from ROS that is generated by oxidative phosphorylation. I propose that this corresponds to basal SulA expression, not capable of delocalizing FtsZ during normal cell division for two of the following possible reasons. 1) The SulA concentration is initially too low (there is evidence showing that SulA must be present at a sufficient concentration to bind and sequester FtsZ), but upon addition of 2,4-D SulA expression increases and is able to sequester FtsZ. We observed increased SulA-GFP signal after overnight exposure, providing evidence for this idea. 2) The presence of a regulator protein may prevent SulA from binding to FtsZ, which then somehow dissociates in the presence of 2,4-D. Again, I have no evidence for these ideas and these are pure speculation. The incomplete molecular description of *E. coli* cell division makes
several observations in this study difficult to explain, but they nonetheless provide valuable insights into how 2,4-D affects microbes, with implications for the impact of other xenobiotics.

Further studies can be used to explore my speculations. It remains unclear whether 2,4-D physically interacts with FtsZ to disrupt the bacterial divisome assembly. There is evidence of 2,4-D obstructing microtubule assembly in vitro (Rosso et al., 2000), a mammalian homologue of bacterial FtsZ, therefore a similar study where FtsZ polymerization is monitored by measuring light scattering in bacteria would help determine whether 2,4-D physically interacts with FtsZ. Our work also shows DNA damage at very short exposure times but more experimental evidence is required to determine the sequence of events involving disruption of divisome assembly, DNA damage and change in localization of SulA. Intermediates of the SOS response can be targeted following artificial induction of DNA damage to help better understand the SOS sequence of events and the time required for SulA expression. Using mutants to examine the change in localization of other divisome complex components (i.e. Min proteins, SlmA, ZipA etc.), individually or simultaneously in combination, using multi-colored FP fusions will also provide a clearer picture of how 2,4-D affects the molecular assembly of the divisome.

6.8.3 Changes to surface ultrastructure in live E. coli during 2,4-D exposure

The biochemical nature of the cell surface changes observed in live E. coli could be further explored using the AFM-precipitation assay for CPS (Noyes et al., 2013), uronic acid assay for EPS (Mojica and Cooney, 2010) and LAL or Limulus assay (Roth et al.,
1990) for LPS post 2,4-D exposure. Each of these polysaccharides can also be isolated and analysed using mass spectrometry to determine any adaptive changes in their structure during 2,4-D exposure. The AFM tip can be biochemically modified using specific linkers such as antibodies to the surface exposed domain of LPS such as the O-antigen and other outer membrane proteins to map the 2,4-D induced surface changes to provide a biochemical basis for the observed changes to surface roughness, elasticity and adhesion. To determine the non-specific effects of 2,4-D, membrane potential changes can be quantified using, for example, the Dis-C2 fluorescent assay. Fluoresceine-labeled 2,4-D (Rogers et al., 1997) can be used to locate the 2,4-D on the bacteria surface, and internal localization can also be determined using confocal assays.

6.9 Concluding remarks

Overall, this research provides a detailed picture of how 2,4-D impacts E. coli and Rhizobia, some of which will be applicable to other bacteria. It is quite clear that this herbicide has undesirable effects on soil bacteria at very low exposure levels, far below that of field application. Some of those undesirable effects potentially emanate from its degradation by-products. Future research should focus on the specific targets of 2,4-D and associated downstream molecular processes that lead to the ultimate phenotypical symptoms during herbicide exposure in E. coli and R. leguminosarum, or alternatively if the effect is simply the result of macromolecular denaturation and a generalized stress response.
6.10 Bibliography


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