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Investigating the relationship between Quorum Sensing, Motility, and the Type 3 Secretion System of *Yersinia pseudotuberculosis*

Robert J. Goldstone

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2011

Declaration

Unless otherwise acknowledged, the work presented in this thesis is entirely my own. No part has been submitted for another degree in the University of Nottingham or any other institute of learning.

Robert Goldstone

September, 2011

Abstract

Over the course of the last two decades, research into the role of quorum sensing (QS) in regulating diverse bacterial behaviours has exploded, and around twelve years ago, a QS network was identified in the enteropathogenic bacterium *Yersinia pseudotuberculosis*, which was shown to control motility and cellular clumping. This thesis seeks to expand this regulatory relationship and explore the causes and consequences of the link between QS and motility, which affects pleiotropic processes including the type 3 secretion system (T3SS) and biofilm formation. Indeed, the clumping phenotype first explored by Atkinson *et al.* (1999), is linked to QS-dependent regulation of the T3SS, since the deletion of several QS genes results in liquid culture biofilm (LCB) formation. This is concomitant with T3S protein secretion into culture supernatant, which occurs under normally non-inducing conditions, while deleting the T3SS structural component *yscJ* prevents secretion and LCB formation. De-repression of the T3SS and the development of LCBs also occurs following mutation of the flagella regulators *flhDC* and *fliA*, revealing that QS and the flagella system co-regulate LCBs. However, interestingly it was found that LCB formation and secretion also occurs following mutation of the flagella structural gene *flhA*. The Δ*flhA* mutant represents a flagella-minus strain, in which the underlying regulatory circuit mediated by FlhDC and FliA is intact, suggesting that an element of the flagella structure that depends on FlhA activity acts as a check-point governing expression of the T3SS.

Both QS and the flagella system positively regulate biofilm formation by *Y. pseudotuberculosis* on the surface of the nematode worm, *Caenorhabditis elegans*. Surprisingly, the up-regulated T3SS was found to be responsible for mediating down-regulation of biofilm formation by *Y. pseudotuberculosis* QS mutants, since subsequent deletion of *yscJ* could restore biofilms to wild-type levels. This suggested that a component of the injectisome was capable of influencing cellular processes in addition to its role in secretion. In light of the link regulatory link between flagella and T3S, this raised the possibility that the injectisome could play a role in the reciprocal regulation of motility. Since the genetic regulatory network underpinning expression of the T3SS is intact in the Δ*yscJ* mutant, like the Δ*flhA* mutant for flagella, the Δ*yscJ* mutant can reveal the role of the injectisome structure in modulating gene expression. By phenotypic observation, it was determined that the Δ*yscJ* mutant displayed aberrant flagella mediated motility, swimming vigorously under conditions in which the wild-type did not, and, similar to the over-production of Yop proteins in the Δ*flhA* mutant, the Δ*yscJ* mutant over-produces flagellin. This suggests that a component of the T3SS injectisome acts as a checkpoint to regulate motility, which appears to be at the level of transcription, since the Δ*yscJ* mutant displays up-regulation of the flagella regulators *flhDC* and *fliA*. Indeed, the relationship between T3S and motility appears to require a direct influence on QS, since subsequent mutation of *ypsI* and *ytbI* abolishes Δ*yscJ*-dependent hyper-motility, the Δ*yscJ* mutant displays altered expression of the QS system genes. Furthermore, for the emerging transcriptional relationship between these systems, the flagella and QS mutants which are up-regulated for the production of Yop proteins also over-express the virulence regulator *virF*, completing the transcriptional regulatory circuit which appears to be crucial for the regulation of lifestyle choices by *Y. pseudotuberculosis*.

Publications arising from this work

Sections of this thesis have appeared in the following publications:

Atkinson S, **Goldstone RJ**, Joshua GWP, Chang C-Y, Patrick HL, Cámara M, Wren BW, Williams P, (2011) Biofilm Development on *Caenorhabditis elegans* by *Yersinia* Is Facilitated by Quorum Sensing-Dependent Repression of Type III Secretion. PLoS Pathogens 7: e1001250.

Goldstone RJ, Popat R, Fletcher MP, Crusz SA, Diggle SP, (2012) Quorum sensing and social interactions in microbial biofilms. In Microbial Biofilms: current research, methods and applications (Ed. Lear & Lewis). Caister Academic Press.

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Robert Goldstone

Table of contents

Title i

Declaration ii

Abstract iii

Publications arising from this work iv

Acknowledgements v

Table of Contents vi

Figures & Tables xv

Abbreviations xix

Chapter 1| Introduction

1.1 Bacterial Communication and Quorum Sensing 2

1.1.1 AHL signalling and QS 3

1.1.2 LuxI family proteins and signal generation 5

1.1.3 LuxR-family proteins and signal transduction 7

1.1.4 AHL signalling in virulence 10

1.2 *Yersinia pseudotuberculosis* 11

1.2.1 Diseases caused by the *yersiniae* 12

1.2.2 The environmental control of virulence 14

1.2.3 The *Yersinia* virulence plasmid 15

1.2.4 The Yop-Ysc type 3 secretion system 16

1.2.4.1 The structure of the Yop-Ysc T3SS 18

1.2.4.2 Sorting proteins for the T3SS 20

1.2.4.3 Chaperones facilitate the T3SS 21

1.2.4.4 The Yop effectors 22

1.2.5 Regulation of the Yop-Ysc T3SS 25

1.2.5.1 The genetic control of the Yop-Ysc T3SS 26

1.2.6 Other *Yersinia* virulence factors 28

1.2.6.1 The Pgm locus 28

1.2.6.2 The adhesins- Inv, Ail*,* the pH 6 antigen and YadA 29

1.2.7 QS in the *yersiniae* 32

1.3 Biofilms- On Surfaces and In Liquid Culture 34

1.3.1 Architecture of biofilms 35

1.3.2 Function of biofilms 37

1.3.2 Biofilm development 39

1.3.2 *Yersinia* biofilms 41

1.3.2 QS in biofilms 42

1.4 Flagella Mediated Motility 45

1.4.1 The structure and function of the flagellum 45

1.4.2 QS and the genetic regulation of flagella 49

1.5 Aims 50

Chapter 2| Materials and Method

2.1 Growth Conditions 53

2.1.1 Growth media 53

2.1.2 Growth conditions 55

2.1.2 Bacterial strains 55

2.2 Genetic Manipulation 60

2.2.1 DNA 60

2.2.2 Plasmids used in this study 60

2.2.3 Restriction enzymes 62

2.2.4 Separation of DNA by agarose gel electrophoresis 62

2.2.5 DNA ligation 63

2.2.6 Polymerase chain reaction conditions 63

2.2.6.1 Synthesis of oligonucleotide primers 63

2.2.6.2 PCR amplification 65

2.2.7 Introducing DNA into bacterial cells 65

2.2.7.1 Preparation of electro-competent cells 65

2.2.7.2 Electroporation 66

2.2.7.3 Conjugation 66

2.2.8 Construction of the *virF* promoter fusion 67

2.2.9 Mutagenesis of *yscJ* 67

2.2.10 Transposon mutagenesis 68

2.2.11 Sequencing of DNA 69

2.3 Extraction and Analysis of Proteins 70

2.3.1 Preparation of supernatant proteins 70

2.3.2 Purification of flagella 70

2.3.3 TCA precipitation 71

2.3.4 SDS-PAGE 71

2.3.5 Protein profiling and sequencing 71

2.4 Phenotypic Assays 72

2.4.1 Liquid culture biofilm assays 72

2.4.2 Congo red binding in liquid culture 74

2.4.3 Measuring biofilms on the surface of *C. elegans* 74

2.4.3.1 Biofilms grown in the agar method 74

2.4.3.2 Biofilms grown in the compost method 75

2.4.4 Determination of luminescence and optical density 76

2.5 Microscopy 77

Chapter 3| QS and liquid culture biofilms

3.1 Introduction 79

3.1.1 Cellular physiology and genetics of LCBs 80

3.1.2 LCBs in the *yersiniae* 83

3.1.3 Chapter 3 aims 86

3.2 Results 88

3.2.1 QS and LCBs 88

3.2.1.1 QS controls LCB formation 88

3.2.1.2 YpsI and YtbI are necessary for LCBs 90

3.2.2 Biofilm matrix components in LCBs 92

3.2.2.1 Extracellular DNA and polysaccharides in LCBs 92

3.2.2.2 DNA is an important structural component of LCBs 97

3.2.3 Supernatant factors influence LCB formation 99

3.2.3.1 Secretion is involved in LCB formation 99

3.2.3.2 Boiling Δ*ypsI* Δ*ytbI* mutant supernatant prevents LCB formation 101

3.2.3.3 Excluding macromolecules from supernatant prevents LCB formation 102

3.2.4 Cell surface factors are involved in LCB formation 103

3.2.4.1 Protease treatment of cell pellets prevents LCBs 103

3.2.4.2 Chromosomally encoded factors are involved in LCBs 104

3.2.5 pYV encodes the supernatant factor required for LCBs 105

3.2.6 T3S is involved in the formation of LCBs 106

3.2.6.1 Yop-related proteins are present in culture supernatants 106

3.2.6.2 Secretion via the Ysc injectisome is necessary for LCB formation 109

3.2.7 Identification of genes involved in LCBs 112

3.2.7.1 Mutants in *flhDC*, *fliA* and *flhA* also form LCBs 112

3.2.7.2 A transposon screen identifies genes involved in LCBs 114

3.2.7.3 An insertion into *psaE* links Congo red binding with LCBs 117

3.3 Discussion 120

3.3.1 Defining cellular aggregation as LCBs 120

3.3.2 QS mediated repression of LCBs 122

3.3.3 A role for T3S in LCBs 123

3.3.4 Pathways to the LCB formation 127

3.3.4.1 The flagella system represses LCB formation and Yop production 127

3.3.4.2 Cell surface factors involved in LCBs 128

3.3.4.3 Transposon insertions can prevent LCB formation 129

3.3.5 Conclusions 133

Chapter 4| Biofilm formation on *Caenorhabditis elegans* is influenced by the T3SS

4.1 Introduction 136

4.1.1 *Caenorhabditis elegans* 136

4.1.1.1 *C. elegans* as a model organism 137

4.1.2 Biofilm related infections in *C. elegans* 138

4.1.3 Significance of the *Yersinia* / *C. elegans* relationship 140

4.1.4 Chapter 4 aims 141

4.2 Results 143

4.2.1 QS regulates biofilm formation on *C. elegans* 143

4.2.2 The biofilm ECM contains extracellular DNA 146

4.2.3 Biofilms form on *C. elegans* in a compost model 149

4.2.4 QS regulated biofilms involve the pYV plasmid 153

4.2.5 QS affects biofilms through deregulation of the T3SS 154

4.2.6 The injectisome is responsible for repressing biofilms 156

4.3 Discussion 158

4.3.1 The significance of QS regulated biofilm formation 158

4.3.2 Extracellular DNA in the biofilm matrix 158

4.3.3 The relationship between QS, T3S and biofilms 160

4.3.4 Biofilm formation on agar and in compost 163

4.3.5 Conclusions 166

Chapter 5| Reciprocal control of motility and the T3SS

5.1 Introduction 168

5.1.1 The biological link between flagella and the T3SS 168

5.1.2 The dichotomy of Flagella and the T3SS in the *Yersinia* 169

5.1.3 Chapter 5 aims 171

5.2 Results 173

5.2.1 Mutation of *yscJ* results in hyper-motility and over-production of flagellin 173

5.2.2 The Δ*yscJ* mutant over-expresses *flhDC* and *fliA* 176

5.2.3 Hyper-motility in the Δ*yscJ* requires YpsI and / or YtbI 180

5.2.4 The expression of QS is modulated by functional YscJ 183

5.2.5 Flagella reciprocally represses the T3SS via *virF* 187

5.2.5 QS represses the T3SS at non-inductive temperatures via an effect on *virF* expression 190

5.3 Discussion 192

5.3.1 The regulation of motility by YscJ and QS 192

5.3.2 A checkpoint possibly controls the reciprocal regulation between motility and T3S 193

5.3.3 Control of QS by the T3SS and consequences for motility 195

5.3.4 Potential links between FlhDC, QS and the T3SS 197

5.3.5 Conclusions 199

Chapter 6| Conclusions and Future Directions

6.1 Conclusions 202

6.2 Future directions 209

References 211

List of tables and figures

Figure or table **Page**

Chapter 1

Figure 1.1|AHLs accumulate in the extracellular environment and trigger behavioural changes at threshold concentrations. 4

Figure 1.2| Some representative chemical structures of the AHL signal family. 6

Figure 1.3| The Yop-Ysc T3SS. 17

Figure 1.4| The flagella machinery of *Y. pseudotuberculosis*. 47

Chapter 2

Table 2.1| Media used in this study. 54

Table 2.2| Strains used in this study. 56

Table 2.3| Plasmids used in this study. 61

Table 2.4| Oligonucleotide primers used in this study. 64

Chapter 3

Figure 3.1| A representation of liquid culture biofilms. 80

Figure 3.2| Methods for cellular aggregation in bacteria. 83

Figure 3.3| Cell clumping, sedimentation and liquid culture biofilm formation are regulated by QS. 89

Figure 3.4| The Δ*ypsI* Δ*ytbI* mutant reverts to a non-clumping phenotype following restoration of *ypsI* and *ytbI* on pSA291. 91

Figure 3.5| Extracellular DNA is enriched around cells of clumping mutants. 93

Figure 3.6| The Δ*ypsI* Δ*ytbI* mutant clumps associate into liquid culture biofilms. 94

Figure 3.7| Biofilm matrix components are abundant in LCBS. 96

Figure 3.8| LCB formation in Δ*ypsI* Δ*ytbI* is prevented by addition of DNAse I. 98

Figure 3.9| The Δ*ypsI* Δ*ytbI* mutant supernatant can induce LBC formation in wild-type grown at 37°C. 100

Figure 3.10| The LCB-inducing factor in Δ*ypsI* Δ*ytbI* mutant culture supernatant is heat-labile. 101

Figure 3.11| Large macromolecules in Δ*ypsI* Δ*ytbI* mutant culture supernatant are required to induce LCBs in the wild-type. 102

Figure 3.12| Proteinase K treated wild-type cells do not form LCBs in response to Δ*ypsI* Δ*ytbI* mutant supernatant. 104

Figure 3.13| Wild-type cells do not require the pYV to be induced to form LCBs by Δ*ypsI* Δ*ytbI* supernatant. 105

Figure 3.14| Liquid culture biofilms require a pYV encoded supernatant factor. 106

Figure 3.15| The secretion of Yop-related proteins is regulated by QS. 108

Figure 3.16| Functional YscJ is required for the formation of LCBs and secretion of Yop-related protein. 111

Figure 3.17| The flagella regulators FlhDC and FliA and the secretion component FlhA affect LCB formation and Yop production. 113

Table 3.1| Loci involved in the LCB phenotype as revealed by a transposon mutagenesis screen. 116

Figure 3.18| Increased pH 6 antigen production probably mediates Congo red binding to Δ*ypsI* Δ*ytbI* mutant cells in liquid culture and on agar. 119

Figure 3.19| The possible regulation of LCB formation by QS in *Y. pseudotuberculosis*. 133

Chapter 4

Figure 4.1| Diagram of the pharynx and anterior digestive tract of *C. elegans,* as revealed by CLSM. 137

Figure 4.2| The biofilms formed by *X. nematophila*, *Y. pseudotuberculosis* and *Y. pestis* on *C. elegans* are morphologically similar and accumulate at the anterior of the worm, around the mouthparts**.** 139

Figure 4.3| Biofilm severity index calculated for wild-type, Δ*ypsI* Δ*ytbI* and the complement strain Δ*ypsI* Δ*ytbI* pSA291. 143

Figure 4.4| Biofilms formed on the cuticle of *C. elegans* by wild-type *Y. pseudotuberculosis* and the AHL negative Δ*ypsI* Δ*ytbI* mutant as revealed by CLSM. 145

Figure 4.5| *Y. pseudotuberculosis* biofilms on *C. elegans* stain positively for eDNA using DAPI, which gives a blue fluorescence, as revealed by CLSM. 147

Figure 4.6| Biofilm severity index calculated for biofilms caused by *Y. pseudotuberculosis* on the surface of *C. elegans* when the bacterial culture is either treated with DNase I or untreated. 148

Figure 4.7| The colonisation of *C. elegans* by *Y. pseudotuberculosis* in compost, as revealed by CLSM. 150

Figure 4.8| QS is important for *Y. pseudotuberculosis* to form biofilms on *C. elegans* in compost, as revealed by CLSM. 152

Figure 4.9| The pYV plasmid determines the reduction in biofilm load in a Δ*ypsI* Δ*ytbI* mutant. 154

Figure 4.10| The attenuation of biofilm formation in the Δ*ypsI* Δ*ytbI* mutant is reversed by mutation of *yscJ*. 155

Figure 4.11| *Y. pseudotuberculosis* grown at 37°C does not form biofilms on *C.* 157

elegans.

Figure 4.12| Three possible mechanisms for biofilm repression by the Ysc injectisome in the Δ*ypsI* Δ*ytbI* mutant. 162

Chapter 5

Figure 5.1| Comparison of the structure of the flagella and injectisome. 169

Figure 5.2| Mutation of *yscJ* results in hyper-motility during growth at 22°C. 174

Figure 5.3| The Δ*yscJ* mutant over-produces flagellin during growth at 22°C. 175

Figure 5.4| The Δ*yscJ* mutant is up-regulated for *flhDC* expression. 177

Figure 5.5| The Δ*yscJ* mutant is up-regulated for *fliA* expression. 179

Figure 5.6| YpsI and YtbI are necessary for the Δ*yscJ* mutant to exhibit hyper-motility. 181

Figure 5.7| YpsI and YtbI are necessary for up-regulation of *flhDC* expression in Δ*yscJ*. 182

Figure 5.8| The expression of the AHL synthase genes *ypsI* and *ytbI* at 22°C is altered in the Δ*yscJ* mutant. 184

Figure 5.9| The expression of the AHL receptor genes *ypsR* and *ytbR* at 22°C is altered in the Δ*yscJ* mutant. 186

Figure 5.10| Mutants in *flhDC*, *fliA* and *flhA* but not *fliC* are up-regulated for *virF* expression at 30°C. 189

Figure 5.11| The QS genes *ypsR*, *ytbI* and *ytbR*, but not *ypsI*, repress the expression of *virF* during growth at 30°C. 191

Chapter 6

Figure 6.1 | A simplified model for the relationship between QS and FlhDC in regulating temperature modulated behavioural decisions in *Y. pseudotuberculosis*. 205

Abbreviations

A list of abbreviations and symbols used in this work:

AD	Anno Domini
AHL	*N*-acyl Homoserine Lactone
AI-2	Autoinducer 2
Ail	Attachment Invasion Locus
Amp^R	Ampicillin (resistance)
BHI	Brain Heart Infusion
bp	Base Pair
CLSM	Confocal Laser Scanning Microscopy
CM	Cytoplasmic Membrane
Cm^R	Chloramphenicol (resistance)
CR	Congo Red
CRP	Cyclic-AMP Receptor Protein
DAPI	4',6-diamidino-2-phenylindol
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynucleotide Triphosphate
EBI	European Bioinformatics Institute
ECM	Extracellular Matrix
e-DNA	Extracellular DNA
EPS	Exopolysaccharide
Flu	Fluffing
FTF	Fructosyltransferse
g	Gravity
GAP	GTPase Activating Protein
GDI	Guanidine Dissociation Inhibitor
GFP	Green Fluorescent Protein
GMP	Guanidine Monophosphate
h	Hour
HHQ	4-hydroxy-2-heptylquinoline
HPI	High Pathogenicity Island
HSL	Homoserine Lactone

HTH	Helix-Turn-Helix
IM	Inner Membrane
Inv	Invasin
IPTG	Isopropyl-1-thio-β-D-galacpyranoside
kb	Kilobase
kDa	Kilodalton
kV	Kilovolts
LB	Luria Broth
LCB	Liquid Culture Biofilm
LCR	Low Calcium Response
LPS	Lipopolysaccharide
M	Molar
MALDI-ToF	Matrix assisted laser desorption ionisation- time of flight
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
Mox	Magnesium oxalate
mRNA	Messenger Ribonucleic Acid
Nal^R	Naladixic Acid (resistance)
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NGM	Nutrient Growth Media
nm	Nanometre
OD	Optical Density
OM	Outer Membrane
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
Pgm	Pigmentation
PNACL	Proteins and Nucleic Acid Centre, Leicester
PQS	*Pseudomonas* Quinolone Signal

Psa	pH 6 Antigen
pYV	Plasmid for *Yersinia V*irulence
QS	Quorum Sensing
RNA	Ribonucleic Acid
Rpm	Revolutions Per Minute
R-WGA	Rhodamine Wheat Germ Agglutinin
SAM	*S*-adenosylmethionone
SDS	Sodium dodecyl Sulphate
SM^R	Streptomycin (resistance)
Spp.	Species
T3S	Type 3 Secretion
T3SS	Type 3 Secretion System
TAE	Tris-acetate EDTA
TCA	Trichloroacetic Acid
TCI	Tissue Culture Invasive
Tet^R	Tetracycline (resistance)
Tp^R	Trimethoprim (resistance)
UK	United Kingdom
UV	Ultraviolet
v	Volume
V	Volts
w	Weight
WT	Wild-type
X	Times
X-gal	5-bromo-5-chloro-3-indolyl β-D-galactoside
Yop	*Yersinia* Outer Protein
^oC	Degrees Centigrade
%	Percent
Δ	Deletion
µF	Microfarad
µg	Microgram
µl	Microlitre
µm	Micrometre
Ω	Ohm

Chapter 1 |

Introduction

1.1 Bacterial Communication and Quorum Sensing

The ability for many species of bacteria to engage in a type of population sensing which couples genetic regulation with growth phase has been termed quorum sensing (QS). QS facilitates co-operation, competition and co-ordinated group behaviours including bioluminescence, virulence, biofilm formation, competence, motility, secondary metabolite (for example antibiotic production) and exoprotein secretion (Williams et al., 2007, Fuqua et al., 1994, Diggle et al., 2007a, Williams, 2007). To achieve this, bacteria typically produce and release small diffusible signalling molecules that accumulate in the environment and at a threshold concentration cause global changes in gene expression, often including auto-induction, whereby the signal induces positive-feedback on its own production (Williams et al., 2007, Salmond et al., 1995, Swift et al., 1996, Williams et al., 2000, Withers et al., 2001, Winzer and Williams, 2001).

There are two extensively characterised QS systems in bacteria- the *N*-acyl-homoserine lactone (AHL) QS group of the *proteobacteria*, and the peptide based QS group in Gram positive bacteria (Kleerebezem et al., 1997, Hardman et al., 1998, Williams et al., 2007, Withers et al., 2001). Another QS system, termed autoinducer 2 (AI-2) has also been discovered which spans the Gram negative / Gram positive divide (Schauder et al., 2001, De Keersmaecker et al., 2006, Surette et al., 1999), however, in some bacteria, it may not function as a QS system but may only play a metabolic role (Doherty et al., 2006). More QS molecules are found in specific bacterial groups, such as the *Pseudomonas* Quinolone Signal (2-heptyl-3-hydroxy-4-quinolone; PQS) and the related 2-heptyl-4-quinolone (HHQ) QS systems found in *Pseudomonas* and *Burkholderia* spp. (Diggle et al., 2006, Diggle et al., 2007b), and the epinephrine /

norepinephrine / autoinducer 3 responsive signalling pathway found to regulate virulence in enterohaemorrhagic *Escherichia coli* (Walters and Sperandio, 2006).

1.1.1 AHL signalling and QS

The QS paradigm is based on bioluminescence in the marine bacterium *Vibrio fischeri.* This species grows to high cell densities in specialised 'light organs' of certain marine squid and generates light via activity of the luciferase (*lux*) operon (Engebrecht et al., 1983). Luciferase expression occurs at high cell-densities through the activity of AHL signalling molecules, usually produced via a member of the LuxI AHL synthase protein family. AHLs constitute a family auto-inducing signalling molecules which diffuse or are exported from the cell and accumulate to a minimal threshold concentration whereupon they activate a cytoplasmic receptor / helix-turn-helix transcriptional activator which is usually a member of the LuxR protein family (Whitehead et al., 2001, Cámara et al., 2002, Kaplan and Greenberg, 1985). The AHL-LuxR complex binds to the promoter of the *lux* genes, and activates bioluminescence (Swartzman et al., 1992). The LuxR/I system can also positively regulate its own expression (Eberhard et al., 1991, Shadel and Baldwin, 1991), and so represents a biochemical switch between the 'quorate' and 'non-quorate' state (figure 1.1) - however, LuxR can also negatively regulate its own expression, which may lead to bistability and hysteresis in the QS network (Williams et al., 2008).

Homologues of *luxI* and *luxR* exist in at least 50 other species of *Proteobacteria* (Williams et al., 2007), and these systems can be classified into two phylogenetic groups: group A, including LuxR/I from *Vibrio* spp. and LasR/I from *Pseudomonas aeruginosa*, are dispersed throughout the α, β, and γ *Proteobacteria* and generally act as transcriptional activators, while group B,

including YpsR/I from *Y. pseudotuberculosis* and EsaR/I from *Pantoea stewartii*, are restricted to the γ proteobacteria and act as transcriptional repressors (Waters and Bassler, 2005, Minogue et al., 2002, Atkinson et al., 1999, Swartzman et al., 1992, Gambello and Iglewski, 1991). In the genome, the *luxI* and *luxR* family genes are typically found in close proximity; in tandem, divergent or even over-lapping pairs (Williams et al., 2007), and have been found on chromosomes, on plasmids (Piper et al., 1999), and carried in transposons (Wei et al., 2006).

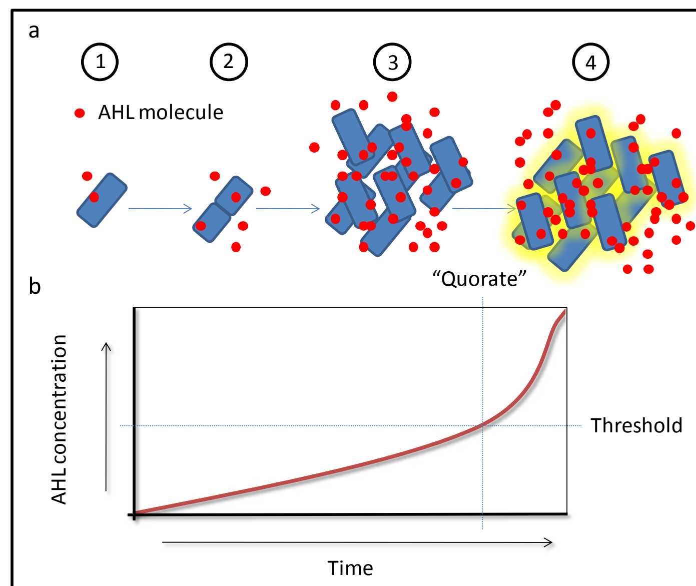


Figure 1.1| AHLs accumulate in the extracellular environment and trigger behavioural changes at threshold concentrations. In the top panel (a), an AHL producing cell is at low cell density (1). Following growth, more AHLs are produced (2) until the cell reaches a high cell density with an abundance of AHLs in the environment (3). As the concentration of AHLs surpasses the threshold, the cells become "quorate" and engage in collective target gene expression, such as the production of bioluminescence (4). The bottom panel (b) shows a graphical representation of the accumulation of AHLs in the extracellular environment that crosses a threshold concentration to induce the "quorate" state and modulate gene expression.

1.1.2 LuxI family proteins and signal generation

The archetypal AHL synthase is LuxI from *V. fischeri*. LuxI is a 22 kDa protein which directs the synthesis of 3-oxo-C_6-homoserine lactone (HSL) from *S*-adenosylmethionone (SAM) and 3-oxo-hexanoyl-acyl-acyl carrier protein (acyl-ACP) (Schaefer et al., 1996). LuxI binds acyl-ACP and forms a covalent intermediate between the acyl-ACP acyl-group and the enzyme (Hanzelka et al., 1997). Subsequent to SAM binding, LuxI catalyses the formation of an amide bond between the carboxyl group of the fatty acid and the amino group of SAM. This intermediate undergoes lactonisation, resulting in the formation of the AHL and 5'methylthioadenosine (Schaefer et al., 1996, Hanzelka et al., 1997).

LuxI-family proteins synthesise a diverse range of AHLs, with acyl-side chain lengths between 4 to 16 carbon atoms which may be fully reduced, or substituted with an oxidized carbonyl or hydroxyl group on the third carbon (Williams, 2007). Long chain AHLs may also be unsaturated (Swift et al., 2001), and recently more AHL groups have been identified, such as the *p*-coumaroyl-HSL, where the side chain is derived from exogenously supplied *p*-coumaric acid derived from a plant metabolite (Schaefer et al., 2008) (Figure 1.2). This diversity in AHL structure is provided by the mechanism of synthesis and the cellular pool of acyl-acyl-ACPs: substitutions on the third carbon depend on fatty acid biosynthesis, and the degree of *N*-acylation is conferred by differences in the fatty acid binding pocket of the LuxI homologue, which either occludes or permits longer acyl chains (Nasser and Reverchon, 2007). This diversity may allow for specificity between the AHL and its cognate receptor and allows bacteria to discriminate signals produced by their own species and reduce 'noise' produced by co-habitants (Nasser and Reverchon, 2007), however, some LuxI homologues are capable of synthesizing more than one type of AHL, though it is

not known if all are biologically relevant (Waters and Bassler, 2005). In contrast to this acyl side chain structural diversity, the AHL produced by *Erwinia carotovora* (3-oxo-C_6-HSL) exists exclusively in the L-isomer, and since synthetic D-enantiomers lack activity, this suggests that other AHLs may also be active in the L-form (McClean et al., 1997).

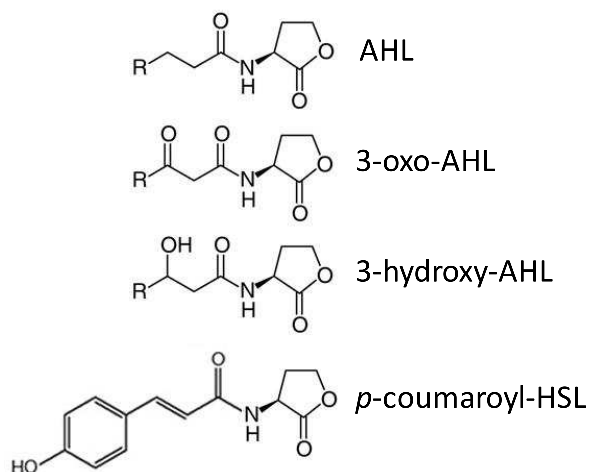


Figure 1.2| Some representative chemical structures of the AHL signal family. *N*-acyl homoserine lactones (AHLs), where the acyl side chain (R) can be C_1 to C_{15} are commonly unsubsituted, or substituted to form 3-oxo-AHLs (*N*-3-oxoacyl-homoserine lactone) or 3-hydroxy-AHL (*N*-3-hydoxyacyl-homoserine lactone). One or more double bonds may also be present in the acyl side chain. More recently, derivatives of AHLs including the *p*-coumaroyl-HSL, where the side chain is provided by a plant-derived molecule, have been identified. Figure adapted from Williams et al., 2007 and Schaefer et al., 2008.

Reflecting on this AHL structural diversity, LuxI family proteins often share relatively little sequence homology, despite the presence of highly conserved regions (Fuqua et al., 1996). Mutational analysis of LuxI has however revealed two segments of the protein which are required for its function- a region within the N-terminus between residues 25 – 70 which includes the active site involved in amide bond formation, and residues 133 – 164 in the C-terminus which form

the acyl-acyl-ACP binding pocket, especially a threonine residue at position 140 (Hanzelka et al., 1997) which is conserved in, LuxI, LasI and EsaI from *Vibrio* spp., *P. aeruginosa* and *P. stewartii* respectively, which all preferentially bind 3-oxo-acyl-ACP and produce 3-oxo-HSL molecules (Watson et al., 2002). These threonine resides are also found in the *Y. pseudotuberculosis* AHL synthase enzymes YpsI and YtbI which direct the synthesis of both 3-oxo and unsubstituted AHLs (Ortori et al., 2007).

However, AHLs can also be synthesized by enzymes other than the LuxI-family, and at least two other families of AHL synthase proteins exist: the *luxM* / *ainS* family present only in *Vibrio spp.* (Milton et al., 2001, Gilson et al., 1995) and *hdtS* from *Pseudomonas fluorescens* (Laue et al., 2000). Both these enzymes synthesize the core HSL ring from SAM, and differ only in the sources they use for the acyl side chain. However both are characterized to a lesser extent than the LuxI family (Williams et al., 2007).

1.1.3 LuxR family proteins and signal transduction

Typically, AHL signals are received by receptor proteins of the LuxR family, but while LuxR homologues all share a similar domain structure, like the LuxI family proteins they share relatively little homology with one another (Fuqua et al., 1996). The *V. fischeri* LuxR protein, as with all other studied homologues, contains two functional domains- an N-terminal binding site for its cognate AHL and a C-terminal helix-turn-helix (HTH) DNA binding motif which binds a region in target promoters termed the '*lux* box'- a 20 bp palindromic sequence of DNA (Egland and Greenberg, 1999). LuxR may bind DNA either synergistically with (Stevens et al., 1994) or independently of (Egland and Greenberg, 2000) the RNA polymerase (RNAP) complex. LuxR binds as a homodimer, each monomer

contacting one half of the dyadic *lux* box and acts as an ambidextrous activator of RNAP (Egland and Greenberg, 1999)- though other LuxR proteins may form homodimers and heterodimers with other LuxR homologues encoded in the same bacteria (Ledgham et al., 2003, Medina et al., 2003, Ventre et al., 2003).

When no AHLs are present, the N-terminal-domain of LuxR blocks dimerisation and the DNA binding capacity of the HTH motif. Upon AHL binding, a conformational change releases this auto-inhibition (Poellinger et al., 1995). AHL binding to TraR from *Agrobacterium tumefaciens* and MrtR from *Mesorhizobium tianshanense* also promotes dimerisation and activation (Qin et al., 2000, Yang et al., 2008). Specificity in the response to AHLs is conferred by discrete differences in the C-terminal-domain AHL binding pocket; this is formed from regions of hydrophobic and aromatic amino acids that are highly conserved through the LuxR homologues. This creates differences in the acyl binding tunnel which accommodate acyl chains of different lengths. Longer chain AHLs also appear to be irreversibly bound to LuxR homologues, while affinities decrease with decreasing chain length (Nasser and Reverchon, 2007). Indeed, one *A. tumefaciens* AHL, 3-oxo-C_8-HSL, is thought to be completely embedded in a narrow channel within the AHL binding domain of TraR, can be removed only following dialysis in the presence of detergent (Zhu and Winans, 1999, Zhang et al., 2002), and TraR can retain activity for up to 8 h after AHLs have been removed (Luo et al., 2003). In contrast, in *V. fischeri,* 3-oxo-C_6-HSL can be readily and reversibly disassociated from LuxR by dilution, suggesting a much weaker interaction (Urbanowski et al., 2004).

Orphan LuxR receptors are encoded in genomes throughout the *proteobacteria*, which lack a *luxI* homologue in the genomic vicinity (Williams et al., 2007). *P. aeruginosa* encodes two of these orphan receptors, *qscR* and *vqsR*. These modulate virulence and the timing of QS by repressing transcription of *lasI* and

rlhI (Chugani et al., 2001, Fuqua, 2006) and affecting the production of AHLs (Li et al., 2007). VsqR selectively activates *lasI* expression without affecting *lasR* and also regulates virulence factors (Juhas et al., 2004, Juhas et al., 2005). It may also be a target of AHL QS regulation since a *lux* box is present in the promoter of *vqsR* and has been shown to drive two-fold more expression in the presence of LasR (Li et al., 2007).

However, it should be noted that while AHL QS is involved in the virulence of many bacterial species, its production is absent from a number of obligate and opportunistic pathogens including *Nesseria meningitidis, Haemophilus influenzae, Helicobacter pylori, E. coli* and *Salmonella* spp. (Williams et al., 2007), however the latter two encode a *luxR* homologue (*sdiA*) which responds to AHLs in the growth environment of AHL producing bacteria, maximally from 3-oxo-C_6-HSL and 3-oxo-C_8-HSL (Williams et al., 2007), and becomes active during transit through the gastrointestinal tract in the presence of AHL producing bacteria (Smith et al., 2008, Dyszel et al., 2010). This raises the possibility that signal interception by these bacteria could contribute to their success as enteric commensals and pathogens. In addition, SdiA in *E. coli* has been shown to respond to an endogenous signal, and over-expression represses cell division, virulence factor production and flagella (Kanamaru et al., 2000). However, no gene has been shown to be regulated by chromosomally encoded *sdiA* alone, and *sdiA* mutants fail to show expected abnormality in cell division (Ahmer, 2004).

1.1.4 AHL signalling in virulence

Several lines of evidence point to QS being actively involved in disease. In a mouse model of *Y. enterocolitica* infection, AHLs have been detected (Jacobi et al., 2003), while 3-oxo-C_{12}-HSL can activate a *gfp* reporter in the lungs of mice infected with *P. aeruginosa* (Wu et al., 2000). Physiological concentrations of AHLs have also been detected in the sputum of cystic fibrosis patients infected with *P. aeruginosa* (Middleton et al., 2002b, Chambers et al., 2005), while the virulence of several bacteria in chronic and acute infections is reduced following mutation of their QS systems (Wu et al., 2001, Imamura et al., 2005, Nelson et al., 2009, Pearson et al., 2000, Rumbaugh et al., 1999, Sokol et al., 2003, Valade et al., 2004). QS regulates a range of virulence determinants including exoprotease production (Lewenza et al., 1999, Pearson et al., 1997, Valade et al., 2004, Swift et al., 1999, Jones et al., 1993), and secretion systems of the type 1 (Liu et al., 2008), type 2 (Corbett et al., 2005, Liu et al., 2008), type 3 (Bleves et al., 2005, Henke and Bassler, 2004, Liu et al., 2008), type 4 (Delrue et al., 2005), type 5 (Chambers et al., 2006) and type 6 (Liu et al., 2008, Aubert et al., 2008) classes. Interestingly, AHLs (predominantly 3-oxo-C_{12}-HSL) also function directly as virulence determinants, and can modulate mammalian physiology and the immune response (Smith et al., 2002, Telford et al., 1998, 2004, Skindersoe et al., 2009, Boontham et al., 2008, Tateda et al., 2003, Hooi et al., 2004), acting as a vasorelaxant, and inhibiting contraction of porcine arterial smooth muscle (Lawrence et al., 1999). This AHL can also accelerate apoptosis of macrophages, neutrophils, dendritic cells and CD4+ cells, but not epithelial cells or CD-8+ cells (Tateda et al., 2003, Boontham et al., 2008).

The regulatory relationship between QS and virulence is not always one-way, and several examples have been found where virulence associated pathways

affect the expression of QS. In *P. aeruginosa*, Vfr is a member of the cyclic-AMP receptor protein (CRP) family of proteins and regulates a range of virulence determinants including exotoxin A production, elastase, type IV pili and type 3 secretion (West et al., 1994, Beatson et al., 2002, Wolfgang et al., 2003). Vfr also positively regulates QS in *P. aeruginosa* by binding to specific DNA sequences in the promoter of *lasR* (Albus et al., 1997). CRP-family proteins also regulate virulence and QS in other bacteria, including *V. cholerae*, where CRP positively regulates the AHL response regulator *hapR* along with swimming motility and several genes involved in intestinal colonisation (Liang et al., 2007).

1.2 *Yersinia pseudotuberculosis*

Yersinia pseudotuberculosis is a Gram negative bacillus, but can be pleiomorphic ranging from shortened cocobacilli to elongated rods, with this pleiomorphism being greatly influenced by environmental conditions such as nutrient availability and temperature (Bottone and Mollaret, 1977, Rollins et al., 2003, Deacon et al., 2003, Rowan, 1999, Wanger, 2010). There are at least fifteen documented species within the genus *Yersinia*, but only three of these are considered to be pathogenic to humans; *Y. pseudotuberculosis*, *Yersinia enterocolitica* and *Yersinia pestis* (Pujol and Bliska, 2005, Perry and Fetherston, 1997, Shivaji et al., 2000). Given the genomic similarity between the two species- *Y. pestis* is thought have emerged from *Y. pseudotuberculosis* some time before the first plague epidemic in 541 AD (Achtman et al., 1999).

Most *Yersinia* are capable of psychrophilic adaptation and grow well in cold temperatures (Bergann et al., 1995, Somov and Varvashevich, 1984), while cold-enrichment is often used for the isolation of *Yersinia* species (Greenwood et

al., 1975). In fact the most prolonged and severe *Y. pestis* pandemic, the 'black death' and associated epidemics, stretched from 1346 to the beginning of the 19th century, coincident with the 'little ice-age', a period of global cooling that occurred between 1400 and 1800 AD. Furthermore Justinian's plague (541 – 767 AD), although occurring shortly after a cold period, originated in East or Central Africa before spreading northwards towards colder regions on the world (Achtman et al., 1999). Reflecting this geographically, high anti-*Yersinia* antibody titres are also frequently found in areas such as Scandinavia (Bottone, 1999), and *Yersinia* reservoirs tend to be in cold to temperate regions of the world (Adesiyun and Krishnan, 1995, Vincent et al., 2008, Rimhanen-Finne et al., 2009) while most infections are reported in winter (Long et al., 2010).

1.2.1 Diseases caused by the *yersiniae*

The diseases caused by *Y. pseudotuberculosis* and *Y. enterocolitica* range between mild, self-limiting enteritis or more serious mesenteric lymphadenitis and appendicitis resulting from ingestion of contaminated food or water (Pujol and Bliska, 2005, Hubbert et al., 1971). However, deadly disease resulting from systemic infection and septicaemia, while rare, carries a 75 % mortality rate despite the use of antibiotics (Butler, 1994, Ljungberg et al., 1995, Deacon et al., 2003). Autoimmune disorders such as reactive arthritis are also common sequelae to infection by both *Y. pseudotuberculosis* and *Y. enterocolitica* (Hannu et al., 2003, Jalava et al., 2006), but erythema nodosum, glomerulonephritis and myocarditis can also be after-effects of infection (Bottone, 1999).

Interestingly, *Y. pseudotuberculosis* has also been implicated in several other diseases- starting in the 1950s, an epidemic spread along the pacific coastal region of the then USSR, symptoms of which presented as erythematous skin

rash and desquamation, exanthema, hyperaemic tongue, and a toxic shock syndrome, similar to scarlet fever caused by Group A Streptococci. A *Y. pseudotuberculosis* strain (IP31758) was identified as the causative agent of this outbreak, and the scarletinoid-like fever caused by this strain may be linked to the acquisition of strain-specific virulence plasmids unrelated to any other *Yersinia* plasmid, including an icm/dot type IV secretion system, which carry immunosuppressive and antiphagocytotic properties and is shared only with the intracellular pathogens in the order *Legionellales* (Eppinger et al., 2007).

Y. pestis, the aetiological agent of bubonic, pneumonic and septicaemic plague- still causes disease worldwide (Titball et al., 2003, Perry and Fetherston, 1997). Infection is usually transmitted via the bite of an infected flea- this causes the bubonic form of the disease, where bacteria disseminate to local draining lymph nodes in the armpit or groin (Titball et al., 2003). This can result in systemic infection and septicaemic plague, followed by dissemination to the lungs and secondary pneumonic plague infection which is highly contagious and can be transmitted via the airborne route in cough droplets, causing primary pneumonic infection in other individuals (Titball et al., 2003).

Interestingly all three *yersiniae* display marked tropism for lymphoid tissue (Brubaker, 1991). The enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* selectively target M-cells (a type of antigen sampling cell which overlays the follicle associated epithelium of the Peyer's patches) to cross the intestinal epithelial barrier, resulting in the destruction of the Peyer's patches and the formation of abscesses in the mesenteric lymph nodes (Autenrieth and Firsching, 1996). The bacteria may then disperse via the lymphatic vessels to the spleen, other lymph nodes and the liver (Carter, 1975, Autenrieth and Firsching, 1996, Clark et al., 1998, Grützkau et al., 1990, Hanski et al., 1989).

1.2.2 The environmental control of virulence

To cause disease, the three pathogenic *yersiniae* possess sophisticated mechanisms to switch on particular sets of genes during infection. The most important signals for this are temperature - where the shift in temperature from those typical of ambient conditions to those prevalent in the mammalian body signals to the bacterium that they have entered a mammalian host; and the availability of calcium, which is thought to signal the proximity of the bacterium to host cells, perhaps by the competitive interaction of calcium with an unidentified *Yersinia* cell surface receptor (Cornelis, 2002b). Temperature regulates the expression of several virulence factors (Straley and Perry, 1995), while pathogenic *yersiniae* require calcium concentrations in excess of 2.5 mM for growth at 37°C. In the absence of calcium, the growth of *Y. pestis* becomes restricted after approximately two generations, which can only be recovered by shifting the cells to growth at 26°C, and not simply by supplying calcium to the growth media (Zahorchak et al., 1979). The growth restriction is, however, less severe in the enteropathogenic *yersiniae* (Perry et al., 1998). This is known as the low calcium response (LCR) which, along with temperature, affects the production of a significant number of virulence related proteins (Chromy et al., 2005).

1.2.3 The *Yersinia* virulence plasmid

One of the important virulence determinants for the pathogenic *yersiniae* is a 70 kb virulence plasmid, termed pYV (plasmid for *Yersinia* Virulence, also called pCD1 or pIB1) which is required for full virulence in mammals (Cornelis et al., 1998a). This plasmid encodes the Yop-Ysc Type 3 Secretion System (T3SS), along with other virulence determinants including the adhesin YadA (El Tahir and Skurnik, 2001). The pYV plasmid is present in all three pathogenic *Yersinia*, but has evolved differently, particularly between the *Y. pseudotuberculosis* (pIB1) / *Y. pestis* (pCD1) branch and that present in *Y. enterocolitica* (pYV) which are distinguished by rearrangements in the replication and partition region which has inverted the *yopE*-*yadA* region (Biot and Cornelis, 1988), several insertions (Snellings et al., 2001, Neyt et al., 1997), and some smaller yet important mutations including frame-shift mutations which abolish the production of YadA and the lipoprotein YlpA from pCD1 from *Y. pestis* (Perry et al., 1998, Hu et al., 1998), and YlpA from pIB1 and pCD1 (Cornelis et al., 1998a, Hu et al., 1998, Perry et al., 1998).

Most of the genes encoded on the pYV are expressed maximally at 37°C, and several are also regulated by the LCR, such that the plasmid can be regarded as an anti-mammalian genome (Cornelis et al., 1998b). The pYV plasmid is important for the resistance of the *yersiniae* to phagocytosis by macrophages and other phagocytic cells, and mediates the behaviour necessary to cause apoptosis in macrophages (Mills et al., 1997, Ruckdeschel et al., 1997), although the *yersiniae* may also persist within phagocytes intracellularly and disrupt the normal phagocytic process (Pujol and Bliska, 2005, Grant et al., 1999). Intracellular persistence of *Y. pestis* in murine peritoneal macrophages, however, does not appear to depend on the pYV (Straley and Harmon, 1984a, Straley and

Harmon, 1984b), and other virulence determinants are also important in *Y. enterocolitica*, since the absence of virulence in low pathogenicity *Y. enterocolitica* isolates is not simply a consequence of the absence of pYV, since if the pYV plasmid is provided to these strains, it does not markedly affect their virulence (Gaede and Heesemann, 1995, Heesemann et al., 1984).

1.2.4 The Yop-Ysc Type 3 Secretion System

The *Yersinia* Yop-Ysc T3SS (Figure 1.3) is a sophisticated multi-protein nanomachine, and can be divided into structural components such as the basal body, the needle, the tip structure and translocon, chaperone and effector proteins. This system can inject (or translocate) Yop (for *Yersinia* outer protein) effectors through a hollow conduit (or needle) into target eukaryotic cells where they can disrupt the eukaryotic cytoskeleton and cellular processes, preventing phagocytosis, inducing apoptosis and subverting the immune response (Cornelis, 2002b).

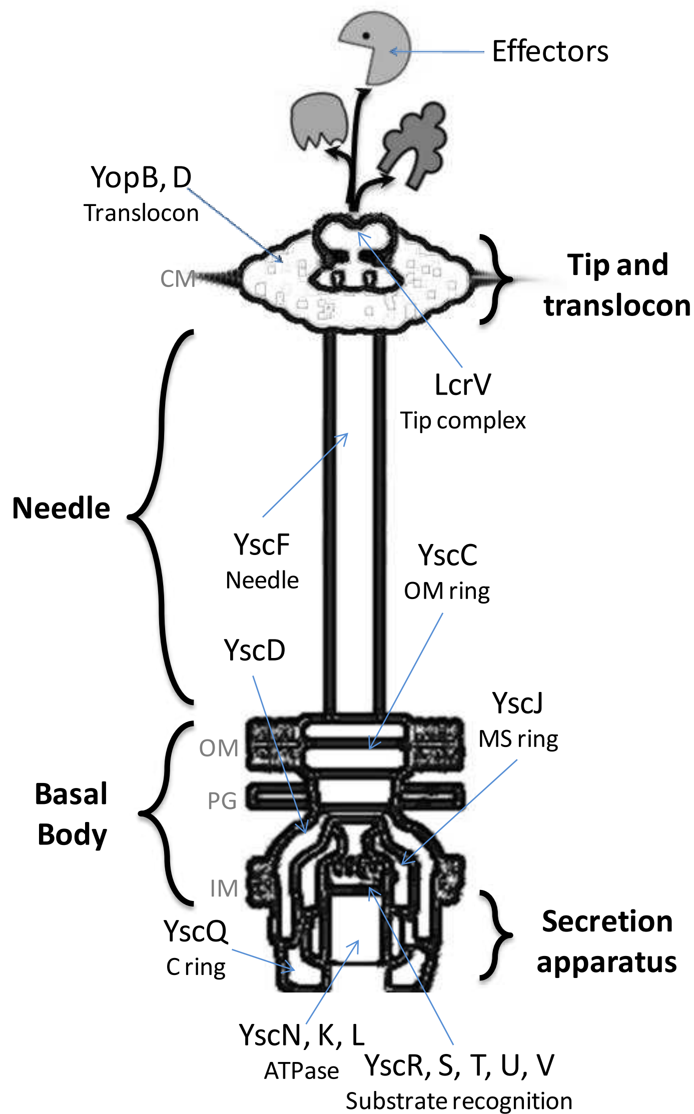


Figure 1.3| The Yop-Ysc T3SS. This multiprotein nanomachine spans the bacterial inner membrane (IM), peptidoglycan (PG) and outer membrane with a basal body structure. A needle protrudes from the basal body which is capped with a tip structure composed of LcrV. The T3SS uses the secretion apparatus to secrete and insert a YopBD translocon pore into target eukaryotic cell membranes (CM), and then translocates effector proteins directly into the cytoplasm. Figure adapted from Diepold et al., 2010.

1.2.4.1 The structure of the Yop-Ysc T3SS

The base of the injectisome is composed of a number of proteins which adopt a cylindrical structure similar to that of the flagella basal body, and are secreted into the membrane by the Sec dependent pathway (Diepold et al., 2010). This structure includes two membrane rings termed the MS (inner membrane) and OM (outer membrane) rings. The MS ring is an assembly of 24 copies of the lipoprotein YscJ, which is anchored to the periplasmic side of the inner membrane via an amino-terminal lipid group and a carboxy-terminal transmembrane domain (Silva-Herzog et al., 2008). YscD connects YscJ to the OM ring (Spreter et al., 2009), which consists of 13 copies of the secretin-family protein YscC (Burghout et al., 2004). These two membrane rings are connected to five integral membrane proteins (YscR, S, T, U and V) which play a role in exporting proteins (Edqvist et al., 2003, Sorg et al., 2007). The export apparatus is flanked by YscQ, which facilitates the binding of the ATPase YscN and secretion substrate-chaperone complexes (Jackson and Plano, 2000, Morita-Ishihara et al., 2006). YscN forms hexameric rings which are activated by oligomerisation, and contributes to providing the energy necessary for the secretion of Yop effectors (Woestyn et al., 1994, Zarivach et al., 2007, Diepold et al., 2010). Secretion through the Ysc injectisome requires a proton motive force, and can be blocked by the addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (Wilharm et al., 2004).

Emanating from the basal body, protruding into the extracellular space, is a 60-80 nm long hollow needle formed by the helical polymerisation of 150 - 300 copies of YscF with an external diameter of 6-7 nm and an internal space of approximately 2 – 3 nm (Diepold et al., 2010, Cornelis, 2006, Hoiczyk and Blobel, 2001). YscF is a substrate for the T3SS, and is exported and polymerised in a T3S-dependent manner along with YscP, a protein which

determines the length of the needle via the helical architecture of the N-terminal domain, possibly acting as a molecular ruler (Wagner et al., 2009, Payne and Straley, 1999, Stainier et al., 2000). Secretion of YscF may be aided by YopR, which is secreted into the extracellular milieu during the stages of injectisome formation (Blaylock et al., 2010, Lee and Schneewind, 1999a). The distal needle is capped with structure composed of four copies of the hydrophilic protein LcrV (Broz et al., 2007, Mueller et al., 2005). LcrV aids in directing the formation of a pore in the membrane of target cells (Hakansson et al., 1993).

The Yop translocon consists of a tripartite pore which is inserted into eukaryotic cell membranes and permits the translocation of Yop effectors into the target cell cytoplasm. The pore is composed of the transmembrane proteins YopB and YopD (Hakansson et al., 1993) and the injectisome tip complex LcrV (Holmström et al., 2001, Bröms et al., 2003, Neyt and Cornelis, 1999a). Bacteria lacking the tip and translocon proteins are still able to secrete effectors into growth supernatant, but are defective in the translocation of these proteins into eukaryotic cells (Lee et al., 2000, Lee and Schneewind, 1999b, Cheng and Schneewind, 2000, DeBord et al., 2001). YopD may, however, only associate with the pore transiently, as it can be found in the cytoplasm of infected HeLa cells following integration of the translocon (Francis and Wolf-Watz, 1998b) where it is thought to play a role in regulating translocation of Yop effectors (Francis and Wolf-Watz, 1998a).

1.2.4.2 Sorting proteins for the T3SS

Included in the injectisome is an in-built ability to discriminate between secretion substrates, providing an order in T3S so the needle is polymerised before the translocon is released, which precedes Yop effector secretion (Cornelis and Wolf-Watz, 1997b). The secretion of early substrates (including the proteins required to complete the needle complex) requires YscU, YscP (Blaylock et al., 2010, Wood et al., 2008). The middle substrates (including the tip complex and translocon) also require YscU and YscP, alongside YopR (Lee et al., 2000, Lee et al., 2001, Schubot et al., 2005a), and the negative regulator LcrQ, which acts partly to prevent the premature secretion of the tip and translocon (Rimpilainen et al., 1992). LcrQ (YscM1 and YscM2 in *Y. enterocolitica*) plays a central role in the hierarchy of Yop secretion (Wulff-Strobel et al., 2002), is normally required to down-regulate the LCR and growth-restriction at 37°C (Rimpilainen et al., 1992), and secretion of LcrQ via the T3SS relieves the repression of *yop* expression (Pettersson et al., 1996). It is also part of the third switch (known as the secretion gate), which regulates secretion of late substrates, the Yop effectors, and only permits expression and transit of the Yop substrates through the T3SS when LCR conditions are encountered. LcrQ cannot function to suppress Yop secretion without LcrG (Wulff-Strobel et al., 2002), a protein which forms part of a 'gatekeeper' complex at the cytoplasmic face of the injectisome and prevents the secretion of Yop effectors until it is displaced or titrated away, perhaps by LcrV (Nilles et al., 1998, Nilles et al., 1997, Matson and Nilles, 2001, Skryzpek and Straley, 1993). The proteins YopN and TyeA also function as part of this gate- YopN binds to the C-terminal domain of TyeA, preventing YopN secretion, until calcium depletion or host cell contact (Cheng et al., 2001, Day et al., 2003, Iriarte et al., 1998, Schubot et al., 2005b).

Within the later acting substrates there is a hierarchy of secretion, with the tip and translocon components loaded into the injectisome before the effector Yops, while YopH also appears to be injected before YopE (Phan et al., 2004, Cambronne et al., 2000, Wulff-Strobel et al., 2002). This final level of substrate selection also involves LcrQ, and may associate with the YopH chaperone SycH at the injectisome gate. SycH is required for the secretion of LcrQ, and may displace LcrQ, loading its ligand and causing secretion of YopH prior to YopE (Phan et al., 2004, Cambronne et al., 2000, Wulff-Strobel et al., 2002). This observation may extend to other chaperones, since Yops with cognate chaperones have been shown to be preferentially secreted by the T3SS (Anderson and Schneewind, 1999, Boyd et al., 2000, Feldman and Cornelis, 2003).

1.2.4.3 Chaperones facilitate the T3SS

The construction and timely secretion of the T3SS requires specific chaperones. These are typically small proteins (12 – 19 kDA) which dimerise, and can be subdivided into three classes: class I chaperones (i.e. SycE, SycO, SycT and SycH) bind to the effector proteins and often share high structural conservation (Locher et al., 2005, Phan et al., 2004, Trame and McKay, 2003, Birtalan et al., 2002, Birtalan and Ghosh, 2001, Büttner et al., 2005); class II chaperones (i.e. SycD and LcrG), which associate with the translocon proteins YopB, YopD and LcrV respectively (Büttner et al., 2008, Matson and Nilles, 2001, Lawton et al., 2002, Wang et al., 2008, Johnson et al., 2007); and class III chaperones (i.e. YscE and YscG), which tend to form heterodimers and facilitate structural components of the injectisome (Sun et al., 2008). The chaperones may serve several functions: stabilising the ligand against degradation, as seen with the YopE chaperone SycE (Woestyn et al., 1996, Cheng and Schneewind, 1999); preventing the activity of the protein- seen where SycD binding to YopB and

YopD prevents premature oligomerisation (Neyt and Cornelis, 1999b), the heterodimeric chaperone YscE / YscG prevents YscF polymerising in the bacterial cytoplasm (Sun et al., 2008), and catalytically inactive YopT binds less effectively to YscT (Locher et al., 2005). Chaperones may also usher their partners to the injectisome, which may be important for effectors synthesised and stored prior to secretion, such as YopE (Lloyd et al., 2001). However, not all these functions are shared by the chaperones (Birtalan et al., 2002, Letzelter et al., 2006, Sory et al., 1995, Wattiau et al., 1994, Cambronne et al., 2000, Cheng et al., 1997), and no chaperones have been identified for YopM or YopP (Cornelis et al., 1998a).

1.2.4.4 The Yop effectors

The Yop effector proteins are virulence factors translocated into eukaryotic cells. Four (YopH, YopE, YopT and YpkA) are involved in disrupting the proper functioning of the cytoskeleton. Several of these target an important group of eukaryotic cell signalling components- the RhoA family of small GTPases. In the active GTP-bound state, RhoA family proteins contribute to several signalling pathways, including directing cytoskeletal rearrangements necessary for phagocytosis following receptor mediated activation. YopE is a functional mimic of eukaryotic GTPase activating proteins (GAPs) (Evdokimov et al., 2002), and disrupts the actin cytoskeleton by inducing the GTPase activity of RhoA family proteins (Black and Bliska, 2000, Aili et al., 2006, Andor et al., 2001). By hydrolysing GTP to GDP, RhoA family proteins are inactivated (Van Aelst and D'Souza-Schorey, 1997) and phagocytosis by macrophages and dendritic cells is prevented (Fahlgren et al., 2009). In *Y. pseudotuberculosis* YopE is critical for virulence in mice, and *yopE* mutants are rapidly cleared from Peyer's patches, and do not colonise the liver and spleen (Holmstrom et al., 1995, Viboud et al., 2006). YopT affects RhoA family signalling by acting as a mimic of eukaryotic

cysteine proteases (Shao et al., 2002), and can cleave within the C-terminal domain of RhoA, Rac1 and Cdc42, liberating the GTPases from the membrane and preventing their functioning (Shao et al., 2002, Shao et al., 2003). The suppression of RhoA-mediated signalling by YopT prevents the formation of the phagocytic cup for internalisation of the bacteria, and also inhibits the formation of focal adhesion complexes required for the formation of pseudopodia and migration of macrophages (Aepfelbacher et al., 2003). Another effector which interrupts RhoA family signalling is YpkA (YopO in *Y. enterocolitica*), a multi-domain protein which structurally mimics serine / threonine protein kinases in the N-terminal domain, while the C-terminal domain bears similarity to eukaryotic GDI-like GTPase-binding domain proteins. YpkA is localised to the inner side of the eukaryotic cell membrane (Håkansson et al., 1996, Dukuzumuremyi et al., 2000), and catalyses the phosphorylation of Gαq, a component of heterotrimeric G-proteins, which inhibits the binding of GTP and prevents signalling which would normally result in stimulation of phospholipase C-β and RhoA mediated pathways (Navarro et al., 2007). The C-terminal domain, meanwhile, associates to RhoA family proteins and inhibits phagocytosis (Barz et al., 2000, Groves et al., 2010).

YopH is one of the most multi-functional Yop proteins, disrupting pathways involved in both innate and adaptive immunity and is essential to the virulence of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* in mice, where *yopH* mutant bacteria fail to colonise the mesenteric lymph nodes and do not infect the liver or spleen (Cantwell et al., 2010, Logsdon and Mecsas, 2003, Trulzsch et al., 2004). YopH is a 51 kDA protein which possesses a C-terminal domain that potently mimics eukaryotic protein tyrosine phosphatases (PTPase) (Rosqvist et al., 1988, Zhang et al., 1992), and an N-terminal domain which contains a cleft reminiscent of eukaryotic SH2 domain proteins, responsible for binding a variety of target host proteins and causes widespread tyrosine dephosphorlyation to

proteins (Khandelwal et al., 2002, Bliska et al., 1991, Guan and Dixon, 1990) in epithelial cells (Black and Bliska, 1997) macrophages (Hamid et al., 1999) and T-cells (Gerke et al., 2005). This inhibits autophagy following binding of Invasin or YadA to β1-integrins (Deuretzbacher et al., 2009), and prevents phagocytosis in macrophages (Rosqvist et al., 1988, Bölin and Wolf-Watz, 1988)- indeed, high expression of YopH alone is sufficient to prevent phagocytosis (Persson et al., 1997). Interestingly, a *Y. pseudotuberculosis yopE* / *yopH* double mutant shows the same level of impairment in the ability to resist phagocytosis as a pYV^- strain (Rosqvist et al., 1990).

The remaining effectors (YopJ, YopM and YopK) down-regulate elements of the immune system such as inflammation and leukocyte recruitment (Grosdent et al., 2002, Shao, 2008, Cornelis, 2002b, Matsumoto and Young, 2009, Cornelis, 2002a). YopJ (YopP in *Y enterocolitica*) is a serine / threonine / lysine acetyltransferase that catalyses the acylation of target proteins, a process which prevents subsequent activation by phosphorylation (Mukherjee et al., 2006). YopJ acetylates the kinases MEK1 and MEK2, and the IKK complex, inhibiting their ability to activate the release of NF-Kβ, which would otherwise induce pro-inflammatory cytokine production (Orth et al., 2000, Mittal et al., 2006, Ruckdeschel et al., 1998, Boland and Cornelis, 1998). YopM is a highly acidic protein, which is translocated into macrophages (Boland et al., 1996), but may also be able to self-deliver into human RSAF cells (Bertrand et al., 2010). Inside eukaryotic cells, YopM interacts with, and stimulates, two cellular kinases, RSK1 and PRK2, assembling into a trimeric complex (McDonald et al., 2003, Hentschke et al., 2010). YopM is also localised to the nucleus by means of a vesicle-associated pathway in yeast and mammalian cells (Skrzypek et al., 1998, Skrzypek et al., 2003, Benabdillah et al., 2004), and once inside the nucleus, YopM may influence the expression of a range of genes, down-regulating many pro-inflammatory cytokines (Bertrand et al., 2010, Ye et al., 2009) and may

counteract the innate immune system by causing depletion of natural killer cells in the liver, spleen and blood (Kerschen et al., 2004). YopK is 21 kDa effector found in all three pathogenic *Yersinia*, but has no known homologue in any other bacterial T3SS. Little is known about the function of YopK, although it is known that *yopK* mutants produce enlarged translocation pores and over-translocate Yops- however these mutants are still avirulent in mice and are rapidly cleared from Peyer's patches (Holmstrom et al., 1995, Holmström et al., 1997). It has been proposed that YopK interacts with the translocon to inhibit immune system recognition, which prevents activation of the inflammasome- a complex of the innate immune system which promotes production of inflammatory cytokines (Brodsky et al., 2010).

1.2.5 Regulation of the Yop-Ysc T3SS

The synthesis and utilisation of the Yop-Ysc system is regulated at multiple levels. Firstly, the system is activated by temperature-dependent mechanisms, and during growth at 37°C the injectisome is constructed at the cell surface, the needle is produced and the LcrV tip complex is installed. The translocon components YopB and YopD are also synthesised, and may be secreted constitutively in low amounts along with LcrV (Lee et al., 2000, Lee et al., 2001). However, full activation of Yop expression and secretion through the injectisome requires the LCR, during which effector proteins are maximally expressed and are secreted into the extracellular environment (Michiels et al., 1990, Zahorchak et al., 1979). However, while many mutants which are calcium 'blind' (i.e. secrete Yop proteins during growth at 37°C in the presence of calcium) show a growth restriction in the presence or absence of calcium at 37°C (Ramamurthi and Schneewind, 2002), the bacteriostasis that accompanies the LCR and Yop secretion can be uncoupled from the production of Yop effectors, since a *Y.*

enterocolitica mutant in *virG*, encoded immediately upstream of the positive regulator *virF*, grows normally under LCR conditions while still secreting Yops into the culture supernatant (Allaoui et al., 1995a). Additionally, single-cell studies have shown that the expression of *yopE* in *Y. pseudotuberculosis* is not related to the cell-cycle (Wiley et al., 2007).

It is interesting to note that the structure of the injectisome may also auto-regulate the T3SS. In *Y. pestis*, *yscC*, *yscD* and *yscG* mutants can only express *lcrV* and *yopM* at basal levels, regardless of calcium (Plano and Straley, 1995), in *Y. pseudotuberculosis* a *yscJ* mutant is calcium-blind for Yop production at 37°C (Holmstrom et al., 1995), and Yop effectors are auto-regulated by negative feedback, whereby the intracellular accumulation of Yops when the injectisome is closed leads to their down-regulation (Allaoui et al., 1995b, Plano and Straley, 1993, Plano and Straley, 1995). This has been suggested to be analogous to FlgM/FliA in controlling flagella biosynthesis (Cornelis and Wolf-Watz, 1997a), and has been shown to involve the Cpx two-component system and an extracytoplasmic function sigma factor (Carlsson et al., 2007a). Other regulatory proteins including YopN, LcrQ, SycH and LcrG are also involved in down-regulating Yop production (Cheng et al., 2001, Wulff-Strobel et al., 2002, Forsberg et al., 1991, Skryzpek and Straley, 1993).

1.2.5.1 The genetic control of the Yop-Ysc T3SS

The major regulator of injectisome assembly and for the synthesis of Yop effectors is the pYV encoded regulator VirF (also called LcrF), which is often used as a diagnostic marker for virulent *yersiniae* (Lambertz et al., 1996). VirF activates expression of the *yopH, yopE, lcrGVH-yopBD* and *virC* operons, and also promotes expression of other virulence factors including pYV encoded adhesin *yadA,* the lipoprotein *ylpA* (China et al., 1990) and the chromosomally

encoded catalase *katY* (Garcia et al., 1999). Like the regulators controlling diverse bacterial T3SS, VirF is an AraC-type regulator which, in the absence of the binding of particular ligand (i.e. arabinose in the case of AraC from *E. coli*), binds to DNA and causes looping between distant sites which prevents gene expression (Egan, 2002, Plano, 2004). However, no endogenous ligand has yet been identified for VirF. VirF probably functions by binding to inverted repeats of the consensus motif TTTaGYcTtTat, although low-affinity binding is also observed in the presence of only one copy of the motif (Wattiau and Cornelis, 1994).

The production of VirF is regulated by temperature and is maximal at 37°C. In *Y. enterocolitica,* this is thought to involve DNA topology changes, whereby hairpin bends in the DNA of the promoter region melt at 37°C and allow transcription to occur (Rohde et al., 1994, Rohde et al., 1999). In *Y. pestis*, thermoregulation of *virF* may be post-transcriptional, since *virF* is expressed at both 26°C and at 37°C, however a stem-loop structure sequesters the Shine-Delgano sequence of the *virF* mRNA at low temperatures, which is relaxed as the temperature increases (Hoe and Goguen, 1993). Factors in addition to DNA topology also influence the production of the T3SS. The histone like protein YmoA also negatively regulates *virF* (Cornelis et al., 1991, de Rouvroit et al., 1992), while the regulator RovA may also repress several T3SS genes through an unknown mechanism (Yang et al., 2010). The cAMP receptor protein (CRP) specifically represses the *sycO-ypkA-yopJ* operon (Zhan et al., 2009), whereas the RNA chaperone Hfq activates the synthesis and secretion of Yop effector proteins (Schiano et al., 2010), as does the CpxA / CpxR two-component system via the activity of an extracytoplasmic function sigma-factor (Carlsson et al., 2007a). Finally, the O-antigen status of the cell also affect the production of the T3SS (Bleves et al., 2002, Perez-Gutierrez et al., 2007) as does Dam dependent DNA methylation (Julio et al., 2002, Falker et al., 2006).

AHL-dependent QS has a role in regulating the T3SS in several bacteria, including *Y. pestis*, where antibody based protein profiling suggested several virulence factors, including LcrV, to be positively regulated by QS (Chen et al., 2006). The plant pathogens *Pectobacterium atrosepticum* and *Ralstonia solanacearum* also use AHL-mediated QS to activate expression of the T3SS (Liu et al., 2008). Conversely, in *P. aeruginosa*, the RhlI/R QS system negatively regulates the T3SS (Bleves et al., 2005), whereas in *Vibrio harveyi* and *Vibrio parahaemolyticus*, both AHL-mediated and AI-2 dependent QS modulate the expression of the T3SS (Henke and Bassler, 2004).

1.2.6 Other *Yersinia* virulence factors

1.2.6.1 The Pgm locus

In addition to the pYV plasmid, the pathogenic *yersiniae* contain several pathogenicity islands which contribute to their virulence. Among these is the Pgm locus, which contains the *hms* genes, originally identified as involved in the adsorption of haemin or haem-analogues such as Congo red to the cell surface (giving a pigmented phenotype to colonies growing on agar supplemented with haemin or Congo red, or Pgm^+) (Perry et al., 1990), but now considered to encode genes responsible for the production of a *β*-1,6-*N*-acetylglucosamine-like biofilm matrix exopolysaccharide (Bobrov et al., 2008). However while this locus is important for the infectivity of both *Y. pseudotuberculosis* and *Y. pestis* in insects and nematodes (Hinnebusch et al., 1996, Darby et al., 2002), it is missing in the high-pathogenicity *Y. enterocolitica* 1B group (Fetherston et al., 1992).

Genetically linked to the *hms* genes is another chromosomal island only present in pathogenic *Yersinia*- the high pathogenicity island (HPI), encoding the yersiniabactin (yersiniaphore) iron acquisition siderophore system (Carniel et al., 1996, Bearden et al., 1997) which plays a key role in the systemic spread of *yersiniae* during infection and reduces the oxidative stress response of macrophages (Paauw et al., 2009, Bearden et al., 1997). Together, the *hms* genes and the HPI island form the Pgm locus. The Pgm locus is thought to have been acquired by the pathogenic *yersiniae* by horizontal gene transfer (Fetherston et al., 1992).

1.2.6.2 The adhesins- Inv, Ail, pH 6 antigen and YadA

The genomes of the pathogenic *Yersinia* encode several virulence factors which act as adhesins and invasins. These factors include chromosomally encoded Invasin (inv), the attachment and invasion locus protein (Ail) and the pH 6 antigen (Psa), and virulence plasmid encoded adhesins such as the auto-transporter YadA (El Tahir and Skurnik, 2001). There is a great degree of seeming redundancy in the number of putative adhesins encoded by the *Yersinia*, highlighted by the fact that a frameshift mutation in the *Y. pestis yadA* coding region renders YadA non-functional, and that in some strains of *Y. pestis inv* is interrupted by the insertion of an IS*200*-like element (Simonet et al., 1996). In their places, *Y. pestis* may use other adhesins including the auto-transporter protein, YapC (Felek et al., 2008) and the Ail homologue OmpX (Kolodziejek et al., 2007). YapC mediates bacterial auto-aggregation and biofilm formation, OmpX provides resistance to serum-dependent killing, and both have been shown to facilitate entry into cultured HEp-2 cells (Felek et al., 2008, Kolodziejek et al., 2007). The *Y. pestis* specific plasmid pPCP1 also aids in promoting invasiveness in this species (Cowan et al., 2000). The pPCP1 plasmid encodes three genes for pesticin, the pesticin resistance gene and the Pla

plasminogen activator, and subsequently Pla was found to be the adhesin involved in inducing invasion into eukaryotic cells (Lähteenmäki et al., 2001).

Invasin (Inv)

Despite the absence of *inv* in *Y. pestis*, invasin is an essential invasion factor for *Y. pseudotuberculosis* and *Y. enterocolitica*, both *in vitro* and *in vivo* (Simonet and Falkow, 1992, Grassl et al., 2003). Invasin fulfils a role in binding to β1-integrins on eukaryotic cell surfaces (Clark et al., 1998, Leong et al.), which are enriched on the apical surface of M-cells in Peyer's patches (Schulte et al., 2000), and causes internalisation following activation of several host cell signal transduction proteins such as Rac-1 and N-WASP (McGee et al., 2001). Furthermore, invasin can confer the ability to bind to and invade tissue culture cells to a non-invasive *E. coli* strain (tissue culture invasive, TCI^+) (Miller and Falkow, 1988). Invasin probably plays a role in the initial establishment of *Y. pseudotuberculosis* and *Y. enterocolitica* infection in the mammalian gastrointestinal system, since this protein is expressed maximally at temperatures below 28°C, at early stationary phase, but also at 37°C in acidic conditions (Pepe et al., 1994), and at least in *Y. pseudotuberculosis*, *inv* mutants fail to translocate across M-cells and instead colonise the luminal intestinal epithelium (Marra and Isberg, 1997).

Attachment Invasion Locus protein (Ail)

Ail is a 17 kDa membrane bound adhesion which was also identified alongside Inv in conferring a TCI^+ phenotype to *E. coli* (Miller et al., 1990, Miller and Falkow, 1988). However this adhesin exhibits more discrimination in its ability to bind to cells than does Inv (Miller and Falkow, 1988), and may promote bacterial adhesion to migrating cells, facilitating dissemination to the lymph

nodes and spleen (Isberg, 1990). Ail also contributes to resistance against serum-dependent killing (Pierson and Falkow, 1993, Bartra et al., 2008, Yang et al., 1996). However, the role of Ail appears to differ slightly between the pathogenic *Yersinia*. While it enhances virulence towards mice in *Y. pestis* (Felek and Krukonis, 2009), it has not been found to have this effect in *Y. enterocolitica* (Wachtel and Miller, 1995), and despite that it aids *Y. enterocolitica* and *Y. pestis* attachment and invasion into eukaryotic cells, it does not seem to play this role in *Y. pseudotuberculosis*, with the pH 6 antigen apparently replacing this function (Yang et al., 1996).

The pH 6 Antigen (Psa)

The pH 6 Antigen (Psa) was originally identified in *Y. pestis* as a surface antigen which is expressed at mammalian body temperatures and at pH values similar to that in phagolysosomes, and is important for infections in mice (Benefraim et al., 1961). Further investigation revealed a cell surface complex composed of aggregates of a 15 kDa protein (PsaA) that requires the regulators PsaE and PsaF for maximal induction (Lindler et al., 1990, Yang and Isberg, 1997). The Psa is a flexible fimbrial structure, with accessory proteins (PsaB and PsaC) similar to *E. coli* Pap, K88, K99 and CS3 fimbriae, which is highly expressed during infection in macrophages (Lindler and Tall, 1993). Biochemical examination of the Psa reveals that it binds to β1-linked galactosyl residues in glycosphingolipids (Payne et al., 1998), mainly of the type found in apolipoprotein B containing lipoproteins in human plasma, such as LDL and lipid rafts in macrophage membranes (Makoveichuk et al., 2003). Furthermore, Psa also acts as a bacterial Fc receptor, binding human immunoglobulin (IgG), but not reacting with rabbit, mouse or sheep IgG (Zav'yalov et al., 1996) and causes the agglutination of erythrocytes (Bichowsky-Slomnicki and Ben-Efraim, 1963).

YadA

YadA is an important adhesin for the enteropathogenic *yersiniae* (El Tahir and Skurnik, 2001), although it is not produced by *Y. pestis* (El Tahir and Skurnik, 2001, Simonet et al., 1996). This auto-transporter is positively controlled by the virulence regulator VirF (LcrF) (Skurnik and Toivanen, 1992), negatively controlled by YmoA (Cornelis et al., 1991), and exhibits classic *Yersinia* thermo-regulation, being maximally expressed at 37°C (Bolin et al., 1982). YadA promotes cellular aggregation, the binding to various cell types and connective proteins, such as fibronectin, and inhibits serum and complement dependent killing (El Tahir and Skurnik, 2001, Kirjavainen et al., 2008). The association between YadA and fibronectin also promotes internalisation of bacteria into eukaryotic cells by binding to β1-integrins exposed on the cell surface (Bliska et al., 1993, Heise and Dersch, 2006), while binding to integrins also facilitates the docking of the T3SS injectisome and Yop translocation into eukaryotic cells (Mejía et al., 2008).

1.2.7 QS in *Yersinia*

Like many other Gram negative bacteria, *Y. pseudotuberculosis*, *Y. pestis* and *Y. enterocolitica* all utilise AHL-mediated QS. *Y. pseudotuberculosis* and *Y. pestis* share a homologous QS network, consisting of two interlinked synthase / receptor systems termed YpsI/R and YtbI/R in *Y. pseudotuberculosis*, and YpeI/R and YspI/R in *Y. pestis*, which are encoded as convergent and overlapping pairs (Atkinson et al., 1999, Atkinson et al., 2008). *Y. enterocolitica*, however, produces a single synthase termed YenI, which shares homology with YtbI. The synthase *yenI* is encoded with the receptor *yenR* although an additional receptor, termed *ycoR*, is encoded elsewhere on the chromosome (Ng, R.,

personal communication). The YpsI/R and YtbI/R QS systems in *Y. pseudotuberculosis* are hierarchically related, where YpsI/R positively controls *ytbI/R* expression. However, while YtbR positively regulates *ytbI*, the YtbI/R system down-regulates the *ypsIR* (Atkinson et al., 2008).

The *Yersinia* synthase enzymes produce a range of AHLs. At least 24 AHLs are produced by *Y. pseudotuberculosis*, predominantly C_6-, 3-oxo-C_6-, 3-oxo-C_7-, C_8- 3-oxo-C_8-, 3-oxo-C_{10}-, 3-oxo-C_{12}-, and 3-oxo-C_{14}- HSL (Atkinson et al., 1999, Yates et al., 2002, Ortori et al., 2007). All these AHLs can be produced by YtbI, which is solely responsible for the production of the long-chain AHLs (Ortori et al., 2007), however 3-oxo-C_6-HSL, is mainly produced by YpsI (Atkinson et al., 1999, Ortori et al., 2007). *Y. pestis* synthesises similar AHLs, with 3-oxo-C_6-HSL, 3-oxo-C_8-HSL as the major signals (Kirwan et al., 2006), while 18 AHLs are produced by YenI (Ng, R., personal communication), predominantly C_6-HSL, 3-oxo-C_6-HSL (Throup et al., 1995), and 3-oxo-C_{10}-HSL, 3-oxo-C_{12}-HSL and 3-oxo-C_{14}-HSL (Atkinson et al., 2006). Environmental factors such as temperature play a large role in determining the extracellular AHL profile produced by *Y. pseudotuberculosis,* with differences in profile during growth at 22°C, 28°C or 37°C, with increased levels of long-chain AHLs at 37°C. At 37°C, AHL production may rely on YtbI, since the *ypsI* mutant produces the same range of AHLs as the wild-type at this temperature while at 28°C YpsI provides 3-oxo-C_6-HSL. Oddly however, this importance of YpsI was not found during growth at 22°C (Atkinson et al., 1999, Ortori et al., 2007).

QS influences several behaviours in the *Yersinia*, most notably motility, which is affected by QS in *Y. pseudotuberculosis* and *Y. enterocolitica* (Atkinson et al., 1999, Atkinson et al., 2006). However, the processes underlying the effect of QS on motility appear to be different, since the expression of the flagella regulators *flhDC* and *fliA* are affected by QS in *Y. pseudotuberculosis* (Atkinson

et al., 2008), whilst they are not in *Y. enterocolitica* (Atkinson et al., 2006). Instead, in *Y. enterocolitica*, QS appears to influence the expression of the flagella structural component *fleB* (Atkinson et al., 2006). In *Y. pseudotuberculosis*, QS also influences the growth of bacteria in clumps during growth at 30°C or 37°C, where a *ypsR* mutant (but not a *ypsI* mutant) forms dense bacterial aggregates in liquid culture (Atkinson et al., 1999). QS may also impact on other virulence factors. In *Y. pestis,* high, non-physiological, concentrations of exogenous C_8-HSL or oxo-C_8-HSL results in the down-regulation of LcrV and several other proteins suggesting QS negatively regulates the T3SS (Gelhaus et al., 2009), while growth phase has, along with temperature, been shown to regulate several other virulence factors including the pH6 antigen in *Y. enterocolitica* (Iriarte et al., 1995) and type 6 secretion in *Y. pestis* (Pieper et al., 2009).

1.3 Biofilms- on surfaces and in liquid cultures

Bacterial biofilms are ubiquitous in the microbial world, occurring in almost every studied environment, from deep-sea hydrothermal vents to the human body during infection (Guezennec et al., 1998, Hall-Stoodley et al., 2004, Costerton et al., 1995, Costerton et al., 1999, Schaber et al., 2007, Costerton et al., 1987, Beveridge et al., 1997). Biofilms are populations of bacteria adherent to each other and to surfaces, growing in an extracellular matrix (ECM) composed of a variety of polymers including lipids, proteins, polysaccharides and DNA (Flemming et al., 2007, Sutherland, 2001, Whitchurch et al., 2002). This mode of bacterial growth is considered to be the principle survival mechanism for bacteria in the environment (Costerton et al., 1995). Many bacterial species also engage in a multi-cellular behaviour variously known as clumping, fluffing,

flocculation, or auto-aggregation, in which bacterial cells adhere tightly to one another to create dense aggregates in a way that is often recognised as a type of liquid culture biofilm (LCB) (Håvarstein et al., 2006, Allesen-Holm et al., 2006, Seidl et al., 2008, Godefroid et al., 2010). These types of aggregates are recognised in industrial applications such as sludge bed reactors, or the marine environment, where they are termed 'sludge', 'flocs' (Bura et al., 1998) or 'marine-snow' (Costerton et al., 1995), however a wide variety of bacterial species from across the genera have been shown to form LCB-like biofilms naturally or *in vitro*, including *Myxococcus xanthus* (Arnold and Shimkets, 1988), *Klebsiella oxytoca* (Zhou et al., 2001) *Sphingomonas* sp., *Acinetobacter* sp. (Singh and Vincent, 1987), some strains of *Lactococcus lactis* (Godon et al., 1994), *Xylella fastidiosa* (Bi et al., 2007), pathogens such as *Brucella melitensis* (Uzureau et al., 2007), *Erwinia chrysanthemi* (Hussain et al., 2008), *Vibrio* sp. (Seki, 1971), enteroaggregative *E. coli* (Albert et al., 1993), *Salmonella* spp. (Römling and Rohde, 1999), *Yersinia pestis* (El Tahir and Skurnik, 2001), *Streptococcus* spp. (Dunny et al., 1978, Handley et al., 1984) and the pneumococci (Tomasz and Zanati, 1971).

1.3.1 Architecture of biofilms

The biofilm matrix is built from a secreted assortment of polysaccharides, proteins, lipids, and nucleic acids known collectively as the ECM (Flemming et al., 2007, Sutherland, 2001, Whitchurch et al., 2002), which help generate a framework around which a structured microbial community can grow. Water probably contributes most significantly to the biofilm matrix, and provides a means to regulate viscosity of the matrix depending on dissolved solutes and may also transport nutrients and waste into and out of the biofilm through structural channels, or voids (Costerton et al., 1995, Stewart, 2003).

For many bacteria, exopolysaccharides (EPS) play a critical role in the matrix of biofilms, providing anchorage between cell and surface, contributing to the viscoelastic properties of the ECM (Mayer et al., 1999) and acting as a rigid structural element of the matrix (Mayer et al., 1999, Sutherland, 2001, Costerton et al., 1995). EPS secreted by bacteria can differ in their composition and chemical properties, though most are polyanionic (Sutherland, 2001). Several bacteria are known to produce β-1,6-linked *N*-acetyl glucosamine (PNAG) including *Staphylococcus epidermidis*, *P. fluorescens*, *Y. pseudotuberculosis*, *Y. pestis*, and *E. coli*, and biofilms from these species can be disrupted by the degradation of this polysaccharide (Itoh et al., 2005, Wang et al., 2004, Tan and Darby, 2004).

In addition to EPS, many bacteria form surface attached biofilms and LCBs that contains extracellular DNA (eDNA), which can function as a cell-to-cell interconnecting scaffold (Whitchurch et al., 2002, Watanabe et al., 1998). In many cases, eDNA originates from the lysis of a subpopulation of cells in the biofilm (Allesen-Holm et al., 2006, Berne et al., 2010, Spoering and Gilmore, 2006), although eDNA can occur in very high concentrations, raising doubt over the source (Flemming et al., 2007). Grids or networks of eDNA have been observed in the biofilm matrix (Allesen-Holm et al., 2006, Böckelmann et al., 2006), and eDNA contributes to the stability and structure of biofilms, since in many cases addition of a DNase to the biofilms reduces biomass and integrity (Whitchurch et al., 2002, Petersen et al., 2004, Tetz et al., 2009, Nijland et al., 2010). Interestingly, bacteria may move along the eDNA grid, suggesting that the network may be used for mobility within the biofilm (Flemming et al., 2007).

1.3.2 Function of biofilms

The properties of the biofilm mode of growth confer enhanced resistance in embedded cells to a diverse range of environmental insults including antibiotic pressure, changes in temperature, UV irradiation and periods of desiccation (Stewart, 1996, Stewart and William Costerton, 2001, Decho, 2000). By providing anchorage to surfaces, biofilms also provide bacteria with access to nutrients which may be dilute in the bulk-phase but concentrate on surfaces by hydrophobic or electrostatic interactions (Beveridge et al., 1997), and can also act as a reservoir of infection (Hall-Stoodley and Stoodley, 2005). It has also been proposed that biofilms may stabilise nutrient supplies by concentrating and storing substrates which are used for growth in periods of starvation (Freeman and Lock, 1995), and some EPS may serve as a nutrient store (Flemming et al., 2007). In addition, many natural complex sources of energy require a consortia of bacteria to release nutrients (Nielsen et al., 2000), and biofilms provide the ability for such structured communities to develop.

An important function of biofilms in the environment is that they aid bacterial survival against predation by bacteriovorous protozoans and metazoans such as amoeba, flagellates and nematodes (Matz and Kjelleberg, 2005, Pickup et al., 2007, Drace and Darby, 2008). Biofilms are considered too large to be consumed by some protozoan predators (Matz and Kjelleberg, 2005), although others such as *Acanthamoeba castellanii* and *Colpoda maupasi* specialise to feed on biofilms (Huws et al., 2005, Weitere et al., 2005). Larger predators such as the nematode *Caenorhabditis elegans* can also feed on bacteria, and biofilms formed by bacteria such as *Y. pestis*. *Y. pseudotuberculosis* and *Xenorhabdis nematophila* block the mouthparts of the worm and prevent feeding (Darby et al., 2002, Drace and Darby, 2008, Atkinson et al., 2011), whereas other bacteria

such as *Staphylococcus epidermidis* use biofilm exopolysaccharides to protect against the *C. elegans* innate immune system and kill the worm following a biofilm-related infection of the intestines (Begun et al., 2007).

LCBs may also represent a discrete yet important biofilm in mammalian infections, since many areas of the mammalian body where bacteria grow can be thought of as liquid cultures. For example in hypertrophied adenoids, floc biofilms are more predominant than surface attached biofilms, forming in areas of stagnation with low mucus flow (Winther et al., 2009). LCBs may influence initial infection, and in *V. cholerae,* the adoption of a LCB-phenotype massively increases infectivity, between 12 – 145 fold, compared with planktonic cells (Faruque et al., 2006). Some species of bacteria, including *Staphylococcus aureus* and *Enterococcus faecalis,* use LCBs biofilms to prevent uptake by, or inhibit the function of macrophages (Goldstein et al., 1978, Sumuth et al., 2000), while both *Mycobacterium* spp. and *Yersinia* spp. can grow as aggregates inside macrophage phagosomes (Lee et al., 2008, Lindler and Tall, 1993). This suggests that LCB formation contributes to pathogenicity towards mammals, and can be an adaptive virulence mechanism as well as being important for survival in the environment. Conversely, the aggregation of bacteria may also be utilised by the commensal microflora in the competitive exclusion of pathogens, as described for the interaction between certain *Lactobacillus* strains with uropathogenic and enteropathogenic *E. coli* (Reid et al., 1988, Spencer and Chesson, 1994, Kmet and Lucchini, 1997). Together this suggests a diverse role for LCBs in both pathogenic and mutualistic relationships.

1.3.3 Biofilm development

The established model of biofilm formation describes a multi-stage developmental series by which cells attach to a surface (Costerton et al., 1995). The biofilm, once established, grows and matures, and individual cells or clumps of biofilm may eventually disperse and propagate new communities. The initial attachment stage involves the initial contact between a bacterial cell and a surface, and in many species this requires motility in the form of flagella mediated swimming (Pratt and Kolter, 1998, Watnick et al., 2001, Klausen et al., 2003b). Swimming is not however a precondition for biofilms *per se*, since many non-motile species such as *Yersinia pestis* (Jarrett et al., 2004) and *Staphylococcus aureus* (Hall-Stoodley et al., 2004), also form biofilms, and some non-motile derivatives of other species such as *P. aeruginosa* can be hyper-adherent (Deziel et al., 2001).

Once cells have attached, growth of the biofilm can occur by at least 3 mechanisms (Stoodley et al., 2002). Cells attached to a surface will divide, and this division will increase the size of the biofilm if these cells remain attached to one another. Species such as *Pseudomonas putida* develop biofilms in this manner (Heydorn et al., 2000). Secondly, cells which have attached to a surface in one location may also translocate to new areas. In *P. aeruginosa*, both flagella-driven 'swimming' and type-IV pili mediated 'twitching' motility are critical for adherence to abiotic surfaces and microcolony formation respectively (O'Toole and Kolter, 1998). The third mechanism involves the recruitment of cells from the environment or bulk phase to a developing biofilm, and it has been proposed that *Yersinia* biofilms grow on *C. elegans* in this way (Tan and Darby, 2004).

A growing biofilm subsequently enters into a developmental process of maturation. This is identified by the development of species-characteristic multi-cellular structures, such as the mushroom-like structures observed in *P. aeruginosa* biofilms (Klausen et al., 2003a) and filamentous-chain and aggregate clusters in *Serratia* (Rice et al., 2005). In some species, a division of labour in bacteria within the biofilm allows the development of cell types such as persister cells, which exhibit suppressed metabolism and growth rate yet are particularly resistant to antimicrobials (Keren et al., 2004), wall-formers which provide a physical support to the biofilm community (Klausen et al., 2006), and hyper-motile populations adept at dispersal (Costerton et al., 1995). Built into the developmental structure of biofilms are discrete spaces free of bacteria, which have been described as 'pores' or 'water-channels' (Beer et al., 1994). These water-channels develop in many cases in both mono- and poly- species biofilms (Beer et al., 1994, Stoodley et al., 2002), and appear to facilitate the movement oxygen and nutrients throughout the biofilm (Sternberg et al., 1999). Studies have shown that within biofilms, oxygen and nutrient gradients fall sharply in dense areas, leading to the shut-down of metabolism in deeply entrenched cells (Stewart, 2003). However, the addition of fresh nutrients can stimulate the metabolism of these deep biofilm cells and water channels have been shown to aid the supply of oxygen and nutrients throughout the biofilm (Sternberg et al., 1999). Biofilm maturation is heavily influenced by environmental parameters such as nutrient availability, and fluid-sheer forces, however in some species such as *P. aeruginosa* maturation is also a discrete molecular process governed by specific genetic loci which often have no identifiable role in other stages of biofilm formation, such as motility, attachment or biofilm growth (Kuchma et al., 2005).

Bacteria exit the biofilm mode of growth via mechanisms of dispersal; either when the particular conditions that is favourable to the biofilm pass, or by the

shedding of cells from a mature biofilm for the colonisation of new areas (Costerton et al., 1995). Dispersed biofilm bacteria may also retain higher infectivity than their planktonic counterparts, as has been shown for *V. cholerae* (Faruque et al., 2006), making them especially important in human pathogenesis (Hall-Stoodley and Stoodley, 2005).

1.3.4 *Yersinia* biofilms

The ability for *Y. pestis* to form biofilms within the flea proventriculus during transmission of plague from the flea vector to a mammalian host is well known (Jarrett et al., 2004). *Y. pseudotuberculosis* does not form biofilms in fleas, but is capable of causing acute oral infections and can also adhere to the flea midgut (Erickson et al., 2007). Both *Y. pestis* and *Y. pseudotuberculosis* form *hmsHRFS*-dependent biofilms on the cuticle of the nematode worm *C. elegans* (Darby et al., 2005, Joshua et al., 2003), which involves the EPS β-1,6-*N*-acetyl glucosamine (Drace and Darby, 2008, Joshua et al., 2003, Jarrett et al., 2004). The *hmsT* locus, which is not genetically linked to the *hmsHRFS* genes, is also required for biofilm formation (Kirillina et al., 2004, Jones et al., 1999). HmsT is a member of the diguanylate cyclase family, and produces the secondary messenger cyclic-di-GMP (Cotter and Stibitz, 2007). High cyclic-di-GMP levels are correlated with increased biofilm formation in several different bacteria (Kirillina et al., 2004, Cotter and Stibitz, 2007, Bobrov et al., 2005, Simm et al., 2005). The action of HmsT is opposed by the phosphodiesterase, HmsP, which inactivates cyclic-di-GMP, and *hmsP* mutants form biofilms with increased biomass (Kirillina et al., 2004, Bobrov et al., 2005).

In *Y. enterocolitica*, mutation of the flagella structural genes also reduces the growth of biofilms on abiotic surfaces (Kim et al., 2008). Motility itself, rather

than just the presence of flagella was shown to be critical, since a *motA* mutant (defective for the ion channel responsible for coupling the proton motive force to torque generation in the flagellum) also forms poor biofilms (Kim et al., 2008). However for *Y. pseudotuberculosis*, while elements of the flagella regulon including the master regulator *flhDC*, the flagella sigma-factor *fliA*, and the structural component *flhA* are required for both swimming and biofilm formation, motility *per se* is not required since a non-motile *fliC* mutant is capable of forming biofilms on *C. elegans* which are as severe as those formed by the wild-type (Atkinson et al., 2011).

1.3.4 QS in biofilms

AHLs have been identified in biofilms growing on surfaces in many environmental and clinical settings (McLean et al., 1997, Gram et al., 2002, Taylor et al., 2004, Bachofen and Schenk, 1998, Stickler et al., 1998, Huang et al., 2007), revealing that bacteria use QS in this mode of growth. AHLs have been found in sputum of cystic fibrosis patients infected with *P. aeruginosa* (Erickson et al., 2002, Middleton et al., 2002a) where they are produced *de novo* (Singh et al., 2000). *Y. pseudotuberculosis* biofilms growing on *C. elegans* have also been shown to concentrate AHLs (Atkinson et al., 2011). Since AHLs can be extracted from biofilms, it is not surprising to find QS genes actively expressed in the biofilm mode of growth. Transcripts for *lasI* and *lasR* have been isolated from *P. aeruginosa* biofilms (Middleton et al., 2002a), and expression of *lasI* and *rlhI* has been investigated using a *gfp* reporter, revealing that expression is maximal in cells at the substratum, and decreases with biofilm height (De Kievit et al., 2001b). The authors of the study also found that *lasI* expression reduces over time, but *rhlI* expression remains constant (De Kievit et al., 2001a). Comparatively, another study using a *lacZ* reporter found the *las* system to be

more active during the early stages of biofilm attachment, while the *rhl* system appears to be more active during the maturation stage (Sauer et al., 2002).

It is largely acknowledged that QS plays a central role in regulating biofilm development, even though other sensory inputs such as nutrient cues modulate the role for QS in biofilms (De Kievit et al., 2001a). In many species, such as *P. aeruginosa, Burkholderia cepacia* and *Serratia* spp., QS appears to influence the maturation stage of biofilm development rather than initial attachment, where QS mutants form flat, undifferentiated biofilms (Davies et al., 1998, Huber et al., 2001, Labbate et al., 2004, Rice et al., 2005). Differentiation and microcolony formation is also prevented in an *Aeromonas hydrophila*, *ahyI* mutant, however the *ahyR* mutant does not share this phenotype, and indeed biofilms formed by the *ahyR* mutant were shown to exhibit increased surface coverage (Lynch et al., 2002). Bacteria can also negatively regulate biofilm formation using AHL-dependent QS. For example, *Pseudomonas aureofaciens* and *Pantoea stewartii* use the CsaR/I and EsaR/I systems respectively to repress the synthesis of biofilm matrix components (Zhang and Pierson, 2001, von Bodman et al., 1998).

Several bacterial species also use QS to regulate LCBs, including *Y. pseudotuberculosis* (Atkinson et al., 1999). The marine bacterium *Rhodobacter sphaeroides* also forms such aggregates following deletion of *cerI*, and in addition overproduces an exopolysaccharide (Puskas et al., 1997). In *Brucella melitensis, a vjbR mutant* grows as an LCB encased in a matrix of exopolysaccharides and eDNA (Uzureau et al., 2007), which is also observed following over-expression of an AHL degrading acylase (AiiD) (Godefroid et al., 2010). QS can also enhance LCB formation. *Erwinia chrysanthemi* grows in aggregates which can be prevented by mutation of *expI* but recovered by the addition of AHLs (Hussain et al., 2008). Additionally, in late-log phase cultures of *P. aeruginosa*, substantial cell clumps develop in a way that is not observed in

the *lasI* and *rhlI* synthase mutant (Allesen-Holm et al., 2006). Together this indicates a species specific role for QS in regulating the formation of LCBs.

QS regulates a number of genes involved in the biofilm mode of growth. Probably the best example of this comes from *P. aeruginosa*, where the RhlR/I system regulates the production of rhamnolipid biosurfactants (Ochsner and Reiser, 1995, Brint and Ohman, 1995), and *S. liquifaciens*, where the SwrR/I system regulates a lipopeptide biosurfactant (Lindum et al., 1998). Biosurfactants affect biofilm architecture (Davey et al., 2003) by influencing swarming motility (Lindum et al., 1998, Caiazza et al., 2005) and mediating detachment and dispersal of cells (Boles et al., 2005). Rhamnolipids appear to be spatially and temporally localised in *P. aeruginosa* biofilms, and are synthesised primarily in the stalks rather than in the caps of the characteristic mushroom-like structures (Lequette and Greenberg, 2005). In *S. liquifaciens*, QS also regulates the genes *bsmA* and *bsmB*, which are involved in the development of the highly differentiated, filamentous biofilms characteristic of this species (Labbate et al., 2004). However, these genes are also involved in biofilm formation independent of QS, when the surface colonised is biotic rather than abiotic (Labbate et al., 2007).

1.4 Flagella mediated motility

1.4.1 The structure and function of the flagellum

Many bacterial species move by employing a sophisticated nanomachine termed the flagellum, which is composed of approximately 25 proteins which, by using a proton motive force, drives the rotation of a rigid helical filament to generate thrust (Macnab, 2003). The structure of the flagellum can be subdivided into several parts, including the basal body; the motor; the export apparatus; the hook and filament (figure 1.4). The flagella is a self-assembling structure which, for the most part, arranges sequentially with proximal structures being incorporated prior to distal ones (Macnab, 2003, Macnab, 2004).

The production of flagella can be subdivided into four stages (Macnab, 2004). In the first stage the motor proteins MotA and MotB are inserted into the cytoplasmic membrane along with a basal body component, the MS ring, composed of approximately 26 copies of FliF, which is inserted into the cytoplasmic membrane (Macnab, 2004, Jones et al., 1990), and the rotor/C-ring structure, composed of FliG/FliM and FliN, which is attached to the MS-ring (Macnab, 2003). These components are exported to the membrane via conventional Sec-dependent secretion along with the secretion apparatus components FliO, FliP, FliQ, FliR, FlhA and FlhB, which occupy the centre of the MS ring (Macnab, 2003). The secretion apparatus probably assembles co-ordinately with the MS-ring, since the export component FlhA has been shown to interact with FliF (Kihara et al., 2001). This structure functions in subsequent stages of flagellum production as a type 3 secretion system. Approximately six

proteins are thought to be essential to the T3S apparatus- FliI, FliP, FliQ, FliR, FlhA and FlhB (Macnab, 2004).

The second stage of flagella synthesis involves the T3S-dependent export of the proximal rod (Macnab, 2004), a complex of several proteins, connected to the MS ring by the junction and export gate protein FliE, which spans the periplasm (Macnab, 2003) and acts as a driveshaft which is connected to the cytoplasmic rotor (Chevance and Hughes, 2008). FliE is thought to bridge the annular symmetry of the MS ring with the helical symmetry of the axial structure, and is required for secretion of the rod, while the rod is terminated by a complex consisting of approximately 26 copies of the distal rod protein FlgG (Macnab, 2003).

The third stage of flagellum production involves Sec-dependent export of the remaining elements of the basal body- the periplasmic P-ring, consisting of 24 copies of FlgI; and the outer-membrane ring, termed the L-ring, which is formed by approximately 28 copies of the lipoprotein FlhH (Macnab, 2003). The fourth stage involves the T3S-dependent export of the remaining distal elements of the flagellum including the hook, associated junction elements, the filament and cap proteins. The flagellum filament is a helical polymer of around 20,000 copies of the flagellin monomer, FliC (Chevance and Hughes, 2008). For the filament to be properly polymerised at the surface, FliC interacts with a self-assembling cap composed of approximately 5 copies of FliD (Macnab, 2003, Ikeda et al., 1996).

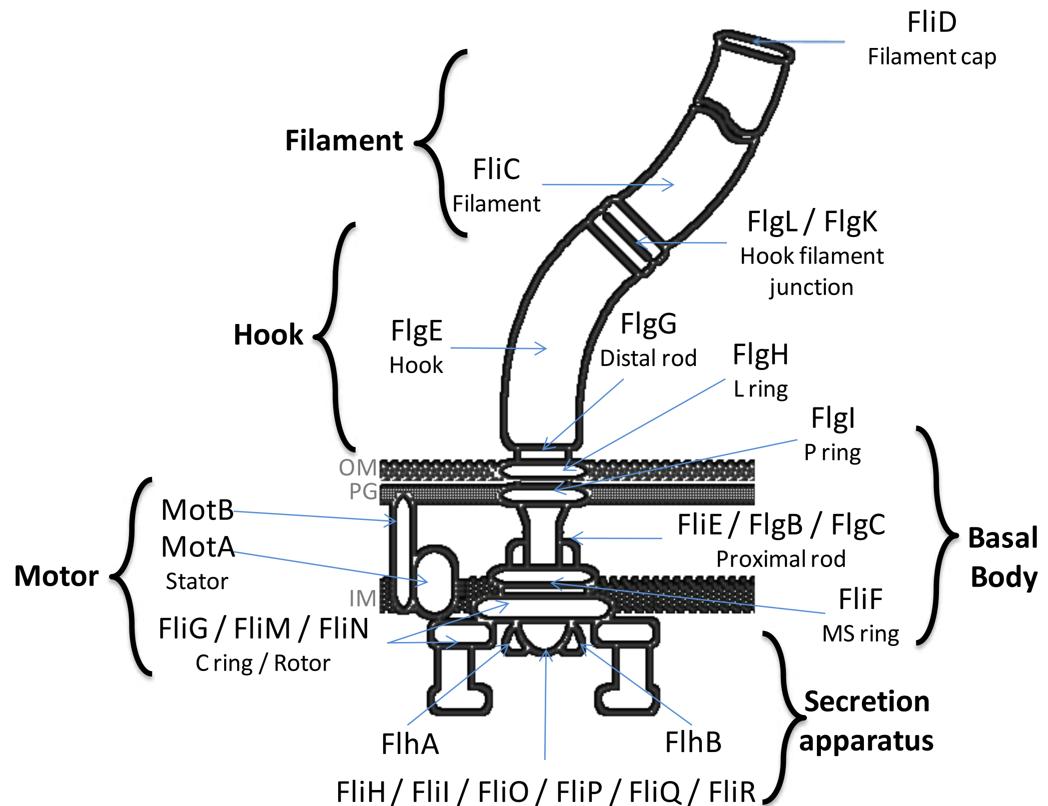


Figure 1.4| The flagella machinery of *Y. pseudotuberculosis*. The flagellum is multiprotein nanomachine that secretes and rotates a helical filament, connected to a hook and basal body, in order to drive bacterial motility. Figure modified from http://www.genome.jp/kegg/pathway/ypy/ypy02040.html.

The motor, which powers the rotation of the flagellum, can be further subdivided into two parts- the stator and a reversible rotor (Minamino et al., 2008). The stator is an integral membrane complex probably composed of four copies of MotA and two of MotB, which are arranged surrounding the basal body (Macnab, 2003). The stator is stationary in reference to the cell, as it is attached non-covalently to the peptidoglycan layer. When protons cross the membrane, they are suspected to bind to a conserved aspartate residue within the stator protein MotB. This causes a conformational change in the stator, involving a conserved proline residue in MotA, which drives rotation of the rotor (Kojima and Blair, 2001). The rotor is a complex of FliF, FliG, FliM and FliN- FliF as the MS-ring, while FliG sits in the C-ring alongside FliM and FliN, together these proteins form

a switch structure which can modify the direction in the spin of the flagellum between clockwise and counter-clockwise rotation (Minamino et al., 2008). In bacteria such as *Salmonella* and *Yersinia*, the filament has a left-handed asymmetry, and so anti-clockwise movement of the flagellum causes the helical filament to create thrust away from the cell, driving motion forwards. Clockwise movement of the filament causes polymorphic structural changes to the filament, generated by the change in torsional load, which can create semi-coiled flagella with a handedness opposite to normal. This produces chaotic fluid dynamics and causes the cell to re-orientate, or 'tumble' (Macnab and Ornston, 1977, Turner et al., 2000).

Most species of *Yersinia*, including *Y. pseudotuberculosis* and *Y. enterocolitica*, but with the exception *Y. pestis*, achieve motility by means of peritrichous arranged flagella (Bercovier and Mollaret, 1984, Bottone and Mollaret, 1977, Hurst et al., 2010, Wauters et al., 1988, Murros-Kontiainen et al., 2010, Bercovier et al., 1984). Even the most distantly related species, *Y. ruckeri* and *Y. nurmii* (Chen et al., 2010), possess peritrichous flagella (Furones et al., 1993, Murros-Kontiainen et al., 2010). It should be noted, however, that Ding *et al.* (2009) noted a single polar flagellum in *Y. pseudotuberculosis* (Ding et al., 2009), and some strains of *Y. ruckeri* have been shown to be non-motile, but still capable of causing red-mouth disease (Fouz et al., 2006).

1.4.2 QS and the genetic regulation of flagella

The genetic regulation of the typical flagella systems of enteric bacteria is organised into three hierarchical classes (McCarter, 2006). The class I gene products include FlhD and FlhC, which form heterodimer complexes constituting what is regarded as the master flagella regulator (often termed FlhDC), and can be regulated by several systems including CRP (Soutourina et al., 1999, Stella et al.), H-NS (Soutourina et al., 1999, Goyard and Bertin, 1997), CsrA (Wei et al., 2001), OmpR (Kim et al., 2003, Hu et al., 2009) and the Rcs phosphorelay system (Francez-Charlot et al., 2003). FlhDC binds to specific recognition sequences in gene promoters and activates expression of the flagella specific sigma factor *fliA* (Ohnishi et al., 1990, Helmann and Chamberlin, 1987) and Class II flagella genes encoding proteins of the flagellum basal body and hook (Liu and Matsumura, 1994). Together, FlhDC and FliA activate expression of the remaining Class III elements of the flagellum, including the flagellum filament and cap (*fliC* and *fliD*), motor proteins and chemotaxis genes (Kutsukake and Iino, 1994, Liu and Matsumura, 1995, Ide et al., 1999).

Interestingly, several bacteria also regulate the expression of *flhDC* using AHL-mediated QS, including *Burkholderia glumae* (Kim et al., 2007) and *Yersinia* spp. (Atkinson et al., 2006, Atkinson et al., 2008), while the expression of several flagella genes is affected by QS in *Sinorhizobium meliloti* (Hoang et al., 2008). In *Y. pseudotuberculosis* and other bacteria, such as *Erwinia chrysanthemi*, mutation of AHL synthase genes results in hyper-motility (Hussain et al., 2008). However, in other bacteria, such as *A. hydrophila*, QS does not appear to play a role in regulating motility (Khajanchi et al., 2009).

1.5 Aims

Atkinson *et al.* (1999) demonstrated that motility and clumping were regulated by QS in *Y. pseudotuberculosis* (Atkinson et al., 1999), and it has since been shown that QS modulates the expression of the flagella regulators *flhDC* and *fliA* (Atkinson et al., 2008). In addition to their role in controlling motility, FlhDC and FliA are also implicated in the environmental control of virulence. For example, a *Y. enterocolitica flhDC* mutant over-produces T3SS proteins under normally non-inducing conditions (Bleves et al., 2002), forms cell clumps reminiscent of a *Y. pseudotuberculosis ypsR* mutant (Bleves et al., 2002), and is attenuated for biofilm formation on abiotic surfaces (Kim et al., 2008). This suggests that, at least in *Y. enterocolitica*, the flagella regulatory cascade is embedded in the lifestyle decision making process- that is, whether to express motility, virulence traits, or to adopt multicellular, aggregative or biofilm-like behaviour under particular conditions. In light of the aggregative phenotype of the *ypsR* mutant, this thesis aims to explore the contribution of QS to other phenotypes associated with the flagella system. In doing so, this work:

- Describes cellular clumping, which is repressed by QS in *Y. pseudotuberculosis*, as a form of liquid culture biofilm (LCB), which is also repressed by the motility system.
- Correlates the formation of LCBs with the up-regulation of the T3SS in several *Y. pseudotuberculosis* QS and motility mutants.
- Reveals that the formation of biofilms by *Y. pseudotuberculosis* on the surface of *C. elegans* is promoted by QS, and is inversely correlated with the activity of the T3SS.
- Demonstrates a reciprocal feedback whereby the T3SS modulates the expression of *flhDC* and motility

- Provides evidence to suggest that T3S-associated modulation of motility requires an effect on the expression of QS.
- Links the up-regulation of the T3SS to over-expression of the virulence regulator, *virF.*

From these objectives, this thesis aims to build on an emerging picture of how QS is intricately involved in the cellular decision making process of *Y. pseudotuberculosis*, which allows this bacteria to behave adaptively in response to specific environmental signals.

Chapter 2 |

Materials and method

2.1 Growth Conditions

2.1.1 Growth media

The media used in this study, their abbreviations and reference or source are listed in Table 2.1

Name	Abbr.	Composition	Ref / source
Luria Broth	LB		(Luria and Delbruck, 1943)
Luria agar	LB agar		(Luria and Delbruck, 1943)
Luria Broth buffered with 3-(*N*-morpholino) propanesulfonic acid	LB_{MOPS}	LB supplemented with 10 mM 3-(*N*-morpholino) propanesulfonic acid	(Yates et al., 2002)
Blue/White selection plates for identification of interrupted *lacZ*	Blue/white	LB supplemented with 100 µg/ml, IPTG and 64 µg/ml X-gal	
Congo red Luria Broth	LB_{CR}	LB supplemented with Congo red 0.1 % (v/v)	This study
Semi-solid Swimming Motility agar	SwMA	(10 g/l tryptone [Oxoid], 5 g/l $NaCl_2$ [Sigma], 0.3 % (w/v) Difco Bacto agar [Difco]	(Atkinson et al., 1999)
Typtone Soy Agar	TSA		Oxoid
Congo red Tryptone Soy agar	TSA_{CR}	TSA supplemented with Congo red 0.1 % (v/v)	This study
Congo red Magnesium Oxalate Tryptone Soy agar	CR_{MOX}	TSA supplemented with 20 mM Sodium Oxalate [Sigma], 20 mM $MgCl_2$ [Sigma], 0.1 % (v /v) Congo red.	(Riley and Toma, 1989)
Worm Nutrient Growth Medium agar	Worm-NGM	NGM supplemented with 5 µg/ml Cholesterol, 1 mM $CaCl_2$, 25 mM KH_2PO_4 (pH6). Lacking $MgSO_4$.	(Epstein and Shakes, 1995)
Autoinducer bioassay medium	AB	17.5 g/l $NaCl_2$, 12.3 g/l $MgSO_4$, 2.0 g/l Casamino acids, 10 mM KPi (pH 7.0), 1 µM L-arginine, 1 % Glycerol. pH 7.5 with NaOH.	(Bassler et al., 1994)

Table 2.1| Media used in this study

Supplements added to the media, abbreviations and working concentrations: Ampicillin (Amp) 50 μg/ml, chloramphenicol (Cm) 30 μg/ml, tetracycline (Tet) 10 μg/ml, streptomycin (Sm) 30 μg/ml, kanamycin (Km) 50 μg/ml, naladixic acid (Nal) 15 μg/ml, trimethoprim (Tp) 100 μg/ml, isopropyl-1-thio-β-D-galacpyranoside (IPTG) 64 μg/ml, 5-bromo-5-chloro-3-indolyl β-D-galactoside (X-gal) 64 μg/ml, L-arabinose at 0.8 % (w/v).

2.1.2 Growth Conditions

Unless otherwise stated, all *Y. pseudotuberculosis* strains were grown at 30°C and all *E. coli* strains were grown at 37°C in LB with agitation at 200 rpm. The growth of the cultures was monitored by reading the absorbance of the culture at either OD_{600} using a Gallenkamp Visi-Spec, or at OD_{405} in a combined spectrophotometer / luminometer (Anthos Lucy I) (Winson et al., 1998) . Unless otherwise stated, bacteria were maintained as colonies on LB agar plates at 4°C and used within 3 weeks. For selection for the presence or absence of the pYV plasmid in *Y. pseudotuberculosis* strains, bacteria were grown on CR_{MOX} agar at 37°C for 48 h, whereby pYV^+ clones grow as small, blood-red colonies, while pYV^- clones grow as large, cream-white colonies. Carriage of pYV was confirmed by PCR directed against pYV encoded *yscU* using primer pairs YscUF and YscUR (Table 2.4). Swimming agar plates were made as previously described (Atkinson et al., 2006). *Y. pseudotuberculosis* cultures were grown overnight and 1 μl inoculated into the centre of the plate. These plates were incubated at the relevant temperature for 48 h or 72 h prior to being examined and photographed.

2.1.3 Bacterial strains

The bacterial strains used in this study are listed in Table 2.2.

Strain	Description	Reference / Source
Escherichia coli		
DH5-α	*E. coli* K-12 cloning strain. [F ϕ80d*lacZ*ΔM15Δ (*lacZYA-argF*) U169 *recAI endAI hsdR17* (r_{K}- m_{K}^{+}) *supE44 thiI gyrI relAI*]	(Hanahan, 1983)
S17-1 λ-*pir*	λ -*pir* lysogen of S17-1 [*thi pro hsdR*– *hsd*M+ *recA* RP4 2-Tc::Mu-Km::Tn*7*]. Permissive host capable if transferring suicide plasmids requiring the Pir protein by conjugation to recipient cells / Sm^{R}.	(Simon et al., 1983)
OP50	Uracil auxotroph nutrient source for *C. elegans* and control for uninfected worms / Tc^{R}.	(Epstein and Shakes, 1995)
S17 pDM4P$_{virF}$::*lux*	S17 containing pDM4P$_{virF}$::*lux* / Sm^{R} Cm^{R}	This study
S17 pSA278	S17 containing pSA278 (P$_{ypsI}$::*lux*) / Sm^{R}	(Atkinson et al., 2008)
S17 pSA279	S17 containing pSA279 (P$_{ypsR}$::*lux*) / Sm^{R}	(Atkinson et al., 2008)
S17 pHP276	S17 containing pHP276 (P$_{ytbI}$::*lux*) / Sm^{R}	(Atkinson et al., 2008)
S17 pHP277	S17 containing pHP277 (P$_{ytbR}$::*lux*) / Sm^{R}	(Atkinson et al., 2008)
Vibrio harveyi BB170	*V. harveyi* bioreporter for AI-2 assays	(Bassler et al., 1994)
Yersinia pseudotuberculosis		
YPIII pIB1	Parent strain of YPIII harbouring the virulence plasmid pYV. Serotype O:3 / Nal^{R}	(Rosqvist et al., 1988)
YPIII pYV^{-}	Parent strain of YPIII cured of the pYV virulence plasmid / Nal^{R}	This study
Δ*flhDC*	YPIII mutant for the Class I flagella regulator *flhDC* / Tet^{R}	(Atkinson et al., 2011)

Δ*flhDC* pGem::*flhDC*	YPIII Δ*flhDC* complemented with functional *flhDC* carried on pGem / Tet^R Amp^R	(Atkinson et al., 2011)
Δ*fliA*	YPIII mutant for the flagella sigma factor *fliA* / Km^R	(Atkinson et al., 2011)
Δ*flhA*	YPIII mutant for the flagella structural component *flhA* / Km^R	(Atkinson et al., 2011)
Δ*fliC*	YPIII mutant for the flagellin filament gene *fliC* / Tet^R	(Atkinson et al., 2011)
Δ*yscJ*	YPIII mutant for the Yop-Ysc T3SS Inner ring component *yscJ* / Tet^R	This study
Δ*yscJ* pHG*yscJ*	YPIII Δ*yscJ* complemented with functional *yscJ* carried on pHG327 / Tet^R Amp^R	This study
Δ*ypsI*	YPIII *ypsI* AHL synthase mutant / Km^R	(Atkinson et al., 1999)
Δ*ypsR*	YPIII *ypsR* AHL receptor mutant / Km^R	(Atkinson et al., 1999)
Δ*ytbI*	YPIII *ytbI* AHL synthase mutant / Cm^R	(Atkinson et al., 2008)
Δ*ytbR*	YPIII *ytbR* AHL receptor mutant / Cm^R	(Atkinson et al., 2008)
Δ*ypsR* Δ*ytbR*	YPIII *ypsR/ytbR* double mutant / Cm^R Km^R	(Atkinson et al., 2008)
Δ*ypsI* Δ*ytbI*	YPIII *ypsI/ytbI* double mutant / Cm^R Km^R	(Atkinson et al., 2008)
Δ*ypsI* Δ*ytbI* pYV^-	YPIII *ypsI/ytbI* double mutant cured of pYV / Cm^R Km^R.	This study
Δ*ypsI* Δ*ytbI* pSA291	YPIII *ypsI/ytbI* AHL synthase double mutant harbouring *ypsI* and *ytbI* on the complementation vector pSA291/ Cm^R Km^R Ap^R.	(Atkinson et al., 2008)
Δ*ypsI* Δ*ytbI* Tn*5*::*psaE*	YPIII *ypsI/ytbI* AHL synthase double mutant with a Tn5 insertion in *psaE* / Cm^R Km^R Tp^R	This study
Δ*ypsI* Δ*ytbI* Δ*yscJ*	Deletion of the *yscJ* type three secretion system inner ring component in the *ypsI/ytbI* double AHL synthase mutant background / Cm^R Km^R Tc^R	This study

Δ*ypsI* Δ*ytbI* Δ*yscJ* pHG*yscJ*	YPIII *ypsI/ytbI/yscJ* triple mutant harbouring *yscJ* on pHG::*yscJ* for complementation. Cm^R Km^R Ap^R.	This study
Promoter fusions		
YPIII pIB1 pDM4P$_{virF}$::*lux*	YPIII wild-type *virF* promoter fusion / Nal^R Cm^R	This study
Δ*ypsI* pDM4P$_{virF}$::*lux*	YPIII Δ*ypsI virF* promoter fusion / Km^R Cm^R	This study
Δ*ypsR* pDM4P$_{virF}$::*lux*	YPIII Δ*ypsR virF* promoter fusion / Km^R Cm^R	This study
Δ*ytbI* pDM4P$_{virF}$::*lux*	YPIII Δ*ytbI virF* promoter fusion / Cm^R	This study
Δ*ytbR* pDM4P$_{virF}$::*lux*	YPIII Δ*ytbR virF* promoter fusion / ^{R} Cm^R	This study
Δ*ypsI* Δ*ytbI* pDM4P$_{virF}$::*lux*	YPIII Δ*ypsI* Δ*ytbI virF* promoter fusion / Km^R Cm^R	This study
Δ*ypsI* Δ*ytbI* pDM4P$_{virF}$::*lux* pSA291	YPIII Δ*ypsI* Δ*ytbI virF* promoter fusion expressing *ypsI* and *ytbI* from pSA291 / Km^R Amp^R Cm^R	This study
Δ*ypsR* Δ*ytbR* pDM4P$_{virF}$::*lux*	YPIII Δ*ypsR* Δ*ytbR virF* promoter fusion / Km^R Cm^R	This study
Δ*flhDC* pDM4P$_{virF}$::*lux*	YPIII Δ*flhDC virF* promoter fusion / Tet^R Cm^R	This study
Δ*flhDC* pDM4P$_{virF}$::*lux* pGem::*flhDC*	YPIII Δ*flhDC virF* promoter fusion expressing *flhDC* from pGem::*flhDC* / Km^R Amp^R Cm^R	This study
Δ*fliA* pDM4P$_{virF}$::*lux*	YPIII Δ*fliA virF* promoter fusion / Km^R Cm^R	This study
Δ*flhA* pDM4P$_{virF}$::*lux*	YPIII Δ*flhA virF* promoter fusion / Km^R Cm^R	This study
Δ*fliC* pDM4P$_{virF}$::*lux*	YPIII Δ*fliC virF* promoter fusion / Tet^R Cm^R	This study
YPIII pIB1 pSA278	YPIII wild-type *ypsI* promoter fusion / Nal^R Sm^R	(Atkinson et al., 2008)
Δ*yscJ* pSA278	Δ*yscJ ypsI* promoter fusion / Tet^R Sm^R	This study

Δ*yscJ* pSA278 pHG*yscJ*	Δ*yscJ ypsI* promoter fusion expressing *yscJ* from pHG*yscJ* / Nal^R Amp^R Sm^R	This study
YPIII pIB1 pSA279	YPIII wild-type *ypsR* promoter fusion / Nal^R Sm^R	(Atkinson et al., 2008)
Δ*yscJ* pSA279	YPIII Δ*yscJ ypsR* promoter fusion / Tet^R Sm^R	This study
Δ*yscJ* pSA279 pHG*yscJ*	YPIII Δ*yscJ ypsR* promoter fusion expressing *yscJ* from pHG*yscJ* / Nal^R Amp^R Sm^R	This study
YPIII pIB1 pHP276	YPIII wild-type *ytbI* promoter fusion / Nal^R Sm^R	(Atkinson et al., 2008)
Δ*yscJ* pHP276	YPIII Δ*yscJ ytbI* promoter fusion / Tet^R Sm^R	This study
Δ*yscJ* pHP276 pHG*yscJ*	YPIII Δ*yscJ ytbI* promoter fusion expressing *yscJ* from pHG*yscJ* / Nal^R Amp^R Sm^R	This study
YPIII pIB1 pHP277	YPIII wild-type *ytbR* promoter fusion / Nal^R Sm^R	(Atkinson et al., 2008)
Δ*yscJ* pHP277	YPIII Δ*yscJ ytbR* promoter fusion / Tet^R Sm^R	This study
Δ*yscJ* pHP277 pHG*yscJ*	YPIII Δ*yscJ ytbR* promoter fusion expressing *yscJ* from pHG*yscJ* / Nal^R Amp^R Sm^R	This study
YPIII pIB1 pSA200	YPIII wild-type *flhDC* promoter fusion / Nal^R Cm^R	(Atkinson et al., 2008)
Δ*yscJ* pSA200	YPIII Δ*yscJ flhDC* promoter fusion / Tet^R Cm^R	This study
Δ*yscJ* pSA200 pHG*yscJ*	YPIII Δ*yscJ flhDC* promoter fusion expressing *yscJ* from pHG*yscJ* / Tet^R Amp^R Cm^R	This study
Δ*ypsI* Δ*ytbI* Δ*yscJ* pSA200	YPIII Δ*ypsI* Δ*ytbI* Δ*yscJ flhDC* promoter fusion / Tet^R Km^R Cm^R	This study
YPIII pIB1 pSA208	YPIII wild-type *fliA* promoter fusion / Nal^R Cm^R	(Atkinson et al., 2008)
Δ*yscJ* pSA208	YPIII Δ*yscJ fliA* promoter fusion / Tet^R Cm^R	This study
Δ*yscJ* pSA208 pHG*yscJ*	YPIII Δ*yscJ fliA* promoter fusion expressing *yscJ* from pHG*yscJ* / Tet^R Amp^R Cm^R	This study

Table 2.2| Strains used in this study

2.2 Genetic Manipulation

2.2.1 DNA

DNA was handled according to standard techniques. Plasmids were purified from bacterial cultures via MiniPrep columns (QIAGEN), and total chromosomal DNA was purified using the Blood and Tissue Kit (QIAGEN), both according to manufacturer's instructions. Unless otherwise stated, all DNA was recovered from *Y. pseudotuberculosis* grown at 30°C, or *E. coli* grown at 37°C, for 16 h with agitation at 200 rpm.

2.2.2 Plasmids used in this study

The plasmids used in this study are listed in Table 2.3

Plasmid	Description	Reference / Source
pGemT-easy	Cloning vector / Amp^R	Promega
pBluescript II KS+	Cloning vector, maintained in *E. coli* DH5-α / Amp^R	Stratagene
pHG327	Low-copy number complementation vector / Amp^R	(Stewart et al., 1986)
pBlueTet	pBluescript II KS+ encoding *tetA* gene from pBR322 / Amp^R	(Atkinson et al., 2008)
pBlueLux	pBluescript II KS+ Vector containing the *luxCDABE* operon / Amp^R	(Atkinson et al., 2008)
pGemP_{virF}	P_{virF} PCR product ligated into pGemT-easy / Amp^R	This study
pBlueP_{virF}	*Apa*I *Not*I insertion of P_{virF} from pGemP_{virF} into pBluescript II KS+ / Amp^R	This study
pBlueP_{virF}::*lux*	*Sac*I insertion of *luxCDABE* excised from pBlueLux into pBluePvirF / Amp^R	This study
pDM4	Suicide vector: *mob*RK2, *ori*R6K (*pir* requiring). *sacBR* of *Bacillus subtilis* / Cm^R	(Milton et al., 1996)
pDM4P_{virF}::*lux*	P_{virF}::*lux* construct excised by *Apa*I *Sal*I and ligated into pDM4 / Cm^R	This study
pGem::*flhDC*	Complementation vector for Δ*flhDC*, *flhDC* PCR product cloned into pGemT-easy / Amp^R	(Atkinson et al., 2011)
pHG::*yscJ*	Complementation vector for Δ*yscJ*, *yscJ* PCR product cloned into pHG327 / Amp^R	(Atkinson et al., 2011)
pSB2020	Constitutively expressing *gfp3* / Amp^R	(Qazi et al., 2001)
pAJD434	Carrying the λred recombinase / Amp^R Km^R	(Derbise et al., 2003)
pSA278	pKNG101::P*ypsI*::*lux* (*ypsI* promoter fusion) / Sm^R	(Atkinson et al., 2008)
pSA279	pKNG101::P*ypsR*::*lux* (*ypsR* promoter fusion) / Sm^R	(Atkinson et al., 2008)
pHP276	pKNG101::P*ytbI*::*lux* (*ytbI* promoter fusion) / Sm^R	(Atkinson et al., 2008)
pHP277	pKNG101::P*ytbR*::*lux* (*ytbR* promoter fusion) / Sm^R	(Atkinson et al., 2008)

pSA200	pDM4::P*flhDC*::*lux* (*ytbR* promoter fusion) / Cm^R	(Atkinson et al., 2008)
pSA208	pDM4::P*fliA*::*lux* (*fliA* promoter fusion) / Cm^R	(Atkinson et al., 2008)

Table 2.3| Plasmids used in this study

2.2.3 Restriction enzymes

All restriction enzymes were purchased from Promega, and used according to manufacturer's instructions. Unless otherwise stated, all restriction reactions were incubated at the appropriate temperature for 3 h to allow for complete digestion.

2.2.4 Separation of DNA by agarose gel electrophoresis

DNA was resolved in TAE-agarose gels (80 mM Tris-acetate pH 7.9, 19 mM EDTA, 0.8 % (w/v) analytical grade agarose (Invitrogen) and contained ethidium bromide at a final concentration of 10 µg / ml. To establish the size of the DNA fragments, 1 kb DNA ladder (Promega) was routinely loaded alongside DNA samples into gels. An appropriate volume of 6 X loading buffer (Promega) was added to DNA samples prior to loading into the gel, and electrophoresis was performed using a horizontal gel apparatus in TAE buffer at 90 V. Where appropriate, DNA fragments were recovered from gel slices using a gel extraction kit (QIAGEN) according to manufacturer's instructions.

2.2.5 DNA ligation

Purified DNA inserts were ligated into appropriate vectors at the ratio of 1 : 3 (vector : insert). Ligations were carried out using 1 µl T4 DNA ligase and the appropriate volume of 1 X T4 reaction buffer (Promega), usually to a final volume of 20 µl and incubated overnight on melting ice.

2.2.6 Polymerase chain reaction conditions

2.2.6.1 Synthesis of oligonucleotide primers

Oligonucleotide primers (Table 2.4) were synthesised by Sigma-Genosys Ltd. (UK). Unless otherwise stated, all primers were designed to the published *Y. pseudotuberculosis* YPIII genome (NCBI, accession: NC_010465.1). Primer sequences are listed in table 2.4. Where appropriate, restriction site sequences were engineered into the oligonucleotides, and these are highlighted in bold and underlined.

Oligonucleotide	Sequence (5'→3')	Notes
YscJaFor	CTGAATTGCGTAGTGTATTGCAGCAG	
YscJupR-Tet	GAGCGCATTGTTAGATTTCATTAGTTTTCACCCCCCCTT CGA	
YscJdownF-tet	GAGCCGGGCCACCTCGACCTGACGTAACACGAGCATA CTGTC	
YscJbRev	CCGCAAGCGAGCGAGAGATTAC	
TetFor	ATGAAATCTAACAATGCGCTC	
TetRev	TCAGGTCGAGGTGGCCCGGCTC	
yscJC_F	TCTAGAGACTGCCGGGCGAATGAG	*Xba*I
yscJC_R	GTCGACCGGCGCCCCGTCTTCGC	*Sal*I
pVirF-ApaI-F	GGGCCCGTTGAATACAAATA	*Apa*I
pVirF-Not1-r	GCGGCCGCATGTTATACTGTCC	*Not*I
RP-1	GACACTCTGTTATTACAAATCG	
YscUF	AAAAGCAAGCGTCGTCAGTT	
YscUR	GCTGTGGCCTCTATTTGCTC	

Table 2.4 | Oligonucleotide primers used in this study

2.2.6.2 PCR amplification

Unless otherwise specified, PCR amplifications were performed using 0.2 µl FailSafe Phusion DNA polymerase (NEB), in a reaction containing 1 µg of each oligonucleotide primer, an appropriate volume of the 5 X reaction buffer supplied with the enzyme, and 2 mM dNTPs. Reactions were carried out in a Hybaid express thermal cycler using the following conditions, unless otherwise stated: 98°C for 5 min for initial denaturation, followed by 35 cycles of melting at 98°C for 30 sec, annealing at 56°C for 30 sec and polymerisation at 74°C for the appropriate length of time (approximately 1 min per kb). A final extension stage at 74°C for 5 min ensured complete polymerisation of all strands. The annealing temperature was also run on a gradient to determine the best temperature for the reaction.

2.2.7 Introducing DNA into bacterial cells

2.2.7.1 Preparation of electro-competent cells

Electro-competent *E. coli* and *Y. pseudotuberculosis* cells to be used for electroporation were prepared as previously described (Sambrook and Russell, 2001). Briefly, bacteria were grown overnight and seeded into 40 ml LB. This was grown to an OD_{600} of 0.6 – 0.8 before cells were harvested by centrifugation (Beckman Avanti 30 Centrifuge CO650) at 5000 *x g* for 10 min at 4°C. Cells were washed 3 times in 40, 20 then 5 ml of ice-cold 10 % (v/v) glycerol. The cells were then re-suspended to a final volume of 60 µl with 10 % (v/v) glycerol. Aliquots were stored at -80°C in sterile 1.5 ml tubes (Eppendorf).

2.2.7.2 Electroporation

DNA for electroporation was routinely dialysed against distilled water for 15 min using a 0.0025 µm nitrocellulose filter (Millipore, UK). Approximately 5 µl of dialysed DNA was added to the 20 µl electro-competent cell aliquot, mixed and electroporated using a Gene Pulsar (BioRad, UK) set to 2.5 kV, 200 Ω, 25 µF. Electroporated cells were recovered in 1 ml LB for 1 h at the appropriate temperature prior to being plated on LB agar supplemented with appropriate antibiotics, and grown overnight at the appropriate temperature. When appropriate, bioluminescent colonies (expressing the *lux* genes) were selected using a photomultiplier camera (Hamamatsu). These colonies can be detected on the basis of light production which can be captured as an image and used to isolate the colony.

2.2.7.3 Conjugation

The vectors pDM4 and pKNG101 were introduced into *Y. pseudotuberculosis* strains from *E. coli* S-17 typically by mating 1: 3 (donor: recipient). Cells were grown for no longer than 16 h prior to being gently washed and combined. Combined cell pellets were re-suspended in 5 µl LB and spotted onto LB agar and incubated at 30°C for 16 h. The resulting colony was recovered from the plate, reconstituted in 1 ml LB and serially diluted 10^6 prior to being plated on LB agar plates with appropriate antibiotics. When appropriate, colonies were selected on the basis of light production following screening using a photomultiplier camera (Hamamatsu).

2.2.8 Construction of the *virF* promoter fusion

An 800 bp PCR product encoding the promoter region of *virF* (from position 52567 to 53366 on the published IP32593 pYV sequence, accession number: NC_006153) was amplified using the primers pVirF-*Apa*I-F and pVirF-*Not*1-R (table 2.4). This product was ligated into pGemT-easy (Promega). The *virF* promoter was removed from pGemT-easy as an *Apa*I and *Not*I fragment, and cloned into similarly digested pBluescript KSII+ to yield pBlue-P_{virF} and clones with interrupted *lacZ* selected on blue/white plates. Subsequently, the Lux biosynthetic operon (*luxCDABE*) was excised from pBlueLux (Atkinson et al., 2008) as a *Sac*I fragment and cloned into similarly digested pBlue-P_{virF} to give pBlue-P_{virF}*::lux*. The P_{virF}*::lux* fusion was excised as an *Apa*I and *Sal*I fragment, and the 8918 bp fragment was cloned into similarly digested pDM4, yielding pDM4-P_{virF}*::lux* and transformed into *E. coli* S17-1 λ-pir, prior to conjugation with the appropriate *Y. pseudotuberculosis* strain.

2.2.9 Mutagenesis of *yscJ*

The *yscJ* gene was deleted in-frame and replaced by a tetracycline resistance cassette following a modified method of Derbise *et al.* (2003), which uses the λred recombinase encoded on the helper plasmid pAJD434 (Derbise et al., 2003). Primer pairs YscJaFor/YscJupR-Tet and YscJdownF-tet/YscJbRev were used to amplify the up- and down- stream regions of *yscJ* (positions 59172 to 59743 and 60344 to 61135 on the published *Y. pseudotuberculosis* YPIII pYV virulence plasmid sequence). YscJupR-Tet and YscJdownF-tet contained 21 bp or 22 bp of sequence homologous to a tetracycline cassette from pBlueTet (O'Neill et al., 2008), which was amplified as an 1191 bp fragment using primer

pair TetFor and TetRev. The deleted *yscJ* fragment containing the tetracycline cassette was then amplified using primer pair YscJaFor and YscJbRev using the 3 newly generated PCR products as a template in one PCR reaction. The resulting 2553 bp fragment was transferred, by electroporation, into YPIII strains carrying the helper plasmid pAJD434, encoding the λred recombinase, which recombines homologous DNA from the PCR product into the genome following induction on LB agar supplemented with arabinose (0.8 % final concentration). The pAJD434 plasmid was then cured from the newly mutagenised strains by growth at 37°C, and mutagenesis confirmed by PCR using the primer pair YscJaFor and YscJbRev to amplify a 2553 bp fragment in the mutants and a 1963 bp fragment in the wild-type.

To complement Δ*yscJ*, an 842 bp product from *Y. pseudotuberculosis* (positions 59686 to 60537 on the IP32953 pYV published sequence) was amplified using primers YscJF-*Xba*I and YscJR-*Sal*I. Following *Xba*I / *Sac*I digestion, the fragment was cloned into similarly digested pBluescript KSII+ and removed as a *Kpn*I / *Pst*I fragment and sub-cloned into the low copy number vector pHG327 (Stewart et al., 1986). The resulting plasmid, pHG*yscJ* was transformed into the Δ*ypsI* Δ*ytbI* mutant.

2.2.10 Transposon mutagenesis

To generate transposon mutant libraries, electrocompetent Δ*ypsI* Δ*ytbI* mutant cells were transformed with the EZ-Tn5 transposome system (trimethoprim resistance, Epicentre). This system is a complex formed between an EZ-Tn5™ Transposon and EZ-Tn5™ Transposase which can reliably generate a library of random gene knockouts. The resulting cells were serially diluted 10^2, 10^3 and 10^4 and plated onto LB agar supplemented with Tp.

To sequence across the region of the transposon insertion, chromosomal DNA from the appropriate mutant was extracted and digested with *Bam*HI and ligated into similarly digested pBluescript KSII+ before being transformed into *E. coli* DH-5α by electroporation. Cells were selected on LB agar supplemented with Tp and Amp. The EZ-Tn5 transposon carries a single *Bam*HI site; therefore positive colonies would carry a fragment containing the transposon and *Y. pseudotuberculosis* DNA to the first available downstream *Bam*HI site. *Bam*HI restriction confirmed the insertion and size of the fragment, and sequencing using the primer RP-1 determined the location of the transposon insertion.

2.2.11 Sequencing of DNA

Sequencing was carried out at the DNA sequencing facility (Queen's Medical Centre, University of Nottingham). Sequences were analysed using LaserGene DNA sequence analysis package (DNAstar Ltd). Subsequent DNA analysis was performed using programs listed by Dr. Andrew Kropinski, Molecular & Cellular Biology, University of Guelph, Ontario, Canada (http://molbiol-tools.ca), and those available from the NCBI (http://www.ncbi.nlm.nih.gov/) or EBI (http://www.ebi.ac.uk/) website.

2.3 Extraction and Analysis of Proteins

2.3.1 Preparation of supernatant proteins

In order to examine proteins present in the supernatant, 10 ml of the appropriate bacterial cultures were grown for 16 h at the appropriate temperature, and cells pelleted by centrifugation at 4000 x *g* for 15 min at 4°C. The supernatant was recovered into a fresh tube and placed on ice and proteins were precipitated via the trichloroacetic acid (TCA) method (see below).

2.3.2 Purification of flagella

Flagella were purified from bacterial cells using the cannular method as previously described (Atkinson et al., 1999). Briefly, bacterial cultures were grown overnight at 22°C, then seeded into 10 ml cultures at an OD_{600} of 0.001 and grown for 24 h at 22°C until the cultures reached an OD_{600} of 2.2. The bacterial cells were washed with fresh media and passed through cannular tubing 20 times. Cells and cell debris was removed by centrifugation, and the proteins present in the resulting filter sterilised supernatant was precipitated by the TCA method.

2.3.3 TCA precipitation

The supernatant was recovered into a fresh tube and incubated on ice with TCA (10 % w/v final concentration) for 1 h and centrifuged at 10,000 x *g* for 30 min at $4^{o}C$. Supernatant was removed and the protein pellet was re-suspended in 500 µl 10 % SDS. To this, 1 ml ice-cold acetone was added, mixed, and incubated on ice for 40 min. Following centrifugation at $4^{o}C$ at 13,000 x *g* for 30 min, the supernatant was removed and the protein pellet was air dried to remove residual acetone and re-suspended in 60 µl PBS and stored at $-20^{o}C$.

2.3.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis was carried out according to standard protocols (Sambrook and Russell, 2001) using 10 % (v/v) polyacrylamide gels. Where appropriate for the purposes of resolution, 4 – 12 % (1 mm) Bis-Tris pre-cast gels (Invirogen) were used according to manufacturers' instructions. Gels were stained with 0.25 % coomassie blue according to standard protocols.

2.3.5 Protein profiling and sequencing

Where stated, proteins present in SDS-gels were identified using either MALDI-ToF or Q-ToF services at the University of Nottingham Sequencing Facility or the Proteins and Nucleic Acid Centre, Leicester (PNACL).

2.4 Phenotypic assays

2.4.1 Liquid culture biofilm assays

To investigate the ability for different *Y. pseudotuberculosis* strains to clump and form LCBs, clumping experiments were performed essentially as previously described (Atkinson et al., 1999). Briefly, cultures were grown overnight at the specified temperature, and 1 ml culture transferred to a spectrophotometer cuvette, which was then statically incubated at room temperature for 20 min to allow LCB formation to occur. Samples were examined by microscopy (see section 2.5).

To determine the role of supernatant in the clumping phenotype, a modified clumping assay was performed. Here, cells of the appropriate culture were pelleted by centrifugation at 5000 x *g* for 10 min, and washed in the same volume of fresh media. Supernatant was filter sterilised, after which 1 ml of culture was pelleted and recombined with sterile supernatant, mixed and left to statically incubate at room temperature for 20 min to allow LCB formation to occur. Where appropriate, to exclude macromolecules from the supernatant prior to the reconstitution of cell pellets, supernatants were centrifuged at 10,000 x *g* for 40 min through concentration columns, which prevent the flow through of large molecular weight molecules, of the appropriate size. The flow through was then used to re-suspend cell pellets which were then used in clumping assays. The large molecular weight molecules were then recovered from the filters by adding 100 µl fresh media to the inverted column, incubating on ice for 30 min and centrifugation at 10,000 x *g* for 30 mins. The resulting elutant was made up to a final volume of 1 ml by the addition of fresh media,

and this was then used in clumping assays. To denature heat-labile molecules in the supernatant, filter sterilised supernatant was heated to 100°C in a boiling water bath for 30 min. The supernatant was cooled to room temperature, and used in clumping assays.

To investigate if proteins present on the cell surface were involved in the clumping phenotype, cell cultures were washed in PBS and incubated with proteinase K (20 µg/ml final concentration) at room temperature for 30 min. Cells were washed three times in PBS and then used in clumping assays.

To determine if extracellular DNA was released by *Y. pseudotuberculosis* into the growth supernatant, cultures were grown and re-suspended in PBS and incubated for up to 16 h at the appropriate temperature with agitation at 200 rpm. Following this, cells were removed by centrifugation and the quantity of DNA in the supernatant measured using the PicoGreen system (Invitrogen) according to manufacturer's instructions.

To determine if extracellular DNA played any role in the formation of LCBs, cell-free supernatant for use in assays was pre-treated with DNase I (20 µg/ml final concentration). Where appropriate, DNase I was also added directly to cultures used in clumping assays. Extracellular DNA was labelled in LCBs by the use of the DNA specific label 4',6-diamidino-2-phenylindol (DAPI 0.5 – 1 µg/ml final concentration) (Böckelmann et al., 2002). Low concentrations of DAPI label the ECM biofilm matrix without penetrating the bacterial cell and staining the intracellular DNA [72]. To determine if exopolysaccharides were present in liquid culture biofilms, cultures for use in clumping assays were stained with Rhodamine-WGA (20 µg/ml final concentration), a lectin which is commonly used to label polysaccharides in bacterial biofilms [12].

2.4.2 Congo red binding in liquid culture

To investigate the binding of Congo red in liquid culture, a modified method of a (reverse) haemolysis experiment was used. Here, Bacteria were cultured overnight and inoculated into LB_{CR} at an OD_{600} of 0.01, and incubated at the appropriate temperature for 16 h with agitation at 200 rpm. Following this, bacterial cells were removed by centrifugation and the optical density (495 nm) of filter sterilised supernatant was measured using a spectrophotometer. The OD_{494} of sterile LB_{CR} (low control) and LB (high control) was also measured, and the amount of Congo red bound by the bacterial cells was calculated using the formula:

$$\boldsymbol{Cell\ bound\ Congo\ red}\ (\%) = \left(\frac{\left[{}^{\boldsymbol{OD\ of\ supernatant}}/_{\boldsymbol{high\ control}}\right]}{\left[{}^{\boldsymbol{high\ control}}/_{\boldsymbol{low\ control}}\right]}\right) \times \mathbf{100}$$

2.4.3 Measuring biofilms on the surface of *C. elegans*

2.4.3.1 Biofilms grown in the agar method

The *C. elegans* wild-type (N2 Bristol) strain was obtained from the *Caenorhabditis* Genetics Centre (University of Minnesota, St. Paul, MN) and maintained on worm-NGM plates (Epstein and Shakes, 1995) lacking $MgCl_2$, seeded with *E. coli* OP50. For biofilm experiments, 1 ml *Y. pseudotuberculosis* overnight culture was spread onto worm-NGM agar plates and air-dried for 1 h. A minimum of 20 *C. elegans* worms were transferred to the *Y. pseudotuberculosis* seeded plates and incubated at 22°C for 16 h. The resulting biofilms which developed on the *C. elegans* surface were examined using a Nikon

SMZ1000 microscope under medium magnification and scored for biofilm severity on a scale of 0 to 3, where 0 represents the absence of biofilm; 1 represents a small biofilm which primarily manifests around the buccal cavity; 2 represents a large, mono-focal biofilm while 3 represent a very large multi-focal biofilm (Atkinson et al., 2011). These data were then used to calculate a biofilm severity index (Tarr, 1972) as:

$$Biofilm\ severity\ index\ (\%) = \left\{\left[\sum\left(\frac{severity\ \times number\ of\ worms\ in\ this\ level}{(highest\ severity\ \times total\ number\ of\ worms)}\right)\right]\right\} \times 100$$

All biofilm experiments were performed 'double-blind'.

For some experiments the presence of EPS and eDNA in biofilms was investigated as described in section 2.4.1. Worms were mounted on concave microscope slides in 30 mM $NaNO_3$ for use in microscopy (see section 2.5).

Where appropriate, to degrade eDNA, DNase I (20 µg/ml) was added to *Y. pseudotuberculosis* cultures prior to spreading onto the NGM plate to be used in biofilm assays.

2.4.3.2 Biofilms grown in the compost method

To simulate *Y. pseudotuberculosis* contamination in the natural environment, approximately 10 g of dried, autoclaved commercial compost (Wilkinson UK, low peat compost) was added to 50 ml falcon tubes, which roughly consumes 5 ml volume space. To this, 3 ml H_2O and 2 ml of *Y. pseudotuberculosis* culture was added, and at least 60 worms transferred into the tubes. This was incubated statically at 22°C for 24 and 48 h. Worms were recovered by gently mixing the soil with 15 ml PBS, the solution was then dispensed into a Petri dish and examined under low magnification using a Nikon SMZ1000 microscope. Worms

can be seen by their swimming motion and were recovered into a fresh Petri dish filled with 20 ml PBS, where they could be separated from the soil detritus. For some experiments EPS and eDNA was labelled as described in section 2.4.1. Worms were mounted on concave microscope slides in 30 mM $NaNO_3$ for microscopy (see section 2.5).

2.4.4 Determination of bioluminescence and optical density

Bioluminescence and optical density were measured simultaneously in a 96-well microtitre plate using a combined spectrophotometer / luminometer (Anthos Lucy I), controlled by the Stingray 2 software package (Dazdaq) as previously described (Atkinson et al., 2008). Briefly, bacteria which had been grown overnight at 30°C were used to seed fresh LB with appropriate antibiotics to an OD_{600} of 0.001. Approximately 200 μl was added to wells of a 96-well microtitre plate, and the plate was incubated in Anthos Lucy I at the appropriate temperature for at least 20 h. Luminescence and OD_{405} of the culture was automatically measured every 30 min, and presented as relative light unit per unit of OD_{405} (luminescence / OD_{405}). For each experiment, the bacteria were incoluated into at least 5 wells each, and at least 3 independent experiments carried out.

Where appropriate, the determination of AI-2 present in culture supernatants followed the previously described method (Bassler et al., 1994).

2.5 Microscopy

Where appropriate, samples were taken at different time courses over the incubation time and visualised by microscopy using either a Nikkon inverted fluorescence microscope or a Zeiss LSM700 inverted confocal microscope. The respective proprietary software packages (Nikkon picture acquisition tool and Zen Light Edition 2009 [Zeiss]) were used for image analysis. For fluorescent detection of on the Zeiss LSM700, laser wavelengths and powers were typically set as 405 nm at 4 % (DAPI), 488 nm at 15 % (GFP) and 561 nm at 15 % (R-WGA). Master gain applied to all channels was approximately 800, and the digital offset approximately 130.

Chapter 3|

Quorum Sensing and the motility regulon co-regulate the Yop-Ysc Type 3 Secretion System, which contributes to the formation of liquid culture biofilms

3.1 Introduction

A number of bacterial species form clusters of clumps of attached cells during liquid culture (Arnold and Shimkets, 1988, Zhou et al., 2001, Singh and Vincent, 1987, Godon et al., 1994, Bi et al., 2007, Uzureau et al., 2007, Hussain et al., 2008, Seki, 1971, Albert et al., 1993, Römling and Rohde, 1999, El Tahir and Skurnik, 2001, Dunny et al., 1978, Handley et al., 1984, Tomasz and Zanati, 1971) which are often recognised as a type of liquid culture biofilm (LCB), since the cells may be surrounded by a complex extracellular matrix (ECM) composed of exopolysaccharides and DNA, similar to surface attached biofilms (Flemming et al., 2007, Sutherland, 2001, Kim et al., 1999, Allesen-Holm et al., 2006, Godefroid et al., 2010, Seidl et al., 2008, Håvarstein et al., 2006, Kreth et al., 2009). This ECM probably provides the encased cells with the benefits of a surface-attached biofilm in resisting antibiotics, and can act as a counter-predation mechanism (Matz and Jürgens, 2003, Matz and Kjelleberg, 2005), while the close proximity of the cells can facilitate genetic exchange (Dunny et al., 1978). Interestingly, LCBs can also assemble quickly, for example some bacteria will form LCB-like biofilms spontaneously in response to antibiotics (Lorian et al., 1978), while the aquatic bacterium *Sphingobium* sp will form LCBs upon detection of secreted molecules from the bacteriovorous flagellate *Poterioochromonas* (Blom et al., 2010).

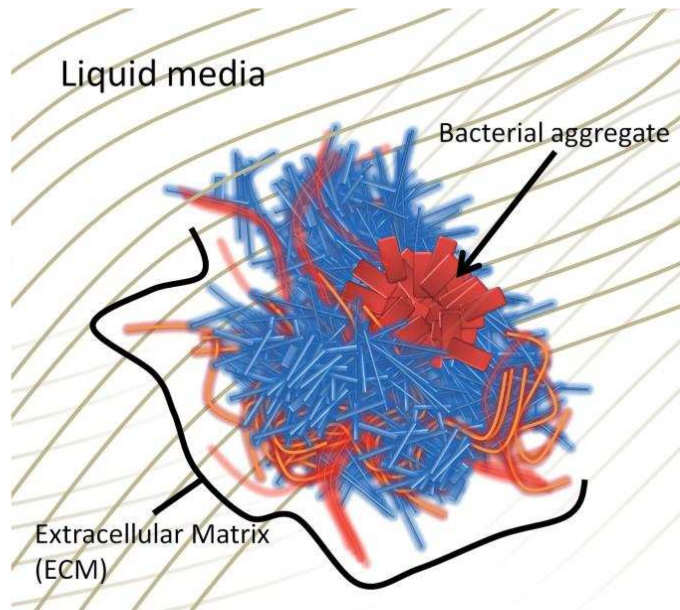


Figure 3.1| A representation of liquid culture biofilms. Here, an aggregate of bacterial cells suspended in liquid media is surrounded by an extracellular matrix (ECM) ECM is typically composed of exopolysaccharides, extracellular DNA and other polymers.

3.1.1 Cellular physiology and genetics of LCBs

LCBs involve a plethora of cellular processes and cell surface properties (Figure 3.2). Early on, it was shown that variation in lipopolysaccharide (LPS) in the outer membrane of *E. coli* and *Salmonella typhimurium* from a full length, 'smooth' form, to a shortened O-chain 'rough' form induced LCB-like aggregates in liquid culture (Diderichsen, 1980). Similarly, 'deep-rough' or O-antigen mutants in *E. coli*, with truncated LPS, also display substantial clumping (Moller et al., 2003, Sheng et al., 2008). Since then, various groups of cell surface proteins have been identified which play a critical role in the formation of cell aggregates. In *E. coli*, the *flu* gene, encoding the Antigen 43 auto-transporter protein, influences fluffing, or the formation of LCBs, (Diderichsen, 1980, Henderson and Owen, 1999). In *E. coli*, *flu* is regulated reciprocally by the methylase Dam and the redox sensor OxyR, which results in Flu+ (LCB forming)

and Flu- (non-forming) cells that are rapidly inter-convertible (Henderson and Owen, 1999, Schembri et al., 2003).

Many other auto-transporters affect LCB formation, including Hap in *Haemophilus influenzae* (Hendrixson and St. Geme, 1998), Cah in enterohaemorrhagic *E. coli* (Torres et al., 2002), YadA and YapC in *Y. pseudotuberculosis* and *Y. pestis* respectively (Bliska et al., 1993, Felek et al., 2008). In most cases, aggregation is thought to be caused by protein-protein interactions between the auto-transporters present on neighbouring cells. Interestingly some auto-transporters have been shown to affect LCB formation beyond protein auto-aggregation, such as in *Azorhizobium caulinodans*, where the auto-transporter AoaA positively influences the secretion of biofilm matrix components (Suzuki et al., 2008). Other families of cell-surface proteins and lipoproteins, are also involved in cell aggregation (Jung et al., 1990). An important group of protein structures are cell surface fimbriae, and in many bacteria including *E. coli* K-12, *Salmonella enteriditis*, *Citrobacter* spp. and *Enterobacter sakazakii*, thin aggregative fimbriae are involved in cell clumping (Vidal et al., 1998, Collinson et al., 1991, Zogaj et al., 2003). However it should be noted that the aggregative effect of fimbriae may depend on other genetic factors, since in some strains of *E. coli*, fimbriae have been shown to block Flu-mediated aggregation, and the ability for Flu+ *E. coli* cells to form LCBs correlates with the absence of fimbrial structures on the cell surface (Hasman et al., 1999, Sherlock et al., 2005). Together, this suggests that the LCB phenotype results from interplay between a number of cell surface attributes that may often be mutually exclusive.

Stress responses, usually controlled by two-component systems, play a large role in regulating the formation of LCBs. In *Streptococcus mutans*, the CovR/S two-component system regulates the expression of a fructosyltransferse (FTF)

and the production of uronic and glucuronic acids important for the biofilm matrix so that under stressful conditions CovS inactivates the repressor CovR and permits the formation of LCBs (Lee et al., 2004, Dalton and Scott, 2004). Mutants in *covR*, in contrast to wild-type, have an abundance of FTF on the cell surface, generate excess glucose and also exhibit substantial auto-aggregation (Lee et al., 2004). This genetic relationship between stress responses and matrix production / multi-cellular aggregation is mirrored in *E. coli* K12, where the enzyme responsible for the production of colonic acid, WcaB is part of the two-component OmpR / EnvZ regulon (Chirwa and Herrington, 2003). Stress also affects the cellular concentration of the second messenger cyclic-di-GMP, which regulates aggregation in a variety of species. In *P. aeruginosa* cyclic-di-GMP is known to affect clumping (D'Argenio et al., 2002), while in *E. coli* AdrA, a diguanylate cyclase which produces cyclic-di-GMP, enhances clumping while YhjH, a phosphodiesterase which degrades cyclic-di-GMP, suppresses this multicellular behaviour (Simm et al., 2005). Together, this indicates that several stress-response systems converge on the regulation of multiple proteins which are involved in the formation of LCBs.

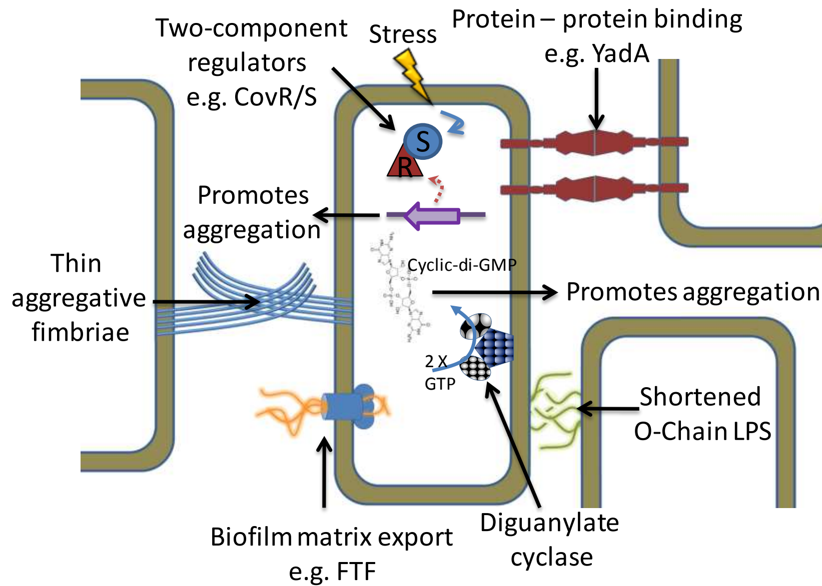


Figure 3.2| Methods for cellular aggregation in bacteria. Bacteria employ several methods to form aggregates and liquid culture biofilms, including cell surface structures such as thin aggregative fimbriae, protein-protein interactions, particularly those involving autotransporters such as YadA. The export of biofilm matrix components and 'rough' LPS with truncated O-chains also enhances the formation of LCBs. LCBs can be regulated by stress responses and two-component regulators, and high cellular cyclic-di-GMP concentrations resulting from the activity of diguanylate cyclases increases cellular aggregation.

3.1.2 LCBs in the *yersiniae*

Y. pseudotuberculosis and *Y. enterocolitica* have long been known to undergo spontaneous agglutination during static culture in tissue culture medium (Laird and Cavanaugh, 1980), which is thought to involve the *Yersinia* adhesin, YadA. YadA is encoded on pYV, and is a critical virulence factor for *Y. pseudotuberculosis* and *Y. enterocolitica*, with roles in binding to a variety of cell types (El Tahir and Skurnik, 2001). The composition of the growth medium influences the production of YadA, with marked suppression in rich media such as BHI and LB compared with minimal media (Kapperud et al., 1985, Bolin et al.,

1982, Eitel and Dersch, 2002). Low calcium or high magnesium concentrations may allow maximal production of YadA (Bolin et al., 1982, Eitel and Dersch, 2002) and this behaviour only occurs following growth at 37°C and not when cells are grown below 30°C (Laird and Cavanaugh, 1980, Perry and Brubaker, 1983, Kapperud et al., 1985), indicating a multi-component regulatory circuit underpinning YadA expression.

Early *e*xperiments hinted at a role for QS in repressing YadA-mediated auto-agglutination, since exponential phase cultures express *yadA* much more than do stationary phase cells (Eitel and Dersch, 2002). However it is not known whether the LCB phenotype observed by Atkinson *et al.* (1999) in response to mutation of *ypsR* (Atkinson et al., 1999) and YadA-mediated auto-agglutination are the same phenotype. In the absence of YadA (El Tahir and Skurnik, 2001), early tests failed to detect auto-agglutination in *Y. pestis* (Perry and Brubaker, 1983). However later auto-agglutination was shown in *Y. pestis*, though this is not dependent on the pYV plasmid, but requires a chromosomal locus identified as YapC (Felek et al., 2008). YapC and YadA share several functional characteristics; both are members of the auto-transporter family of proteins and are involved in adherence to host cells and in biofilm formation (Felek et al., 2008, El Tahir and Skurnik, 2001).

In addition to YadA and YapC, other *Yersinia* cell surface proteins have been shown to affect clumping, including OmpX in *Y. pestis*, a member of the Ail/Lom family of proteins similar to the *Y. pseudotuberculosis* Attachment and Invasion Locus (Ail) protein (Kolodziejek et al., 2007). In other species, fimbrial structures are also associated with clumping; the *Yersinia* Psa is a cell surface fimbriae heavily expressed during infection of macrophages (Lindler and Tall, 1993), and while the Psa has not been previously shown to affect multicellularity *in vitro*, a characteristic of *Yersinia* spp. growing inside macrophages is the

formation of tight cell aggregates (Pujol and Bliska, 2003), suggesting that the Psa could be involved.

Biofilm matrix components are also implicated in aggregation in *Yersinia*, where the *hms* locus of *Y. pestis* causes bacterial aggregation within the blood-meal of the flea vector (Hinnebusch et al., 1996). The *hms* locus drives the production of the biofilm matrix exopolysaccharide β-1,6-*N*-acetyl-D-glucosamine (Bobrov et al., 2008), and is crucial for biofilm formation in both *Y. pestis* and *Y. pseudotuberculosis* (Hinnebusch et al., 1996, Darby et al., 2002). The *hms* locus is also involved in the binding of haem or the haem analogue Congo red, which gives rise to the pigmented (Pgm^+) phenotype (Pendrak and Perry, 1993, Buchrieser et al., 1998). In *Y. pestis* Pgm^+ cells only develop when the bacteria are grown below 28°C (Brubaker, 1991), and it has long been known that Pgm^+ *Y. pestis* forms clumps in a variety of liquid media at this temperature (Perry et al., 1990). In contrast, *Y. pseudotuberculosis* does not generally bind Congo red or form clumps at 28°C, with only one strain, IP32790, known to give a pigmented phenotype (Buchrieser et al., 1998). In contrast, *Y. pseudotuberculosis* and *Y. enterocolitica* take up Congo red following growth in calcium depleted conditions at 37°C in media containing the stain- and this phenotype is dependent on cells harbouring the pYV plasmid (Prpic et al., 1983, Thoerner et al., 2003).

3.1.3 Chapter 3 aims

The clumping of bacteria and the formation of floc-type biofilms has been widely reported, and several species, including *Y. pseudotuberculosis*, regulate this behaviour using QS (Puskas et al., 1997, Godefroid et al., 2010, Uzureau et al., 2007, Atkinson et al., 1999). However, the nature of these aggregates in *Y. pseudotuberculosis* has not been fully investigated- while it is known that a *Y. pseudotuberculosis* Δ*ypsR* mutant forms dense bacterial aggregates that are not observed in the Δ*ypsI* mutant or the parent control (Atkinson et al., 1999), it is not known if the YtbR / I QS system also regulates clumping, nor is it known what cellular processes underlie this phenotype. This chapter presents data to suggest that, in addition to the Δ*ypsR* mutant but unlike the Δ*ypsI* mutant, clumping also occurs following mutation of *ytbR* or *ytbI*, or when both AHL synthase or receptor genes are deleted. These clumps associate together to form large aggregates, which label abundantly for eDNA and EPS- biofilm matrix components more often associated with surface-attached biofilms, which suggests that these aggregates represent a form of LCB. LCB formation requires a pYV-encoded secreted factor that probably interacts with a chromosomally encoded cell surface factor to induce the formation of LCBs. This secreted factor is dependent on the Ysc injectisome, since its production into the supernatant is abolished following subsequent mutation of *yscJ*, a key structural component of the injectisome.

To investigate the role of other systems that influence LCB formation, and since the flagella regulon in *Y. pseudotuberculosis* is also regulated by QS (Atkinson et al., 1999, Atkinson et al., 2008) and has previously been shown to regulate T3S and cell-aggregation in *Y. enterocolitica* (Bleves et al., 2002), the involvement of the flagella regulators FlhDC and FliA in the LCB phenotype was investigated.

The data show that mutation of *flhDC* or *fliA* results in the formation of LCBs, which is correlated with a similar profile of Yop-related proteins secreted into the culture supernatant. Interestingly, a mutant in *flhA*, encoding a component of the flagella secretion system, also over-produces Yop proteins and forms LCBs, suggesting that elements of the structure of the flagella may play a role as checkpoints in the regulation of LCB formation and Yop production. To begin to investigate the genetic basis of LCB formation, a transposon mutagenesis approach has been used to identify loci which, when mutated by insertion of the transposon, are able to revert a LCB forming strain of *Y. pseudotuberculosis* (such as the Δ*ypsI* Δ*ytbI* mutant) to the non-LCB forming phenotype reminiscent of the wild-type. This screen identified nine loci which are necessary for the Δ*ypsI* Δ*ytbI* mutant to form LCBs.

3.2 Results

3.2.1 Quorum sensing and LCBs

3.2.1.1 QS controls LCB formation

Previously, LCBs have been shown to be formed during culture of a *Y. pseudotuberculosis* Δ*ypsR* mutant, maximally following growth at 37°C, but also observed following growth at 30°C. These LCBs are not observed in the wild-type at these temperatures, nor in a Δ*ypsI* mutant, or when any of the strains are grown at 22°C (Atkinson et al., 1999). Since this work by Atkinson *et al.* (1999), several other QS mutants have been constructed, and now include mutants in the *ytbI / R* system, and double AHL synthase (*ypsI* / *ytbI*) and receptor (*ypsR* / *ytbR*) mutants (Atkinson et al., 2008). To investigate whether the strains Δ*ytbI*, Δ*ytbR*, Δ*ypsI* Δ*ytbI*, and Δ*ypsR* Δ*ytbR* clumped and formed LCBs, overnight cultures were grown at 37°C alongside the Δ*ypsI* and Δ*ypsR* mutants and the wild-type, and left statically at room temperature for 20 min prior to being inspected visually (Figure 3.1 a) and by microscopy (Figure 3.1 b) for LCB formation.

Figure 3.3 shows visual inspection of cultures in spectrophotometer cuvettes (a) and phase contrast microscope images of the cultures (b), and demonstrates that, like Δ*ypsR* (ii), the strains Δ*ytbR* (iv), Δ*ytbI* (v), Δ*ypsR* Δ*ytbR* (vi) and Δ*ypsI* Δ*ytbI* (vii) all develop into dense bacterial aggregates which rapidly associate together under static conditions. Conversely, cultures of the wild-type (i) and Δ*ypsI* mutant (iii) do not show this phenotype. The formation of clumps and sediment is a rapid process- initially, Δ*ypsR*, Δ*ytbR*, Δ*ytbI*, Δ*ypsR* Δ*ytbR* and Δ*ypsI* Δ*ytbI* mutant cultures appear homogenous, however within 5 to 10 min

the bacteria can be visually observed to aggregate into macroscopic clumps. Within 20 min, these clumps begin to sediment at the bottom of the culture vessel, forming a dense layer of bacterial cells which appear to be encased in an iridescent material. However, neither the cells nor the iridescent material is present in a confluent layer, with areas of low material density, which creates a patch-work 3-dimensional structure reminiscent of biofilm architecture (Figure 3.3 b ii, iv, v, vi & vii).

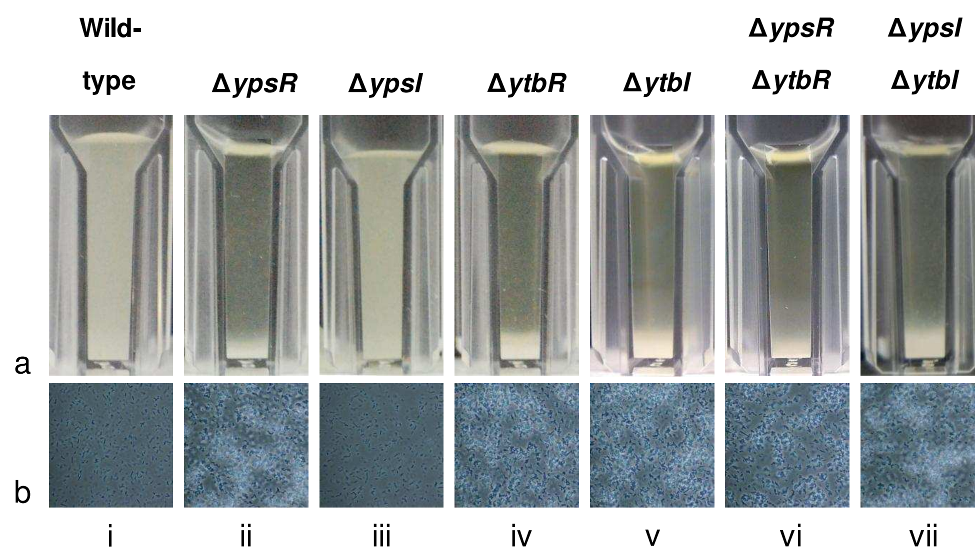


Figure 3.3| Cell clumping, sedimentation and liquid culture biofilm formation are regulated by QS. When wild-type *Y. pseudotuberculosis* (i) and isogenic QS mutants Δ*ypsR* (ii), Δ*ypsI* (iii), Δ*ytbR* (iv), Δ*ytbI* (v), the double mutants Δ*ypsR* Δ*ytbR* (vi) and Δ*ypsI* Δ*ytbI* (vii) are grown overnight at 37°C, and statically incubated in spectrophotometer cuvettes (a), it can be seen that, in contrast to the wild-type and Δ*ypsI*, all other QS mutants clump and form sediment. LCBs form as the cells can be observed to visually aggregate into macroscopic clumps, prior to sedimentation to the bottom of the culture vessel. Phase contrast microscope images of the cultures (b) show a dense network of cells surrounded by an iridescent material. Large areas seem to contain less cells and less material, suggestive of 3-dimenstional biofilm architecture.

These results show that LCBs are not restricted to the Δ*ypsR* mutant, but develop in the Δ*ytbR*, Δ*ytbI*, Δ*ypsR* Δ*ytbR* and Δ*ypsI* Δ*ytbI* mutants, and

suggests that QS is important for repressing the formation of LCBs in *Y. pseudotuberculosis*. These strains also form LCBs at 30°C, albeit more slowly, as shown for the Δ*ypsR* mutant (Atkinson et al., 1999) (data not shown).

3.2.1.2 YpsI and YtbI are necessary for LCBs

Despite the fact that the single Δ*ypsI* mutant does not form liquid culture biofilms, most QS genes clearly play a role in repressing the formation of LCBs. In light of this, the double synthase mutant strain Δ*ypsI* Δ*ytbI* was selected for further investigation, since this strain is unable to synthesise AHLs and thus represents a fully AHL-negative strain (Ortori et al., 2007). In many species such as *P. aeruginosa*, *E. carotovora* and *V. fischeri*, QS-associated phenotypes, discovered through mutagenesis of the relevant AHL-synthase gene can be readily complemented by supplementing the culture with the relevant AHL-signal molecule (Swift et al., 1996, Winzer et al., 2000). However, in *Y. pseudotuberculosis* and *Y. enterocolitica*, the exogenous addition of AHLs does not rescue the known phenotypes associated with QS mutation (Atkinson et al., 2008). To confirm that AHLs are involved in LCB formation, Δ*ypsI* Δ*ytbI* (expressing *gfp3* from pSB2020) was transformed with pSA291, a plasmid harbouring functional copies of *ypsI* and *ytbI* under the control of their native promoters, which restores AHL production and other QS regulated processes in the Δ*ypsI* Δ*ytbI* background (Atkinson et al., 2008). Figure 3.4 shows wild-type (i), Δ*ypsI* Δ*ytbI*, (ii) and Δ*ypsI* Δ*ytbI* pSA291 (iii) expressing *gfp3*, used in LCB assays and shown in spectrophotometer cuvettes (a). To investigate the mass of cells that are formed during growth, cultures were also subjected to CLSM (b).

Both microscopic and macroscopic tests demonstrated that formation of LCBs by Δ*ypsI* Δ*ytbI* could be prevented by supplementing functional copies of *ypsI* and *ytbI* on pSA291 (Figure 3.4, compare ii and iii). Within 5 min Δ*ypsI* Δ*ytbI*

developed into very large cell aggregates (Figure 3.4 b ii), whereas Δ*ypsI* Δ*ytbI* pSA291 is much more similar to the wild-type (Figure 3.4 b, compare iii and i).

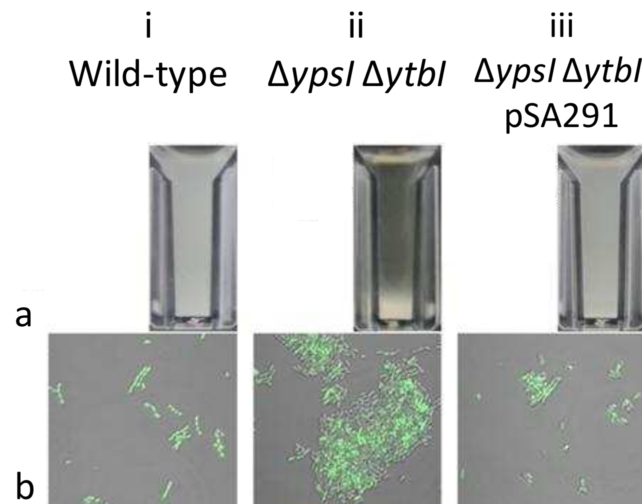


Figure 3.4| The Δ*ypsI* Δ*ytbI* mutant reverts to a non-clumping phenotype following restoration of *ypsI* and *ytbI* on pSA291. The Δ*ypsI* Δ*ytbI* mutant shows LCBs at following growth at 37°C (ii), while this is not observed in wild-type (i), and LCBs are substantially reduced in the Δ*ypsI* Δ*ytbI* pSA291 strain, where functional *yspI* and *ytbI* are provided to the Δ*ypsI* Δ*ytbI* mutant *in trans* on pSA291 (iii). Panels show spectrophotometer cuvettes containing cultures which have been statically incubated for 20 min (a), and microscope images showing LCB formation at the cell level. Bacteria are expressing *gfp3* from pSB2020 and appear green (b).

3.2.2 Biofilm matrix components in LCBs

3.2.2.1 Extracellular DNA and polysaccharides are present in LCBs

Extracellular DNA (eDNA) is an ECM component that provides structural support for liquid culture biofilms and sessile biofilms alike (Whitchurch et al., 2002, Vilain et al., 2009, Allesen-Holm et al., 2006, Godefroid et al., 2010, Seidl et al., 2008, Håvarstein et al., 2006, Kreth et al., 2009). Extracellular DNA may be particularly important in LCBs, since it is present in the aggregates of several bacteria including *P. aeruginosa*, *B. melitensis*, *S. aureus* and *Streptococcus* spp., and these aggregates can be disrupted by the addition of DNase (Allesen-Holm et al., 2006, Godefroid et al., 2010, Seidl et al., 2008, Håvarstein et al., 2006, Kreth et al., 2009). To investigate whether eDNA was present in the cell aggregates formed by the Δ*ypsI* Δ*ytbI* mutant, the DNA probe DAPI was added to cultures immediately after removal from overnight growth at 37°C, and incubated for 5 min prior to examination by CLSM. Figure 3.5 shows DAPI labelling is much more prominent in cultures of the Δ*ypsI* Δ*ytbI* mutant than the wild type, suggesting that extracellular DNA (eDNA) is present in the matrix of LCBs. In addition, DAPI labelling is also reduced in the Δ*ypsI* Δ*ytbI* pSA291 strain when compared with the Δ*ypsI* Δ*ytbI* mutant (compare Figure 3.5, c ii with c i & c iii).

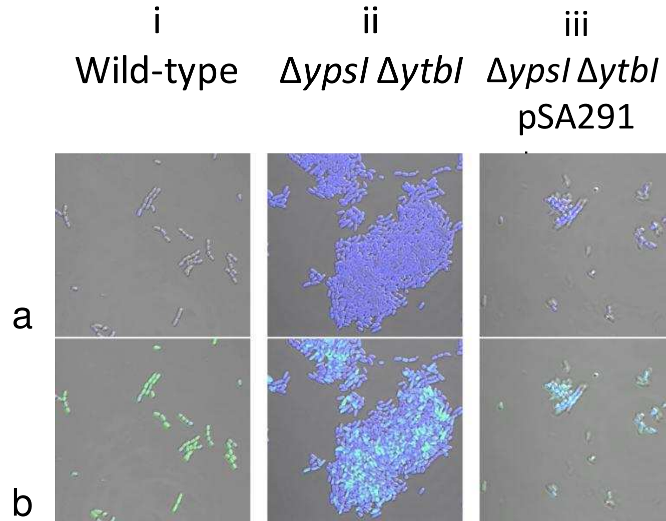


Figure 3.5| Extracellular DNA is enriched around cells of clumping mutants. CLSM reveals that cells of the Δ*ypsI* Δ*ytbI* mutant label heavily for the DNA specific stain DAPI (ii), whereas such heavy labelling is not observed in the wild-type (i), and is reduced when *ypsI* and *ytbI* are provided to the Δ*ypsI* Δ*ytbI* mutant on pSA291 (iii). Panels show DAPI fluorescence (a) and a composite image showing bacteria expressing *gfp3* overlaid with the DAPI fluorescence image (b). This data shows the fluorescence from DAPI in Δ*ypsI* Δ*ytbI* mutant cultures effectively masks the GFP signal (compare ii a and ii b), suggesting an abundance of eDNA surrounds the cells. In contrast, GFP fluorescence from wild-type cells is clear (i b), with very little DAPI labelling (i a), suggesting that the DAPI labelling in the Δ*ypsI* Δ*ytbI* mutant is not due to DAPI entering the cell and staining chromosomal DNA.

As previously stated, *Y. pseudotuberculosis* QS mutant LCBs develop from bacterial aggregates that coalesce and sediment. In order to follow this development by microscopy, and to observe the presence of eDNA, Δ*ypsI* Δ*ytbI* cells were grown at 37°C for 18 h, labelled with DAPI and then moved to static incubation at room temperature. At several time points (5, 10, 15 and 20 min), the culture was viewed by fluorescence microscopy.

Figure 3.6 shows the process of cellular clumping, clump coalescing and LCB formation that occurs in Δ*ypsI* Δ*ytbI*. Cells clump after 5 min; the clumps then

associate into loosely packed aggregates after 10 min and become very dense after 15 min, and finally settle to the bottom of the culture vessel after 20 min. Interestingly, dense areas of DAPI labelling at 20 min coincides with the iridescent material observed under phase microscopy, suggesting that this material is eDNA.

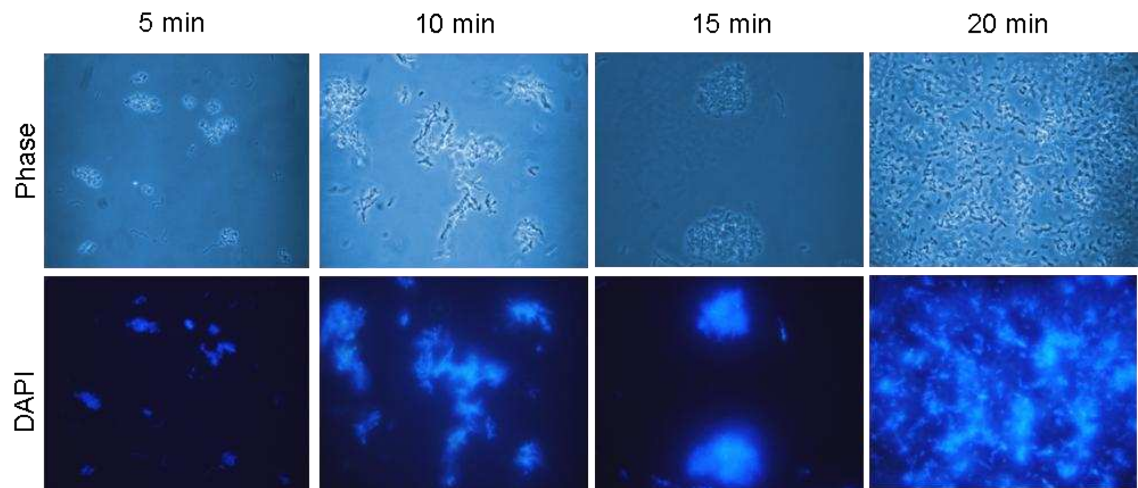


Figure 3.6| The Δ*ypsI* Δ*ytbI* mutant clumps and associate into LCBs. Phase contrast images and DAPI labelling of eDNA over several time points showing the process of liquid culture biofilm formation. After 5 min, small clusters of cells have associated together, forming bigger clusters after 10 min. By 15 min, these clusters have packed into 'grains' which can be observed macroscopically to begin settling to the bottom of the culture vessel. After 20 min, thick sediment has developed, with cells tightly associated into a 3-dimensional structure. DAPI labelling shows these cells to be covered in extracellular DNA. Interestingly, an iridescent material observable by phase microscopy co-localises with intense DAPI labelling, suggesting this material to be eDNA. Wild-type cells label much less strongly with DAPI, suggesting that eDNA release is involved in the LCB phenotype (not shown).

Extracellular polysaccharides (EPS) are also important components of the biofilm matrix (Sutherland, 2001). An important EPS for several bacteria including *Y. pseudotuberculosis* is β-1,6-*N*-acetyl-D-glucosamine, which is produced by the *hms* locus (Bobrov et al., 2008, Itoh et al., 2005), and can be labelled by Wheat

Germ Agglutinin coupled to a fluorophore such as rhodamine (R-WGA) (Tan and Darby, 2004). R-WGA therefore represents a fluorescent marker suitable for the detection of β-1,6-*N*-acetyl-D-glucosamine, although WGA can sometimes also bind to other polysaccharides, glycoproteins and glycolipids (Molin et al., 1986). To investigate whether such polysaccharides were present in the LCBs formed by Δ*ypsI* Δ*ytbI*, overnight cultures of wild-type and Δ*ypsI* Δ*ytbI* grown at 37°C were labelled with R-WGA, and incubated statically for 20 min before examination by fluorescence microscopy. DAPI was also used to label eDNA in order to further examine the qualitative difference in DAPI labelling between Δ*ypsI* Δ*ytbI* and wild-type.

Figure 3.7 shows that both DAPI and R-WGA label the LCBs formed by Δ*ypsI* Δ*ytbI*, however very little labelling is observed in wild-type (compare Figure 3.7 i with ii). For both DAPI and R-WGA, the labels are not dispersed evenly through the Δ*ypsI* Δ*ytbI* biofilm. For eDNA, DAPI labels what appears like a network of biofilm material, with large gaps devoid of cells (Figure 3.7 a ii), while for R-WGA, labelling is pronounced in punctuated spots throughout the biofilm (Figure 3.7 b ii). The eDNA in the LCBs formed by the Δ*ypsI* Δ*ytbI* mutant can also be labelled with ethidium bromide (data not shown).

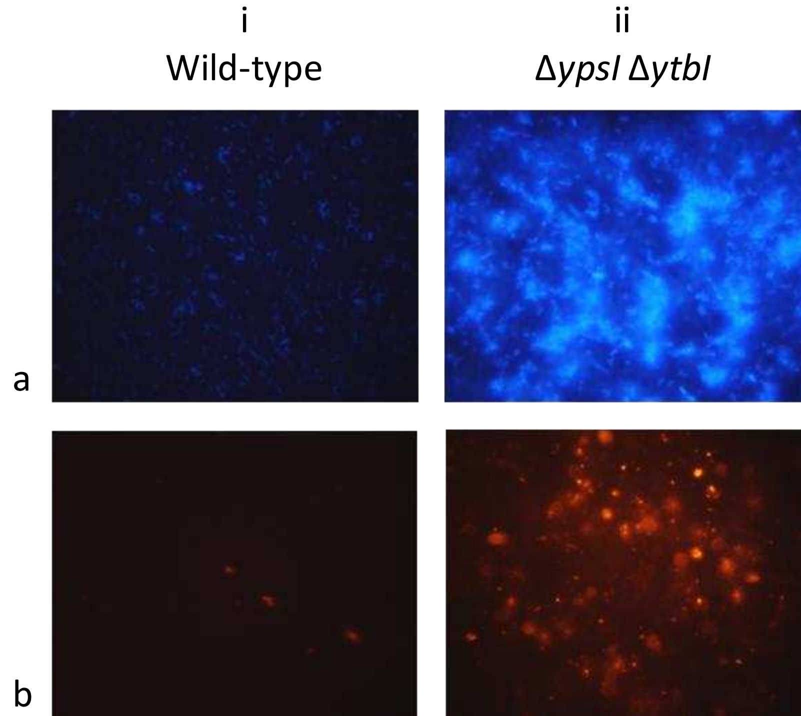


Figure 3.7| Biofilm matrix components are abundant in LCBS. The LCBs formed by the Δ*ypsI* Δ*ytbI* mutant stains abundantly for extracellular matrix components eDNA (labelled with DAPI, ii a) and β-1,6-*N*-acetyl-D-glucosamine (labelled with R-WGA, ii b). LCBs are absent in wild-type and labelling by DAPI or R-WGA is substantially reduced (i a & i b).

Together these results show that following growth at 37°C, Δ*ypsI* Δ*ytbI* cells coalesce into progressively larger clumps- possibly their density causes them to settle and sediment into LCBs. The Δ*ypsI* Δ*ytbI* cells appear incredibly sticky, whereby, under the microscope, clumps passing by one another will readily associate together. These LCBs label heavily for the biofilm matrix components eDNA and EPS, and possess a three-dimensional architecture which is not observed in the wild-type.

3.2.2.2 Extracellular DNA is an important structural component of LCBs

The addition of DNase to liquid cultures of several species of bacteria prevents the formation biofilms similar to the LCBs observed in this study (Allesen-Holm et al., 2006, Godefroid et al., 2010, Seidl et al., 2008, Håvarstein et al., 2006, Kreth et al., 2009). To investigate whether extracellular DNA played a role in maintaining the structure of *Y. pseudotuberculosis* LCBs, Δ*ypsI* Δ*ytbI* LCBs were treated with DNase I, an enzyme which can cleave both double stranded and single stranded DNA, labelled with DAPI and observed by CLSM. The treated and untreated cultures were also left statically for 20 min in spectrophotometer cuvettes to visually assess the formation of LCBs. Figure 3.8 shows that the addition of DNase I prevents the formation of LCBs by Δ*ypsI* Δ*ytbI* (compare Figure 3.8 i and ii). Furthermore DAPI staining demonstrates that most of the DNA observed on Δ*ypsI* Δ*ytbI* is extracellular, since very little DAPI labelling can be observed in the sample treated with DNase I compared with untreated (compare Figure 3.8 c i and c ii).

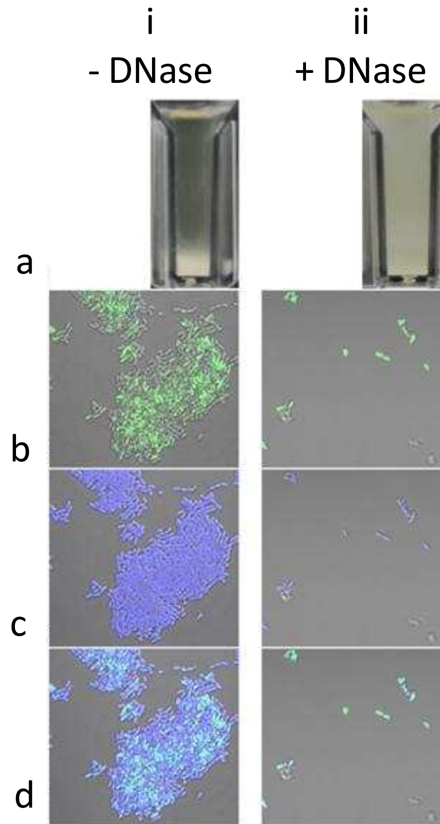


Figure 3.8| LCB formation in Δ*ypsI* Δ*ytbI* is prevented by the addition of DNAse I. When Δ*ypsI* Δ*ytbI* mutant cultures are grown at 37°C overnight, the cells can be observed by CLSM to associate into large clumps (i b-d). These clumps are not observed if DNase I is added to the cultures prior to examination by microscopy (ii b-d). When these cultures are incubated statically at room temperature in spectrophotometer cuvettes, the Δ*ypsI* Δ*ytbI* mutant culture sediments (i a), which is not observed when the culture is treated with DNase I (ii a). CLSM reveals large cell aggregates of bacteria expressing *gfp3* in the DNase I untreated control (i b), which are not present when cells are treated with DNase I (ii b). DAPI labelling is also reduced following DNase I treatment (compare ii c & i c), confirming that the DNA is present outside of the cells. A composite image of DAPI and GFP shows the co-localisation of DNA and cells (i & ii d).

3.2.3 Supernatant factors influence LCB formation

3.2.3.1 Secretion is involved in LCB formation

Biofilms usually involve the synthesis of extracellular matrix components, such as EPS, eDNA and proteins, which are secreted into the extracellular environment. To examine in more detail the contribution of secreted factors to the LCB phenotype, a series of experiments were carried out whereby washed cell pellets of the non-LCB forming wild-type were mixed with sterile-filtered supernatant taken from wild-type or the Δ*ypsI* Δ*ytbI* mutant grown overnight at 37°C or 30°C. In this way, if the propensity for the Δ*ypsI* Δ*ytbI* mutant to form LCBs was carried in the supernatant, it should be possible to induce LCB formation in the wild-type by supplying the cells with culture supernatant from the Δ*ypsI* Δ*ytbI* mutant.

Figure 3.9 shows the LCBs formed by the Δ*ypsI* Δ*ytbI* mutant can be induced in the wild-type by re-suspension in Δ*ypsI* Δ*ytbI* supernatant from growth at either 37°C or 30°C, indicating that factors present in the spent growth medium are important for LCB formation (Figure 3.9, c & d). Interestingly, only wild-type cells grown at 37°C were sensitive to LCB induction by Δ*ypsI* Δ*ytbI* supernatant, whereas wild-type cells grown at 30°C do not show this phenotype when exposed to Δ*ypsI* Δ*ytbI* mutant culture supernatant (compare Figure 3.9, c & g). However, Δ*ypsI* Δ*ytbI* mutant culture supernatant harvested from cultures grown at 22°C does not induce LCB formation in the wild-type (data not shown).

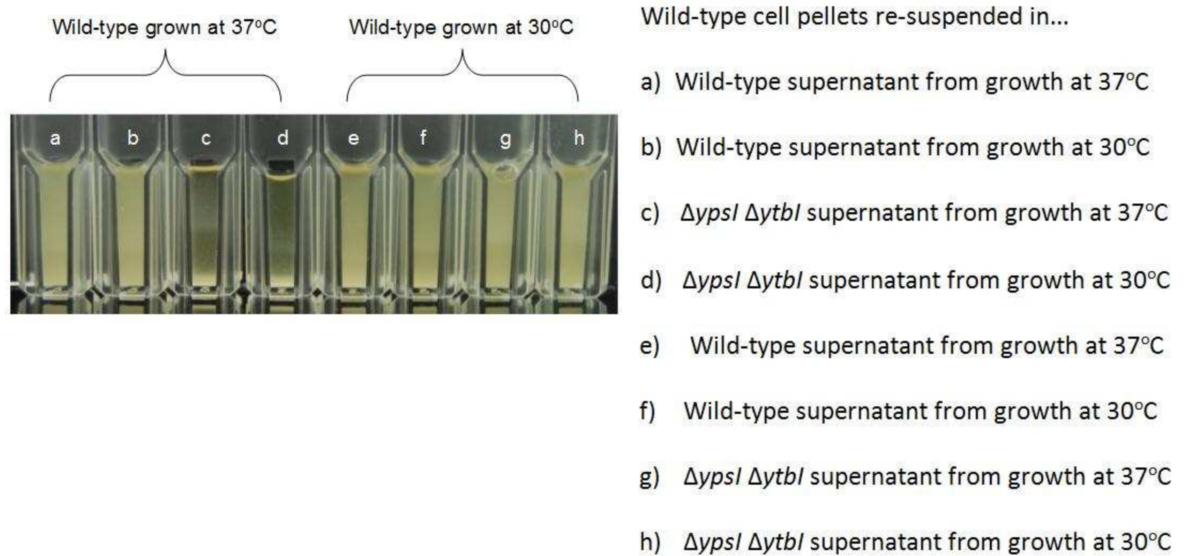


Figure 3.9| The Δ*ypsI* Δ*ytbI* mutant supernatant can induce LBC formation in wild-type grown at 37°C. When sterile filtered supernatants from Δ*ypsI* Δ*ytbI* cultures, grown at either 30°C or 37°C, are used to re-suspend cell-pellets of the wild-type, cells grown at 37°C are induced to form LCBs (c / d). However, wild-type cells grown at 30°C do not respond to Δ*ypsI* Δ*ytbI* supernatant by forming LCBs (g / h). Wild-type supernatant has no LCB-inductive effect on wild-type cells, irrespective of growth temperature (a, b, e, and f).

These results suggest a supernatant factor is produced during growth of the Δ*ypsI* Δ*ytbI* mutant, but not the wild-type, at 30°C and at 37°C and is involved in the LCB phenotype, as it can induce LCB formation in wild-type cells. However, the growth temperature clearly plays a role in determining the ability for wild-type cells to form LCBS, since the wild-type must be grown at 37°C for Δ*ypsI* Δ*ytbI* mutant culture supernatant to induce LCBS, and LCBs cannot be induced by Δ*ypsI* Δ*ytbI* mutant culture supernatant following growth of the wild-type at 30°C.

To investigate the factor in the culture supernatant further, Δ*ypsI* Δ*ytbI* mutant culture supernatant was subjected to heat treatment and filtration through protein concentration columns to give more information on the nature of the

supernatant factor produced by the Δ*ypsI* Δ*ytbI* mutant that could induce LCB-formation in the wild-type. The treated supernatant was then applied to wild-type cells to probe for any effect of these treatments on the ability for Δ*ypsI* Δ*ytbI* supernatant to induce LCB formation.

3.2.3.2 Boiling of Δ*ypsI* Δ*ytbI* supernatant prevents LCBs

Apart from certain heat-stable exotoxins, the proteins from most non-extremophilic bacteria are heat labile. To investigate the effect of heat treatment on the ability of Δ*ypsI* Δ*ytbI* mutant supernatant to induce LCB formation in wild-type cells, sterile filtered Δ*ypsI* Δ*ytbI* mutant supernatant was heated in a boiling water bath for 30 min. This heat-treated supernatant was then used to re-suspend wild-type cells as described earlier. Figure 3.10 shows heat treated Δ*ypsI* Δ*ytbI* supernatant loses the ability to induce LCB formation on wild-type cells (compare Figure 3.10 a & b), which suggests the factor in Δ*ypsI* Δ*ytbI* mutant supernatant is heat-labile.

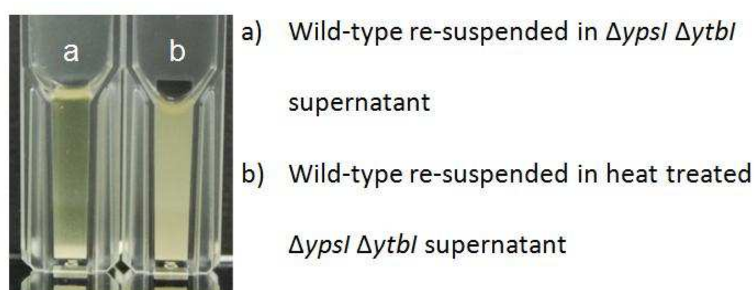
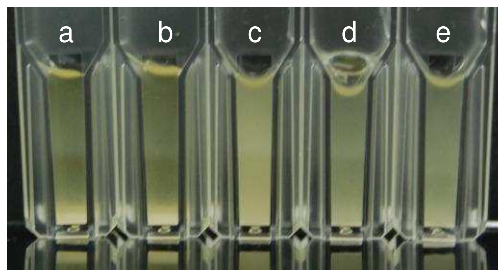


Figure 3.10| The LCB-inducing factor in Δ*ypsI* Δ*ytbI* mutant culture supernatant is heat-labile. When wild-type cells are re-suspended in Δ*ypsI* Δ*ytbI* supernatant, they form LCBs (a). However, the LCB-inductive effect of Δ*ypsI* Δ*ytbI* mutant culture supernatant can be abolished by heating in a boiling water bath for 30 min (b).

3.2.3.3 Excluding macromolecules from Δ*ypsI* Δ*ytbI* mutant supernatant prevents LCB

Given that heat-treating Δ*ypsI* Δ*ytbI* mutant culture supernatants abolishes LCB formation in the wild-type, there was a strong possibility that the supernatant factor produced by the Δ*ypsI* Δ*ytbI* mutant was a large macromolecule. To investigate this possibility, size exclusion experiments were carried out on Δ*ypsI* Δ*ytbI* mutant supernatants by centrifugation through protein concentration columns (10, 30 and 100 kDa), and mixing the resulting flow-through with wild-type cell pellets, as previously described. Figure 3.11 reveals that when macromolecules up to 100 kDA are excluded from the flow through, the resulting supernatant cannot induce LCB formation in wild-type cells when compared with un-filtered controls.



WT re-suspended in Δ*ypsI* Δ*ytbI* supernatant…

a) Unfiltered

b) Filtered through 0.02 μm membrane

c) Filtered through 100 kDa cut-off membrane

d) Filtered through 30 kDa cut-off membrane

e) Filtered through 10 kDa cut-off membrane

Figure 3.11| Large macromolecules in Δ*ypsI* Δ*ytbI* mutant culture supernatant are required to induce LCBs in the wild-type. When unfiltered Δ*ypsI* Δ*ytbI* mutant supernatant and filtered to only remove Δ*ypsI* Δ*ytbI* cells is used to re-suspend wild-type (WT) cell pellets, LCBs form (a, b). However, when large macromolecules are excluded from the supernatant by passing through size exclusion filters, the resulting flow-through cannot induce wild-type cells to form LCBs (c, d, and e).

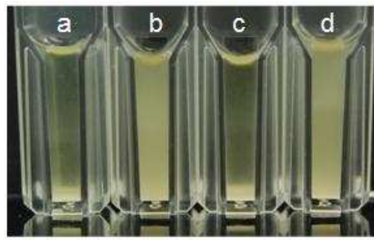
The results of these experiments suggested that the factor in Δ*ypsI* Δ*ytbI* mutant supernatant that is capable of inducing LCB formation in the wild-type, is a large-molecular weight (>100 kDa) heat labile molecule.

3.2.4 Cell surface factors are involved in LCB formation

Wild-type cells only form LCBs following growth at 37°C, which suggests that temperature regulated processes also play a role in LCB formation. Several temperature-regulated proteins in *Y. pseudotuberculosis* are exposed on the cell surface during growth at 37°C, including YadA, Ail, the pH 6 antigen, and the Ysc injectisome (Bolin et al., 1982), leading to the possibility that any of these may interact with the Δ*ypsI* Δ*ytbI* supernatant factor to cause LCB formation. Additionally, *Y. pseudotuberculosis* also switches its LPS structure according to temperature (Rebeil et al., 2004), and since LPS is also known to impact on the LCB phenotype in other bacteria (Diderichsen, 1980, Moller et al., 2003, Sheng et al., 2008), this could also play a role in LCB formation in *Y. pseudotuberculosis*.

3.2.4.1 Protease treatment of cell pellets prevents LCB formation

To investigate the impact of cell surface proteins and LPS on the formation of LCBs, cell pellets of the wild-type and Δ*ypsI* Δ*ytbI* mutant cultured at 37°C were treated with proteinase K and subjected to Δ*ypsI* Δ*ytbI* mutant supernatant as previously described. This would strip surface proteins from the cell, and also removes LPS (Kitchens and Munford, 1998). Figure 3.12 reveals cell surface proteins, or perhaps LPS, are important for the ability of the Δ*ypsI* Δ*ytbI* mutant to form LCBs, and for Δ*ypsI* Δ*ytbI* mutant supernatant to induce LCB formation in the wild-type, since proteinase K treatment of cell pellets prevents these cells from forming LCBs.



a) Wild-type re-suspended in Δ*ypsI* Δ*ytbI* supernatant

b) Protease treated wild-type re-suspended in Δ*ypsI* Δ*ytbI* supernatant

c) Δ*ypsI* Δ*ytbI* re-suspended in Δ*ypsI* Δ*ytbI* supernatant

d) Protease treated Δ*ypsI* Δ*ytbI* re-suspended in Δ*ypsI* Δ*ytbI* supernatant

Figure 3.12| Proteinase K treated wild-type cells do not form LCBs in response to Δ*ypsI* Δ*ytbI* mutant supernatant. When wild-type or Δ*ypsI* Δ*ytbI* mutant cells are re-suspended in Δ*ypsI* Δ*ytbI* mutant culture supernatant, LCBs form (a & d). Conversely, when either wild-type or Δ*ypsI* Δ*ytbI* cells are treated with proteinase K prior to being re-suspended in Δ*ypsI* Δ*ytbI* mutant culture supernatant, LCBs are prevented from forming (b & d).

3.2.4.2 Chromosomally encoded factors, not YadA, are involved in LCB formation

YadA is a critical adhesin for *Y. pseudotuberculosis* with a well-documented role in cellular auto-agglutination (El Tahir and Skurnik, 2001). This virulence factor is encoded on the pYV plasmid, and since cell surface proteins are important for LCB formation, if YadA is involved then a pYV negative derivative of wild-type *Y. pseudotuberculosis* should not be able to form LCBs when exposed to Δ*ypsI* Δ*ytbI* supernatant. In order to test this hypothesis, a pYV-negative (pYV^-) derivative of wild-type YPIII was isolated and cell pellets of this strain were used alongside a pYV^+ control in LCB experiments. Figure 3.13 shows that pYV^- wild-type YPIII forms LCBs in response to Δ*ypsI* Δ*ytbI* mutant supernatant, in a similar manner to the pYV^+ control, indicating that the pYV does not encode the cell surface factor responsible for LCB formation.

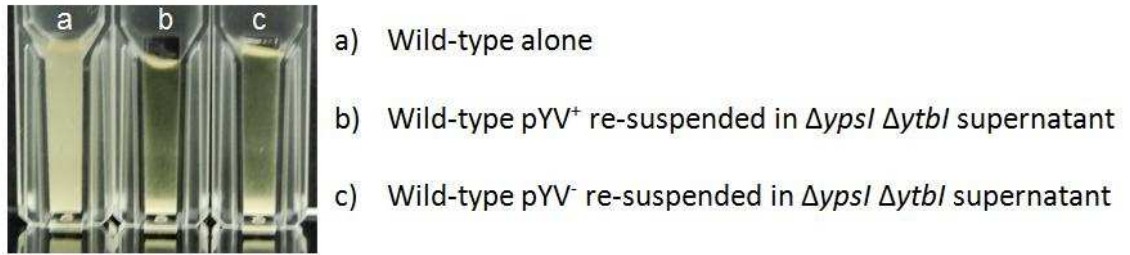


Figure 3.13| Wild-type cells do not require the pYV to be induced to form LCBs by Δ*ypsI* Δ*ytbI* supernatant. Wild-type cells do not normally show LCB formation (a), however LCBs form when wild-type cells are re-suspended in Δ*ypsI* Δ*ytbI* mutant supernatant, and both pYV^{+} and pYV^{-} wild-type strains can be induced to form LCBs (b & c).

These data suggest that the temperature-regulated cell surface factor that is necessary for LCB formation is not encoded on pYV, but is probably located on the chromosome.

3.2.5 The pYV encodes the supernatant factor required for LCBs

Although the cell surface factor involved in LCB formation appears to be encoded on the chromosome, there was a possibility that the supernatant protein involved in inducing LCB formation in wild-type cells was associated with pYV. To investigate this, the pYV plasmid was cured from the Δ*ypsI* Δ*ytbI* mutant (Δ*ypsI* Δ*ytbI* pYV^{-}), and supernatants examined for their ability to induce LCBs in the wild-type when compared with supernatants from the Δ*ypsI* Δ*ytbI* pYV^{+} mutant.

Figure 3.14 shows that the pYV negative Δ*ypsI* Δ*ytbI* mutant does not form LCBs, and supernatant taken from this strain cannot cause LCBs to form in wild-

type cells when compared with supernatants taken from cultures of a Δ*ypsI* Δ*ytbI* pYV^{+} mutant.

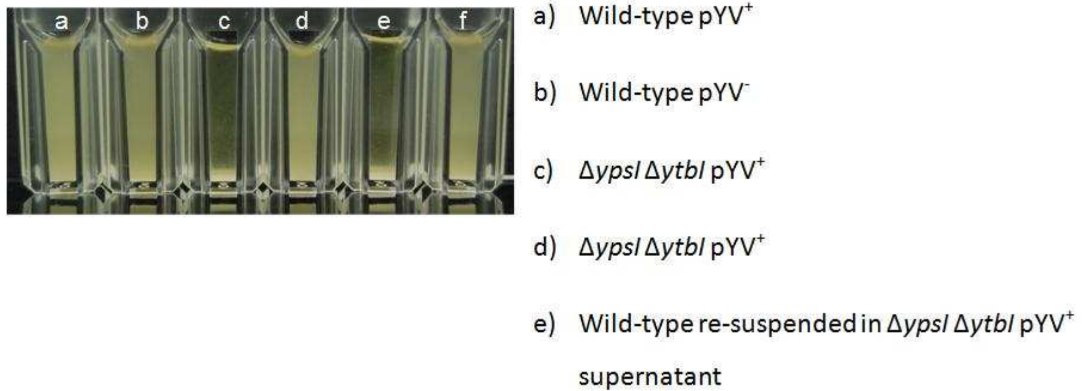


Figure 3.14| Liquid culture biofilms require a pYV encoded supernatant factor. Unlike the LCBs formed by Δ*ypsI* Δ*ytbI* pYV^{+}, when the pYV plasmid is cured from Δ*ypsI* Δ*ytbI*, no LCBs are formed (compare c & d), similar to the wild-type, which does not form LCBs regardless of pYV status (a & b). Similarly, when sterile filtered supernatant is taken from Δ*ypsI* Δ*ytbI* pYV^{-} cultures and applied to wild-type cells, no LCBs form (f) unlike when wild-type cells are re-suspended in supernatant taken from Δ*ypsI* Δ*ytbI* pYV^{+} cultures, which induces LCBs (e).

3.2.6 T3S is involved in the formation of LCBs

3.2.6.1 Yop-related proteins are abundant in the culture supernatants of strains which form LCBs

Since the presence of pYV was essential for the production of the supernatant factor(s) responsible for inducing LCB formation, and the fact the pYV encodes the Yop-Ysc T3SS which plays a large role in protein secretion in *Y. pseudotuberculosis*, it was important to investigate protein differences between the supernatants of the mutants which formed LCBs (the Δ*ypsR*, Δ*ytbI*, Δ*ytbR*, Δ*ypsI* Δ*ytbI* and Δ*ypsR* Δ*ytbR* mutants) and those that did not (wild-type, the

Δ*ypsI* mutant and the complement Δ*ypsI* Δ*ytbI* pSA291 strain). In order to do this, these strains were grown at 37°C or 30°C for use in LCB experiments as previously described, and the proteins present in cell-free culture supernatant precipitated and visualised by SDS-PAGE (Figure 3.15).

When culture supernatants are analysed by SDS-PAGE, the Δ*ypsR*, Δ*ytbI*, Δ*ytbR*, and Δ*ytbI* mutants all produced a range of supernatant proteins during growth at 37°C (Figure 3.15 a) or 30°C (Figure 3.15 b), which were reduced in cultures of the wild-type. When investigated by MALDI-ToF, these protein bands were identified as the Yop regulon proteins YopH, YopM, YopN, and LcrV. Similar to the Δ*ytbI* mutant, the Δ*ypsI* Δ*ytbI* mutant also produces this range of supernatant proteins, which is reduced when functional *ypsI* and *ytbI* are restored to on pSA291 (Figure 3.15 c). The same protein profile was identified in the Δ*ypsR* Δ*ytbR* mutant, but absent when the strains were cultured at 22°C (data not shown). Figure 3.15 (c) shows a 4 – 14 % gradient gel, which can resolve YopH and YopM separately, where they co-migrate in normal 10 % gels shown in Figure 3.15 (a) and Figure 3.15 (b), as has been reported previously (Leung et al., 1990).

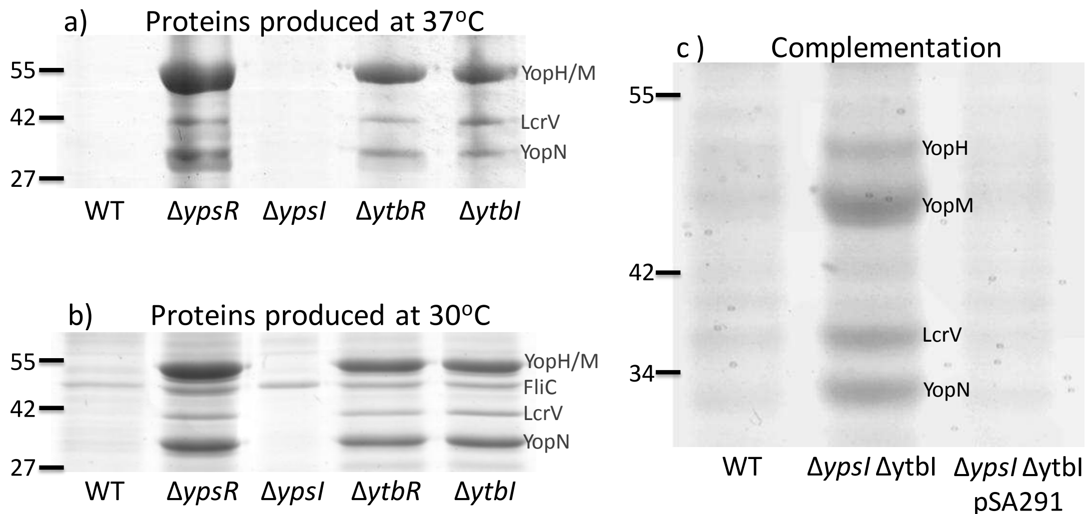


Figure 3.15| The secretion of Yop-related proteins is regulated by QS. Unlike the wild-type and Δ*ypsI* mutant, the Δ*ypsR*, Δ*ytbR* and Δ*ytbI* mutants all produce a range proteins in the supernatant during culture at 37°C (a) and 30°C (b), in the same conditions in which LCBs form. MALDI-ToF identifies these as the Yop-related proteins YopH, YopM, LcrV and YopN. Flagellin (FliC) was also observed in the growth supernatant of all strains during growth at 30°C (b). By separating the supernatant proteins produced by the Δ*ypsI* Δ*ytbI* mutant during growth at 37°C, YopH and YopM can be separately resolved, in addition to LcrV and YopN, and when functional *ypsI* and *ytbI* is restored to the Δ*ypsI* Δ*ytbI* mutant on pSA291, the production of Yop-related proteins into the supernatant is reduced to levels similar to the wild-type (c).

To rule out the possibility that the increased levels of proteins in the Δ*ypsI* Δ*ytbI* mutant culture supernatant was a result of increased cell lysis of the mutant, cultures were stained with propidium iodide. Propidium iodide, used in cell viability assays, enters cells with compromised membranes and fluoresces red (Boulos et al., 1999). No difference was observed in the labelling by propidium iodide between the Δ*ypsI* Δ*ytbI* mutant and the wild-type, suggesting that increased cell lysis does not explain the appearance of Yops in the supernatant (data not shown).

These results demonstrate that the pYV is essential for the production of a supernatant protein which is secreted and can induce LCB formation in wild-type, and suggests that this protein(s) is associated with the Yop regulon, since Yop proteins are present in culture supernatant under the same conditions as promote LCB formation.

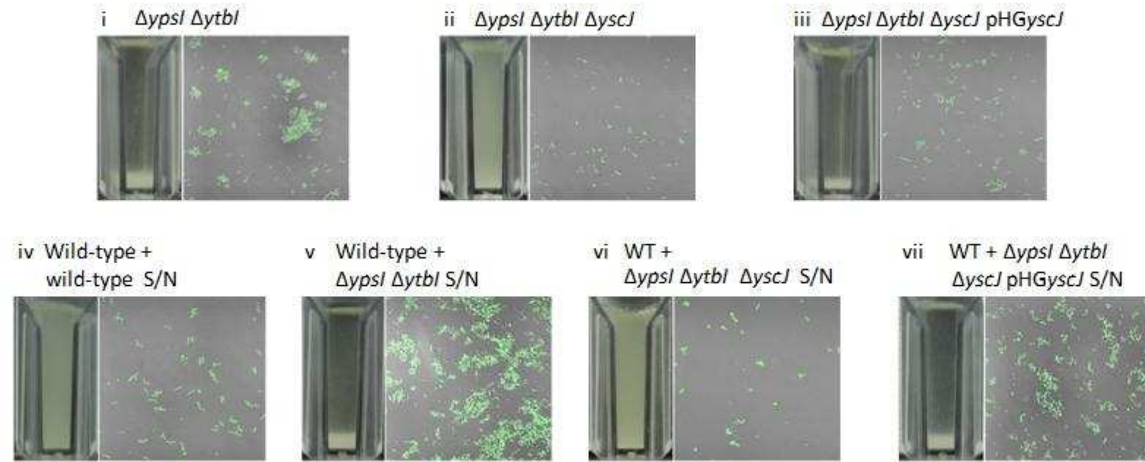
3.2.6.2 Secretion via the Ysc injectisome is necessary for LBC formation

The appearance of Yop-related proteins in Δ*ypsI* Δ*ytbI* mutant culture supernatant led to the possibility that one or more of these proteins was involved in the LCB phenotype. To confirm that LCBs result from the induction of functional T3SS, rather than a result of other genes on the pYV, the Δ*ypsI* Δ*ytbI* mutant was modified by deleting *yscJ*, a structural component of the inner-ring of the Yop-Ysc injectisome (Diepold et al., 2010). This component is essential for the construction of the full injectisome needle, and mutants in *yscJ* cannot secrete Yops (Allaoui et al., 1995b).

To investigate the role of the T3SS, the Δ*ypsI* Δ*ytbI* Δ*yscJ* triple mutant was examined for LCB formation alongside the complemented Δ*ypsI* Δ*ytbI* Δ*yscJ* pHGyscJ strain, in which *yscJ* is restored on pHG327. Sterile filtered supernatant from the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant and Δ*ypsI* Δ*ytbI* Δ*yscJ* pHGyscJ strain was also used to re-suspend wild-type cells in LCB experiments as previously described (Figure 3.16 a). To confirm that YscJ was necessary for the appearance of Yop related proteins in the supernatant of the Δ*ypsI* Δ*ytbI* mutant, the proteins present in culture supernatants of the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant and Δ*ypsI* Δ*ytbI* Δ*yscJ* pHG*yscJ* strain was also investigated by SDS-PAGE (Figure 3.16 b)

Figure 3.16 shows that the presence of a functional copy of *yscJ* is essential for the LCB phenotype of the Δ*ypsI* Δ*ytbI* mutant, the ability for the Δ*ypsI* Δ*ytbI* mutant culture supernatant to cause LCB formation in wild-type cells (Figure 3.16 a); and for the secretion of Yop-related proteins into the culture supernatant (Figure 3.16 b). Unlike the Δ*ypsI* Δ*ytbI* mutant (Figure 3.16 a i & a v), the injectisome-minus Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant could not form LCBs, nor could supernatant harvested from the strain induce LCBs in wild-type cells (Figure 3.16 a ii & a vi). Complementation with functional *yscJ* on pHG*yscJ* partially restores LCBs (Figure 3.16 a iii), and supernatant from the complemented strain could induce LCB formation in the wild-type (Figure 3.16 a vii). Furthermore, by re-suspending Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant cell pellets in supernatants from the ΔypsI Δ*ytbI* or Δ*ypsI* Δ*ytbI* Δ*yscJ* pHGyscJ strains, LCB formation could be restored (data not shown). Similarly, when the proteins present in culture supernatants were investigated, no Yop-related proteins were found in the culture supernatant of the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant, unlike the Δ*ypsI* Δ*ytbI* mutant. When *yscJ* is restored on pHG*yscJ*, the proteins YopH, YopM, LcrV and YopN can be observed (Figure 3.16 c). YopH and YopM have co-migrated in this gel, as has been reported previously in one-dimensional electrophoresis (Leung et al., 1990). Together, these data indicates that a functional T3SS is essential for the secreted protein component of the LCB phenotype.

a) Liquid culture biofilms



b) Proteins produced at 37°C

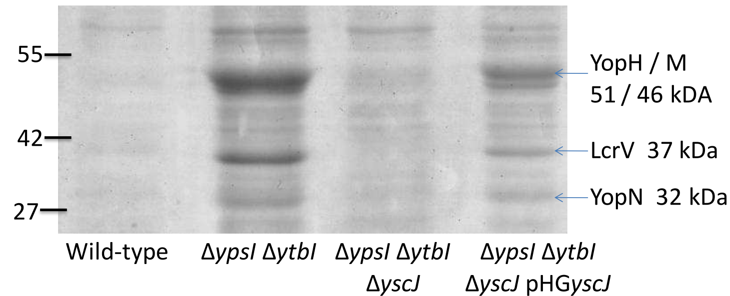


Figure 3.16| Functional YscJ is required for the formation of LCBs and secretion of Yop-related protein. When investigated by the cuvette assays and microscopy of cultures expressing *gfp3* (a), in the absence of functional *yscJ*, LCBs are prevented from forming in the Δ*ypsI* Δ*ytbI* mutant (compare a i & a ii), and LCB formation is recovered in the complemented strain Δ*ypsI* Δ*ytbI* Δ*yscJ* pHGyscJ (a iii). Wild-type supernatant (S/N) has no effect when applied to wild-type cells (a iv), however when wild-type cells are re-suspended in the Δ*ypsI* Δ*ytbI* mutant supernatant, the wild-type forms LCBs (a v). This is not observed when supernatants from the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant is used to re-suspend wild-type cells (a vi) but is when using supernatant from the Δ*ypsI* Δ*ytbI* Δ*yscJ* pHGyscJ strain (a vi). When supernatant proteins are investigated by SDS-PAGE (b), the Yop-related proteins present in the culture supernatant of the Δ*ypsI* Δ*ytbI* mutant, are absent in the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant, but restored when functional *yscJ* is supplemented on pHG*yscJ*.

3.2.7 Identification of genes involved in the formation of LCBs

3.2.7.1 *Y. pseudotuberculosis flhDC, fliA* and *flhA* mutants also form LBCs

In *Y. enterocolitica*, the flagella system has previously been linked to the control of both LCB-like biofilms and the T3SS, where a *flhDC* mutant forms pronounced LCB-like sediments following static incubation and over-produced Yop-related proteins (Bleves et al., 2002), while the over-production of Yop proteins has also been observed in a *fliA* mutant (Horne and Prüß, 2006). Similar to *Y. enterocolitica* (Atkinson et al., 2006), motility is controlled by QS in *Y. pseudotuberculosis* (Atkinson et al., 1999, Atkinson et al., 2008), and so to investigate whether the motility system was involved in controlling T3S and LCB formation in *Y. pseudotuberculosis*, the Δ*flhDC*, Δ*fliA* and Δ*flhA* mutants were cultured overnight at 37°C and used in LCB experiments as previously described (Figure 3.17 a). To investigate the possibility that the flagella system also regulates the T3SS, proteins present in the culture supernatant from strains grown at 37°C (Figure 3.17 b) and 30°C (Figure 3.17 c) were precipitated and analysed by SDS-PAGE.

These results show that LCBs form in the Δ*flhDC*, Δ*fliA* and Δ*flhA* mutants during growth at 37°C, where LCBs are not formed by the wild-type under these conditions (Figure 3.17 a). Furthermore, investigation of the proteins present in the supernatant reveals that several proteins are up-regulated in the mutants compared with the wild-type during culture at 37°C (Figure 3.17 b) or 30°C (Figure 3.17 c). This up-regulated protein profile is similar to that observed in the QS mutants, and when investigated by MALDI-ToF, the proteins were

revealed to be YopH, YopM, LcrV and YopN. YopH and YopM have co-migrated in Figure 3.17 (c), as has been reported previously in one-dimensional electrophoresis (Leung et al., 1990), although they have been resolved separately in Figure 3.17 (b).

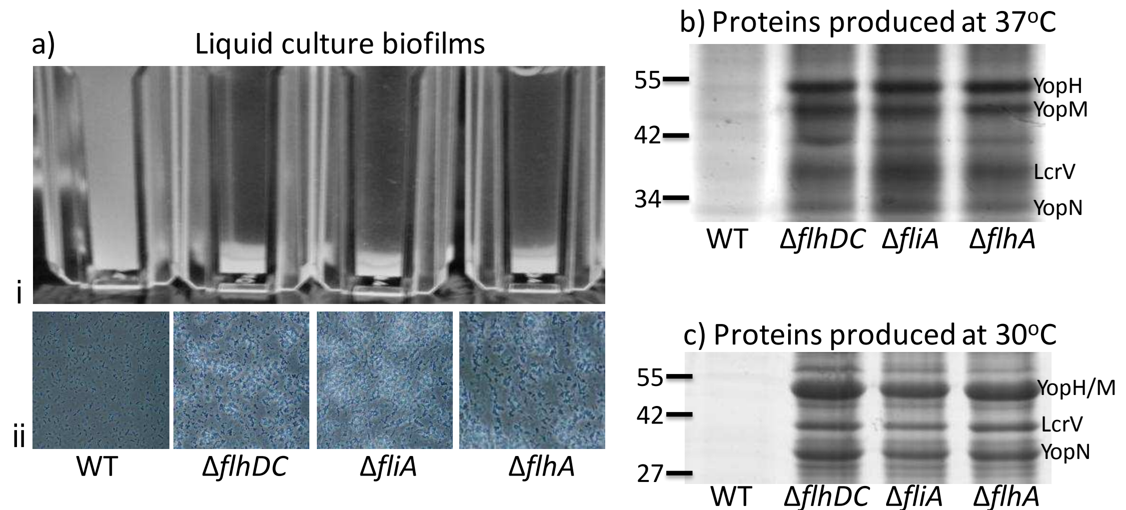


Figure 3.17| The flagella regulators FlhDC and FliA and the secretion component FlhA affect LCB formation and Yop production. The Δ*flhDC*, Δ*fliA* and Δ*flhA* mutants form LCBs following growth at 37°C, as revealed by the cuvette assay (a i) and by phase microscopy (a ii). The formation of LCBs at this temperature correlates with the up-regulated release of several proteins into the growth supernatant which are not present in the wild-type (b). MALDI-ToF identifies these to be the Yop virulon associated proteins YopH, YopM, LcrV and YopN. These proteins are also found in the culture supernatant of the mutants, but not the wild-type, during growth at 30°C (c). Protein marker weights are given in kDa.

Together, these results show that, as for *Y. enterocolitica*, FlhDC and FliA in *Y. pseudotuberculosis* repress T3SS-dependent secretion, and that the appearance of Yop-related proteins in the culture supernatants of the Δ*flhDC* and Δ*fliA* mutants correlates with the formation of LCBs. Interestingly, the flagella secretion component FlhA, which is required for the T3S-dependent export of the flagella (Macnab, 2004), is also required to repress the production of Yop-related proteins and LCB formation.

3.2.7.2 A Transposon mutagenesis screen identifies candidate genes involved in the LCB phenotype

In light of these results, a transposon mutagenesis approach was used to identify *Y. pseudotuberculosis* genes involved in the supernatant and cell-surface factors responsible for the Δ*ypsI* Δ*ytbI* dependent LCB phenotype. The Tn5 transposon would insert randomly into either the Δ*ypsI* Δ*ytbI* chromosome or pYV, and clones which do not exhibit LCBs would be expected to carry mutations in genes responsible for this phenotype.

Approximately 800 Tn5 insertion mutants were individually screened for their ability to form LCBs, and nine clones were determined to be both LCB negative and pYV^+, since PCR directed against pYV encoded *yscU* yielded an amplification product (data not shown). The mutated genes carrying the Tn5 insertion were cloned and mapped to the *Y. pseudotuberculosis* YPIII genome (Table 3.1).

Despite the requirement for the pYV plasmid in the LCB phenotype, all the transposon insertions characterised were in chromosomal loci. Four of these insertionally mutated genes encode putative intracellular proteins: the regulator of the pH 6 antigen, *psaE* (YPK_2671), the auto-inducer 2 processing enzyme *lsrG* (YPK_3655) (Miller and Bassler, 2001), a catalase *katA* (YPK_2855), and an unidentified AraC-type regulator (YPK_3661). The insertions into all these, except *lsrG*, would be expected to exert polar effects since the insertions create frameshift mutations. Four clones attenuated for LCBs possessed insertions in putative membrane proteins. An Ail / Lom family protein gene YPK_2061 and a putative auto-transporter (YPK_0763) both contain in-frame insertions, while a putative membrane protein gene homologous to a family only found in enantomopathogenic bacteria such as *Xenorhabdus* and *Photorhabdus* spp. (YPK_1310), and a subunit of an ATP synthase membrane proton channel

(YPK_4420), both contain insertions which would be predicted to cause frame-shift mutations. The final clone identified possesses a frame-shift insertion into a hypothetical protein encoded by YPK_3644.

After these clones were identified as non-LCB forming mutants, they were further investigated by re-suspending the transposon mutant cells grown at 37°C in supernatant harvested from Δ*ypsI* Δ*ytbI* mutant cultures. Strains which could be induced to form LCBs were designated + and those unable to form LCBs designated -. None of the clones were able to form LCBs after induction with Δ*ypsI* Δ*ytbI* mutant supernatant, which suggested that all the loci influenced the ability for Δ*ypsI* Δ*ytbI* mutant to produce the cell surface factor required for LCB formation. Following this, supernatant was harvested from transposon mutant cultures and used to re-suspend cells of the wild-type. Supernatants from six clones (YPK_0763, YPK_1310, YPK_2061, YPK_2671 / *psaE*, YPK_2855 and YPK_4420) were able to induce LCB formation in the wild-type, suggesting that these clones were only affected in the production of the cell-surface factor or the cellular response required for LCB formation, and not for the production of the supernatant factor. Three clones (YP_3655 / *lsrG*, YPK_3661 and YPK_3644) showed both cells and supernatants to be incapable of forming or inducing LCBs, suggesting that these 3 loci are required for the production of both the cell-surface and supernatant factor involved in LCB formation.

	(Forward / Reverse strand)	Gene name / location	Description / Function	Cells	Super-natant
Cellular proteins	3046398 (F)	*psaE* YPK_2761	Transcriptional regulator of the pH 6 antigen	-	+
	3147814 (F)	*katA* YPK_2855	Catalase (cytoplasm and periplasm)	-	+
	4035883 (F)	*lsrG* YPK_3655	Antibiotic biosynthesis mono-oxygenase, auto-inducer 2 processing enzyme	-	-
	4042343 (R)	YPK_3661	AraC-like transcriptional regulator, HTH-domain protein (near LSR)	-	-
Membrane proteins	861542 (F)	YPK_0763	Outer membrane auto-transporter barrel domain protein	-	+
	1437968 (F)	YPK_1310	Hypothetical protein, putative membrane protein in *Xenorhabdus nematophila*	-	+
	2288753 (F)	YPK_2061	Ail / Lom family protein	-	+
	4659474 (R)	YPK_4420	ATP synthase membrane proton channel subunit A	-	+
Unknown	4023179 (F)	YPK_3644	Hypothetical protein	-	-

Table 3.1| Loci involved in the LCB phenotype as revealed by a transposon mutagenesis screen. Approximately 800 clones from a random transposon mutant library in the LCB-forming Δ*ypsI* Δ*ytbI* mutant were individually screened for clones which showed restoration to the non-LCB phenotype reminiscent of the wild-type. Nine insertions in loci important for the formation of LCBs were identified, and are categorised by their assumed cellular localisation. The mutants were tested for the ability for cell pellets to form LCBs following re-suspension in Δ*ypsI* Δ*ytbI* mutant supernatant (cells: form LCBs = +, do not form LCBs = -), and for the ability of culture supernatant harvested from growth to induce LCB formation in wild-type cells (supernatant: induces LCBs = +, cannot induce LCBs = -).

3.2.7.3 A transposon insertion into *psaE* links Congo red binding with LCBs

Except for the transposon insertion into *psaE*, all the other insertion mutants exhibited normal growth on CR_{MOX} plates at 37°C, growing as small, red colonies typical of those observed in pYV^{+} *Y. pseudotuberculosis* colonies. However, the transposon mutant in *psaE* (Δ*ypsI* Δ*ytbI* Tn5*::psaE*) grew as large, white colonies, reminiscent of pYV^{-} derivatives of *Y. pseudotuberculosis*, yet amplification of *yscU* showed that this strain carried pYV (data not shown). This raised the possibility that Congo red uptake could be correlated with the ability for *Y. pseudotuberculosis* to form LCBs, since Δ*ypsI* Δ*ytbI* pYV^{-} cells do not form LCBs and, like pYV^{-} wild-type cells, do not bind Congo red when grown on CR_{MOX} plates (data not shown). However, unlike the binding of Congo red in the wild-type, the Δ*ypsI* Δ*ytbI* mutant does not require low calcium to form LCBs. To investigate the possibility that Congo red binding could occur in liquid culture, and was correlated with LCB formation, Δ*ypsI* Δ*ytbI* and the *psaE* transposon mutant were grown alongside wild-type and Δ*ypsI* Δ*ytbI* pSA291 in LB_{CR} for 16 h at 22°C, 30°C and 37°C. From these cultures, cell-free supernatant was harvested, and the difference in residual Congo red left in the medium was determined against bacteria-free LB_{CR}. Figure 3.18 (a) shows that Congo red binding in liquid culture follows a very similar pattern to LCB formation, with almost 90 % of available Congo red bound by the Δ*ypsI* Δ*ytbI* mutant during growth at 37°C, and over 50 % at 30°C. During growth at 22°C however, Δ*ypsI* Δ*ytbI* binds only 17 % of the available Congo red. In contrast wild-type cells appear to bind only 20 % of available Congo red during growth at 30°C, 30 % at 37°C, and 13 % during culture at 22°C. Strikingly, and similar to the loss of LCB formation, the *psaE* transposon mutant (Δ*ypsI* Δ*ytbI* Tn5::*psaE*) bound only approximately 10 % of the available Congo red regardless of temperature.

To supplement this data, the ability for the strains to bind Congo red on agar in the presence of calcium was investigated by growing the strains for 48 h at 22°C, 30°C and 37°C on LB_{CRA}. Representative images of single colonies were captured using a plate microscope at medium magnification (Figure 3.18 inserts). These data show that, as in liquid media, the Δ*ypsI* Δ*ytbI* mutant could bind Congo red during growth on agar plates in calcium containing media during growth at 30°C or 37°C, and that this effect was abrogated when *psaE* was inactivated by the insertion of a transposon. At 30°C, Congo red binding in Δ*ypsI* Δ*ytbI* mutant colonies occurs in a dense, clearly delineated region in the centre of the colony, whereas during growth at 37°C Congo red is bound throughout the colony, except at a thin strip around the perimeter. During growth at 22°C, and similar to the wild-type, the Δ*ypsI* Δ*ytbI* mutant does not bind Congo red, however during growth at 30°C or 37°C, the wild-type appears to bind much less Congo red than the Δ*ypsI* Δ*ytbI* mutant, with only limited and diffuse Congo red visible in the centre of the colony at 37°C.

Together, these results show that the ability for the Δ*ypsI* Δ*ytbI* mutant to form LCBs is correlated to the ability for this strain to bind Congo red, and that the pH 6 antigen, which is regulated by PsaE, may play a role in both these phenotypes.

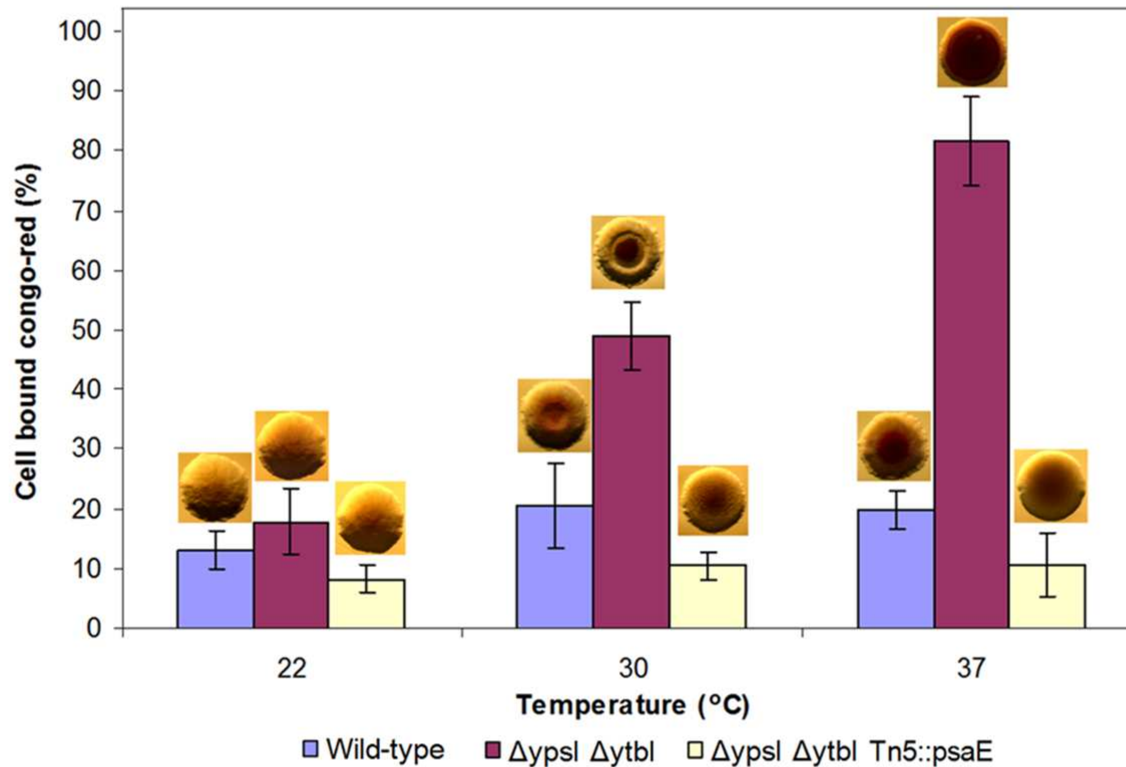


Figure 3.18| Increased pH 6 antigen production probably mediates Congo red binding to Δ*ypsI* Δ*ytbI* mutant cells in liquid culture and on agar. Congo red binding by *Y. pseudotuberculosis* in YLB liquid media and on agar (inserts). On agar and in liquid culture at 37°C and 30°C, the wild-type and the Δ*ypsI* Δ*ytbI* pSA291 strains bind little Congo red, but with some binding in the centre of colonies at 37°C. The Δ*ypsI* Δ*ytbI* mutant binds substantially more Congo red than wild-type on agar and in liquid medium, with the majority of the colony densely stained with Congo red at 37°C, and a central, tightly delineated area of dense Congo red binding at 30°C. In contrast, the Δ*ypsI* Δ*ytbI* Tn5::*psaE* transposon mutant does not bind Congo red on agar plates or in liquid culture at 30°C or 37°C. At 22°C, no strain showed binding of Congo red in colonies, and very little in liquid culture.

3.3 Discussion

3.3.1 Defining cellular aggregation as LCBs

This chapter has elaborated the role for QS in controlling the cell aggregation first described in *Y. pseudotuberculosis* by Atkinson *et al*. (1999), who demonstrated that a Δ*ypsR* mutant displayed substantial clumping following growth at either 30°C or 37°C (Atkinson et al., 1999). The results presented in this chapter show that in addition to the Δ*ypsR* mutant, the Δ*ytbR*; Δ*ytbI*; Δ*ypsR* Δ*ytbR* and Δ*ypsI* Δ*ytbI* mutants all clump during liquid culture in a way not observed in the wild-type or Δ*ypsI* mutant. These bacterial aggregates are surrounded by biofilm matrix components, including eDNA and EPS, similar to that observed following mutation of QS in *R. sphaeroides* (Puskas et al., 1997) and *B. melitensis* (Uzureau et al., 2007), and can be recognised as a type of biofilm. The development of LCBs these mutants shows a remarkable similarity with the development of sessile biofilms, especially those that grow by recruitment of cells from the bulk fluid and interestingly, Tan & Darby (2002) suggested that *Y. pseudotuberculosis* forms biofilms on the nematode worm *C. elegans* in this way (Tan and Darby, 2004). Microscopic examination of these clumps shows that the cells do not settle into a confluent layer, but assemble into a patchwork 3-dimensional structure. Large areas are devoid of cells and matrix components, much like water channels observed in surface attached biofilms which supply water and nutrients throughout the biofilm (Sternberg et al., 1999). The cells do not easily dissociate from the sediment when the biofilm is disturbed; when lightly rotated, strands of biofilm appear; these remain attached to the sediment, and quickly return to the bottom when left static. This suggests that the biofilm possesses some degree of architectural integrity.

This structural integrity may be provided by a mixture of biofilm matrix components including eDNA and EPS. This polysaccharide is probably β-1,6-*N*-acetyl-D-glucosamine, since it displays affinity for the lectin R-WGA, which has previously used to label this polysaccharide in biofilms caused by *Y. pseudotuberculosis* on the nematode worm *C. elegans* (Tan and Darby, 2004, Joshua et al., 2003, Atkinson et al., 2011) and can be found in aggregates of *S. aureus* (Seidl et al., 2008). In LCBs, eDNA appears to be essential for developing and maintaining structure, since DNase I treatment can both prevent LCBs from forming and disrupt developing LCBs; this has also been observed in other bacteria, including *P. aeruginosa* (Allesen-Holm et al., 2006) *B. melitensis* (Godefroid et al., 2010) *S. aureus* (Seidl et al., 2008) and *Streptococcus* spp. (Håvarstein et al., 2006, Kreth et al., 2009). Together, this suggests that biofilm matrix components are commonly used by bacteria to maintain close association of cells when suspended in liquid culture. Interestingly, when investigating cell viability using propidium iodide, there was a possibility that the label would also stain the eDNA surrounding the Δ*ypsI* Δ*ytbI* mutant cells- this did not occur, and Δ*ypsI* Δ*ytbI* cells labelled similarly to the wild-type. Both DAPI and ethidium bromide can label the eDNA surrounding Δ*ypsI* Δ*ytbI* cells, which can bind to single stranded and double stranded DNA. Propidium iodide, conversely, cannot bind to single stranded DNA (Van Erp et al., 1988), and so this suggests that the eDNA involved in LCB formation may be single stranded, which has previously been seen in LCBs formed by the bacterium *Rhodovulum sulfidophilum* (Nishimura et al., 2006).

3.3.2 QS mediated repression of LCBs

The results in this chapter show that QS in *Y. pseudotuberculosis* represses the formation of LCBs, since deletion of *ypsR*, *ytbR*, *ytbI*, or both synthases or receptors results in LCB formation. It is possible that normally, this QS-dependent inhibition acts to cause dissociation of cells from clumps as cell-density increases, thereby preventing the formation of aggregates which, depending on the prevailing environmental conditions, are too large. When cell densities are low, clumps of bacteria may benefit each other by offering protection from predation (Matz and Kjelleberg, 2005), facilitating genetic exchange (Dunny et al., 1978) or perhaps even providing the sufficient density to settle when in aqueous environments. At high cell densities however, clumping may be less useful to bacteria by limiting the diffusion of nutrients or preventing dissemination of the bacteria. However, it is also possible that these LCBs develop under conditions in which AHL-QS is normally repressed- for example, AHLs are known to be inactivated by pH-dependent lactonolysis which is accelerated at 37°C (Yates et al., 2002) and evidence suggests that the QS genes in *Y. pseudotuberculosis* are expressed more at 22°C than at 37°C (Atkinson et al., 2008). This suggests that in certain conditions in the mammalian body, AHLs may be down-regulated and quickly degraded which would result in an AHL-negative state, similar to the Δ*ypsI* Δ*ytbI* mutant phenotype. This could indicate that LCBs can develop and are important *in vivo*.

3.3.3 A role for T3S in LCBs

Initial observations that the pYV plasmid was essential for LCB formation in the Δ*ypsI* Δ*ytbI* mutant raised the possibility that YadA may play a role in the LCB phenotype, since YadA is known to mediate auto-agglutination (El Tahir and Skurnik, 2001). However, YadA is unlikely to play a role in the formation of QS-regulated LCBs, since a pYV negative (pYV$^-$) derivative of the wild-type or the Δ*ypsI* Δ*ytbI* mutant, which would lack *yadA*, can be induced to form LCBs following re-suspension in supernatant harvested from cultures of a Δ*ypsI* Δ*ytbI* pYV$^+$ strain. This indicated that the pYV-dependent factor LCBs was present in the supernatant, rather than cell associated. Although it is possible that a subset of YadA expressed by the Δ*ypsI* Δ*ytbI* mutant detaches from the cell and is carried in the supernatant, it was not identified by SDS-PAGE analysis of supernatant proteins. The pYV plasmid is intricately linked to virulence in *Y. pseudotuberculosis*, carrying in addition to *yadA* the genes encoding several other virulence factors including the Yop-Ysc T3SS (Cornelis et al., 1998a).

When the secreted protein profile of the LCB forming mutants was investigated by SDS-PAGE and MALDI-ToF, it became clear that several Yop-related proteins were present under conditions which could cause the induction of LCBs (37°C or 30°C), but absent in supernatants from cultures of the wild-type. These proteins, including LcrV, YopH, YopM, and YopN, are secreted through the injectisome (Cornelis, 2002b, Fields and Straley, 1999, Forsberg et al., 1991). Interestingly, several other Yop proteins were not found in the growth supernatant, including YopE, which is normally highly produced during the activation of the T3SS and is secreted by both the wild-type and the Δ*ypsI* Δ*ytbI* mutant under Yop inducing conditions (data not shown). YopH has previously been proposed to precede YopE in the secretion hierarchy (Wulff-Strobel et al.,

2002), but while the mechanism of this hierarchy is not clear, these results suggests that QS could play a role.

However *in vitro*, the T3SS is usually controlled both by temperature and calcium concentration, only being expressed at 37°C and secretion occurring under low-calcium conditions (Cornelis et al., 1998a). Finding Yop-related proteins in the supernatant of cultures grown at 30°C or 37°C and in the presence of calcium is significant as it shows that the environmental regulation of Yop production and secretion is mediated, at least in part, by QS and suggests that these proteins are involved in LCB formation. This was confirmed following the mutation of *yscJ*, an essential component of the Ysc-injectisome, in the Δ*ypsI* ΔytbI mutant background. Without YscJ, the injectisome cannot be built and no Yop secretion can occur (Silva-Herzog et al., 2008). In this AHL- and secretion- deficient mutant (Δ*ypsI* Δ*ytbI* Δ*yscJ*), LCBs do not form, Yops do not appear in the supernatant, and culture supernatant cannot induce LCB formation in wild-type cells.

By filtering the Δ*ypsI* Δ*ytbI* supernatant used to re-suspend wild-type cells through pores of various exclusion sizes it was established that ability for this supernatant to confer LCBs on wild-type depended on a large molecular weight factor, in excess of 100 kDa, revealing that a large macromolecule is responsible for augmenting LCBs. However, there are no known Yop proteins which are larger than 100 kDa, the largest being YopO / YpkA at 82 kDa (Galyov et al., 1993). It is possible that this protein was retarded by the 100 kDa pore filter, and that YopO causes the LCB phenotype to be conferred by Δ*ypsI* Δ*ytbI* mutant supernatant, however YopO was not specifically identified in the supernatant proteins. It is possible, then, that protein multimers or aggregates in the supernatant are responsible for the LCB phenotype, which is supported by the fact that some type 3 secretion proteins are known to be able to aggregate and

form complexes in the extracellular environment (Ménard et al., 1994, Michiels et al., 1990).

It is possible that *Y. pseudotuberculosis* could possess a mechanism to detect the extracellular accumulation of Yop proteins to measure the destruction of eukaryotic cells, as the Yop proteins are liberated by cell lysis. Perhaps the lysis of host cells, which would normally be assumed to cause the release of inflammatory cytokines and recruitment of the immune system, acts as a warning for *Y. pseudotuberculosis* to assume a clumped phenotype to aid in resisting phagocytosis. If the LCB phenotype is a response to Yops in the extracellular environment, it is perhaps likely that more than one Yop protein or an aggregate can stimulate this behaviour. Investigation of individual Yop effector deletions in the Δ*ypsI* Δ*ytbI* mutant would shed light on the contribution of each protein.

It is interesting to note that bacterial T3SS have previously been linked to LCB formation. In *Mycobacterium tuberculosis*, the adoption of a clumped phenotype results in an increased ability to infect macrophages, and this has been linked to increased T3S (Brennan et al., 2001), while *E. chrysanthemi* requires the T3SS for aggregative pellicle formation at air-liquid interfaces at 37°C (Yap et al., 2005). In addition, when investigating the regulation of the length of the *S. typhimurium* T3SS needle, Kubori *et al*. (2000) discovered that when over-expressing the transcriptional activator of the T3SS, *hilA*, a mutation in *invJ*, which encodes a needle length regulator, resulted in an abundance of elongated needle structures on the cell surface and bacterial clumping (Kubori et al., 2000). It is possible that hyper-activity of the Ysc system in the Δ*ypsI* Δ*ytbI* mutant leads to an abundance of needle complexes on the cell surface, which in turn may cause aggregation. However, this would not explain why supernatant

harvested from the Δ*ypsI* Δ*ytbI* mutant can induce LCB formation in the wild-type or in pYV$^-$ cells.

Alternatively it is possible that the abrogation of LCB formation in the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant is unrelated to the inability for this mutant to secrete Yops or produce needles, but rather depends on the fact that this mutant may be unable to properly control the LCR. A widely used marker for virulence in *Yersinia* is the ability for *Y. pseudotuberculosis* to bind the dye Congo red, in a low-calcium dependent manner, during growth at 37°C. Previously, needle mutants in *yscF* have been shown incapable of binding Congo red under these conditions (Davis and Mecsas, 2007), although mutants which secrete lower or an altered profile of Yops still bind the dye (Mecsas et al., 2001), as do mutants in the Yop effectors such as YopE and YopH (Logsdon and Mecsas, 2003). The Δ*ypsI* Δ*ypsI* Δ*yscJ* mutant, however, does not bind Congo red in a low-calcium medium during growth at 37°C (data not shown). In light of this, there may be other secreted factors related to the LCR, but un-related to T3S *per se*, which are responsible for the LCB phenotype in the Δ*ypsI* Δ*ytbI* mutant. However, this would not explain why pYV$^-$ cells can respond to Δ*ypsI* Δ*ytbI* mutant supernatant by forming LCBs. It is perhaps more likely that proteins secreted into the extracellular environment through the Ysc injectisome are responsible for the link between the LCB phenotype and pYV.

3.3.4 Pathways to the LCB phenotype

3.3.4.1 The flagella system represses LCB formation and Yop production

In *Y. enterocolitica*, FlhDC and FliA are known to negatively regulate the T3SS, and a *flhDC* mutant forms LCB-like biofilms (Bleves et al., 2002, Horne and Prüß, 2006). In *Y. pseudotuberculosis*, the Δ*ypsI* Δ*ytbI* mutant is repressed for *flhDC* expression when compared with the wild-type (Atkinson et al., 2008), and so finding that Δ*flhDC* and Δ*fliA* mutants also form LCBs and are up-regulated for the production of the same Yop-related proteins as the Δ*ypsI* Δ*ytbI* mutant suggests that the flagella regulon is required down-stream of QS for the repression of LCB formation. Interestingly, the same range of Yop-related proteins was found, lacking YopE, in the culture supernatant of the Δ*flhDC* and Δ*fliA* mutants when compared with the Δ*ypsI* Δ*ytbI* mutant, further suggesting that QS regulates LCB formation via the flagella pathway.

Surprisingly, the flagella secretion system component FlhA was also found to be essential for repressing LCB formation and reducing Yop secretion under normally non-inducing conditions. Without FlhA, the majority of the flagellum cannot be constructed (Macnab, 2003), and the Δ*flhA* mutant is non-motile (Atkinson et al., 2011). However, since both *flhDC* and *fliA* are intact in the Δ*flhA* mutant, this suggests that elements of the flagella structure, and not just the flagella regulators, may play a role as check-points in the regulation of T3S and LCB formation. It could be that FlhA itself plays an important regulatory role in governing cellular decisions, and in other bacteria mutation of *flhA* prevents the secretion of virulence factors (Ghelardi et al., 2002). It also impacts on FlgM production (Ghelardi et al., 2002), an anti-sigma factor which sequesters and inactivates FliA (Kutsukake and Iino, 1994) and also mediates internalisation of

P. aeruginosa into cultured epithelial cells in a manner that cannot be explained by a loss of motility, with an 80 % reduction in invasion in a *flhA* mutant, compared with 50 % observed in a *fliC* mutant (Fleiszig et al., 2001). However, it is perhaps more likely that the effects of *flhA* mutation noted are due to the activity of the developing flagella as a regulatory checkpoint. The developing flagellum is known to feed-back information to the cell regarding its status, including the secretion of the anti-sigma factor FlgM upon completion of the of the basal body and hook, which leads to FliA-dependent transcription of *fliC* (Karlinsey et al., 2000). Interestingly, the Δ*fliC* mutant, unlike the Δ*flhA* mutant, does not secrete Yop-related proteins into the culture supernatant (Atkinson et al., 2011) - FliC is only produced subsequent to FliA activation by FlgM export (Kutsukake and Iino, 1994, Ding et al., 2009), which could suggest that FlgM export represents a critical part of the switch between T3S and motility, and that class III flagella genes are not involved in this regulation.

3.3.4.2 Cell surface factors involved in LCBs

Proteinase K can remove cell surface factors important for the formation of LBCs from pYV$^+$ and pYV$^-$ alike, indicating that a cell surface factor encoded on the chromosome was involved in the LCB phenotype. Wild-type cells can only form LCBs at 37°C, suggesting that this factor is normally regulated by temperature. However, QS may also affect this temperature regulation, since LCBs form in the mutants during growth at 30°C (Atkinson et al., 1999). The binding of *Y. pseudotuberculosis* to mammalian cells involves at least three chromosomally encoded surface structures: invasin (*inv*), the pH 6 antigen (*psaA*) and Ail (*ail)* (Grassl et al., 2003, Yang et al., 1996, El Tahir and Skurnik, 2001). It is unlikely that either Inv or Ail play a role in the LCB phenotype, since these gene are repressed during growth at 37°C (Pierson and Falkow, 1993, Pepe et al., 1994). Although Inv can be expressed at 37°C if the environment is acidic (Pepe et al.,

1994). However, since proteinase K can remove LPS from the bacterial cell surface (Kitchens and Munford, 1998) this does not exclude the possibility that LPS may play a role in LCB formation, especially the type of shortened O-chain LPS typically seen in *Y. pseudotuberculosis* during growth at 37°C which has been associated with aggregation in *E. coli* (Diderichsen, 1980, Moller et al., 2003, Sheng et al., 2008).

3.3.4.3 Transposon insertions in the Δ*ypsI* Δ*ytbI* mutant chromosome can prevent LCB formation

The transposon mutant screen revealed several interesting genes which, when mutated, prevented the Δ*ypsI* Δ*ytbI* mutant forming LCBs, and many of the loci identified in the screen can be linked to already established processes governing LCB formation in other species. The catalase KatA appears to play a role, specifically in regulating the production of the cell surface factor required for cells to form LCBs. In other bacteria KatA is responsible for mediating hydrogen peroxide resistance in biofilms (Elkins et al., 1999), and is regulated by the oxidative regulator OxyR (Han et al., 2008). In *E. coli*, oxidised OxyR causes Flu-mediated aggregation (Waldron et al., 2002). This could suggest interplay between catalases and the oxidative stress response may regulate the production of cell surface factors involved in LCB formation in *Y. pseudotuberculosis*. Interestingly, the expression of *katA* has been shown to be growth phase dependent in *Y. pestis* (Han et al., 2008), indicating a link to QS.

A separate transposon insertion in YPK_3655 also abrogates LCB formation in the Δ*ypsI* Δ*ytbI* mutant background. This gene, *lsrG* is annotated as an antibiotic biosynthesis monooxygenase and in *S. typhimurium*, the function of LsrG is to modify a putative QS signal, AI-2, synergistically with the LsrF protein, encoded upstream. It is thought that this modification by LsrG terminates the

signaling capability of AI-2, by preventing phospho-AI-2 binding, and relaxes the repressive effect of the AI-2 response regulator LsrR on gene transcription (Marques et al., 2011). AI-2 signaling plays a role in cellular aggregation in *V. cholerae* (Miller and Bassler, 2001), and this data suggests it could also be involved in LCB formation in *Y. pseudotuberculosis*. The production of AI-2 in the supernatant was investigated in the *Y. pseudotuberculosis lsrG* transposon mutant, and revealed that this mutant could not produce extracellular AI-2 (data not shown). This is similar to an *E. coli luxO* mutant, which cannot produce AI-2, and like the Δ*ypsI* Δ*ytbI* Tn5*::lsrG* strain, does not form aggregates typical in the wild-type (Miller and Bassler, 2001). This data raises speculation about the possible involvement of AI-2 in co-regulating phenotypic traits in *Y. pseudotuberculosis* alongside AHL-mediated QS.

Six genes downstream of YPK_3655, another transposon insertion into a putative AraC-type regulator, YPK_3661, prevent LCB formation in the Δ*ypsI* Δ*ytbI* mutant. YPK_3661 shares domain structure with the AraC-type regulators RhaR / RhaS, which are involved in *E. coli* rhamnose catabolism, and are repressed by AI-2 signalling (Wang et al., 2005). Although the function of YPK_3655 in *Y. pseudotuberculosis* is unknown, it suggests that multiple regulators may converge on the LCB phenotype in *Y. pseudotuberculosis*. The possibility that YPK_3661 may affect rhamnose metabolism is especially interesting since rhamnose is present in *P. aeruginosa* biofilm matrices (Wozniak et al., 2003).

The transposon mutant in a putative auto-transporter encoded by YPK_0763 highlights the possibility that auto-transporter proteins in addition to YadA may influence cell-aggregation leading to LCB formation in *Y. pseudotuberculosis*. Auto-transporters other than YadA have been identified as involved in clumping in *Y. pestis* (Felek et al., 2008) and in several other species they play a role in aggregation (Suzuki et al., 2008, Torres et al., 2002, Sherlock et al., 2005). It

is possible that several auto-transporters can facilitate cell-cell contact in *Y. pseudotuberculosis* and that they are all regulated to be utilised under different conditions. In the same way, it is interesting to find a transposon mutant in the Ail / Lom family protein encoded by YPK_2061 also prevents LCB formation. In *Y. pestis,* the *ail* homologue OmpX is involved in the formation of cell aggregates (Kolodziejek et al., 2007), and together, this suggests that the role of a variety of other surface proteins converge on the formation of LCBs, perhaps working synergistically to regulate LCB formation.

A transposon insertion into the gene YPK_3644 also resulted in the reversion of the Δ*ypsI* Δ*ytbI* mutant to the non-LCB phenotype. This gene, which encodes a small 77 amino acid hypothetical protein, is a member of the DUF1049 super family of uncharacterised proteins. This protein is predicted to be localised to the cytoplasmic membrane, and a search of the PROSITE database shows YPK_3644 possesses a bacterial IgG-like domain at the N-terminus, and two potential glycosylation sites. The reversion of the transposon mutant in YPK_3644 to a non-LCB phenotype is interesting due to the proximity of this gene to the O-antigen polymerase *wzy* (YPK_3646). In *E. coli* and *S. typhimurium*, rough colony variants or mutants, with altered O-antigen produce LCBs (Diderichsen, 1980, Moller et al., 2003, Sheng et al., 2008). Similarly, in *Y. pseudotuberculosis* and *Y. enterocolitica*, LPS becomes rough at 37°C where it is smooth below 26°C (Krasikova et al., 2000); this differs to *Y. pestis,* where the O-antigen gene cluster is silenced, leading to an LPS which is constitutively rough (Skurnik et al., 2000). In *Y. enterocolitica*, an O-antigen mutant is unable to secrete Yops under conditions usually permissive for Yop secretion (Perez-Gutierrez et al., 2006). This may be due to the over-expression of the flagella master regulator genes *flhDC* in the O-antigen mutant, since FlhDC is known to negatively regulate T3S in *Y. enterocolitica* (Bengoechea et al., 2004, Perez-Gutierrez et al., 2006, Bleves et al., 2002). In light of this, it is interesting to

note that supernatant from the YPK_3644 transposon mutant could not induce LCB formation in the wild-type; suggesting that Yop-production is also affected in this strain and increasing the possibility that LPS biosynthesis is affected by QS and contributes to the LCB phenotype.

This chapter also presents data which suggests that QS in *Y. pseudotuberculosis* affects the synthesis of the Psa via the regulator PsaE. Even though some evidence suggests QS positively regulates the Psa in *Y. pestis* (Chen et al., 2006), in *Y. enterocolitica* it is produced maximally at the transition between exponential phase and stationary phase, with decreased amounts present two hours into stationary phase (Iriarte et al., 1995). The data in this chapter suggest that de-repression of *psaE* in the Δ*ypsI* Δ*ytbI* mutant leads to an abundance of Psa on the cell surface, since i) Congo red binding in *Y. pseudotuberculosis* during culture in liquid is correlated with the LCB phenotype and is dependent on *psaE*, and ii) the Δ*ypsI* Δ*ytbI* Tn5*::psaE* transposon mutant is attenuated for LCB formation. The Psa is known to be expressed in phagolysosome compartments (Perry and Fetherston, 1997), but while the *yersiniae* have been observed to grow as aggregates in phagosomes (Lindler and Tall, 1993), *Y. pseudotuberculosis* YPIII is not thought to be an intracellular pathogen, and so in this strain the Psa may perform other functions. It is interesting that the both the *psaE* transposon mutant and the Δ*yscJ* mutant cannot bind Congo red, which hints at a further regulatory relationship between these two systems- indeed like the Yop effectors, the Psa is an anti-phagocytotic factor (Huang and Lindler, 2004), and has previously been suggested to partly facilitate the contact necessary for the translocation of Yop effectors into eukaryotic cells (Mejía et al., 2008).

3.3.5 Conclusions

This chapter has shown that several factors contribute to the formation of LCBs in *Y. pseudotuberculosis*. The Yop-Ysc T3SS is de-repressed, and secreted proteins presumably act upon cell-surface factors to stimulate the release or binding of matrix components and LCB formation (Figure 3.19).

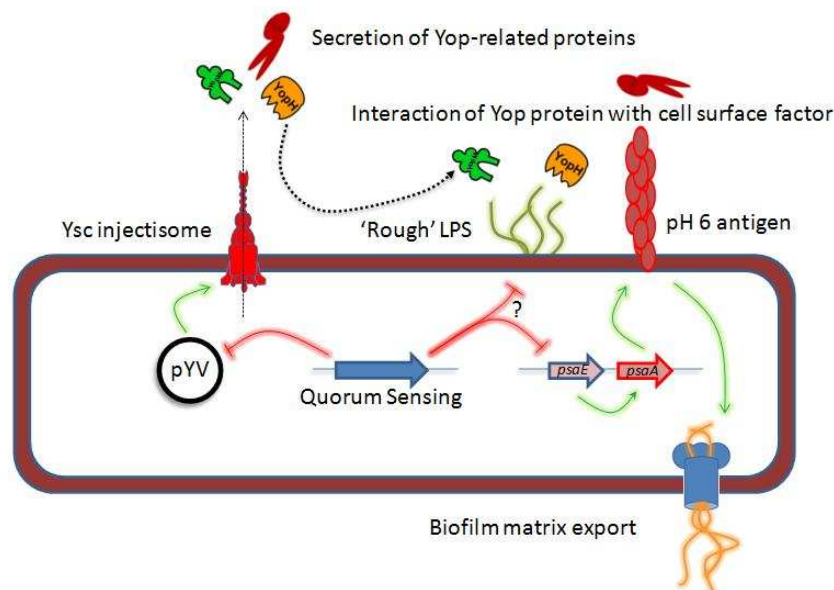


Figure 3.19| The possible regulation of LCB formation by QS in *Y. pseudotuberculosis*. QS acts to repress the production and secretion of Yop proteins, probably via the flagella system. QS also appears to repress temperature regulated surface factors. Yops in the supernatant interact with these elements, and induce the formation of LCBs through and effect on the production or binding of biofilm matrix components.

The adoption of LCBs by bacteria can be viewed as a behaviour where cells can come together in response to hostile environments. In *Sphingobium* sp., it has been suggested that the mere "scent of danger", or rather the detection of factors released into the environment by a predator flagellate, is sufficient to trigger LCB formation (Blom et al., 2010). For *Y. pseudotuberculosis*, experiencing temperature shifts upwards of 30°C is likely to be a cue for the

switch between the environment and mammalian host, since although mammalian body temperature is 37°C, ambient temperatures rarely rise to 30°C in areas prone to being *Yersinia* reservoirs, which tend to be in cold or temperate regions of the world (Adesiyun and Krishnan, 1995, Vincent et al., 2008, Rimhanen-Finne et al., 2009). During the course of such a transition, a low density of cells might associate together as an anti-predatory behaviour (Matz and Kjelleberg, 2005, Pickup et al., 2007, Blom et al., 2010), as opposed to bacteria in a large population, which may have less need for the protective effect of neighbouring cells. It would be interesting to investigate the possibility that *Y. pseudotuberculosis* uses LCBs as an anti-predatory mechanism, potentially against phagocytic cells, and this may provide important detail on how *Y. pseudotuberculosis* survives in the mammalian environment. This may provide important discoveries relevant to infection control.

Chapter 4|

Biofilm formation on *Caenorhabditis elegans* is facilitated by quorum sensing dependent repression of type 3 secretion

4.1 Introduction

4.1.1 *Caenorhabditis elegans*

C. elegans is a small, free-living nematode which can be reliably isolated from compost and rotting vegetable matter (Barrière and Félix, 2005, Félix and Braendle, 2010, Kiontke and Sudhaus, 2006). Little is known about the natural ecology of *C. elegans*, however it is known that it is a coloniser of organic material which is rich in nutrients and microorganisms, but the worm is rarely found in natural undisturbed soil environments, despite extensive sampling (Félix and Braendle, 2010, Kiontke and Sudhaus, 2006). *C. elegans* is thought to feed primarily on bacteria and small eukaryotes, and all isolated species can grow using *E. coli* as a food-source (Félix and Braendle, 2010, Kiontke and Sudhaus, 2006), however it is not known which microbes *C. elegans* feeds on in nature, but *C. elegans* can also feed on single cells of the slime-mould *Dictyostelium discoidium* (Kessin et al., 1996). To feed, *C. elegans* uses the pharynx as a muscular pump to suck up bacterial cells (Albertson and Thomson, 1976). The pharynx rhythmically contracts to ingest bacteria into a terminal bulb, where an organ termed the grinder with specialised abrasive extensions of the cuticle disrupts the cells and passes them through pharyngeal-intestinal valve to the digestive tract (Figure 4.1) (Albertson and Thomson, 1976).

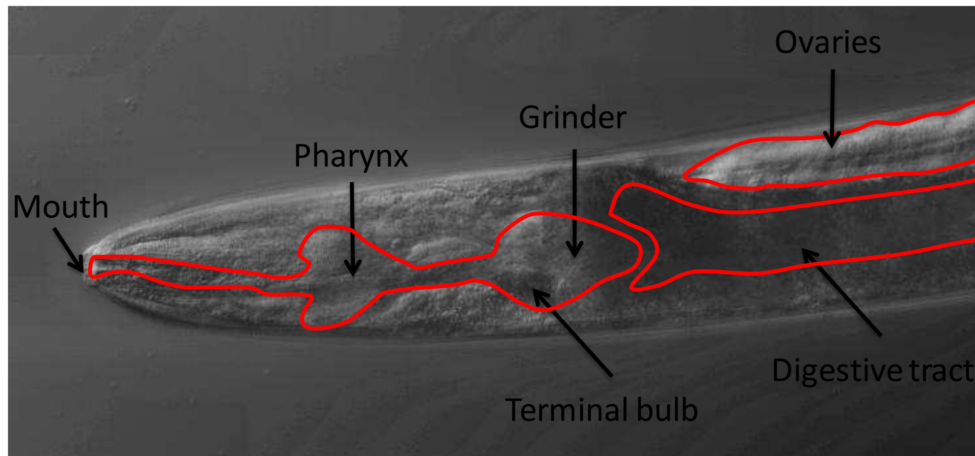


Figure 4.1| Diagram of the pharynx and anterior digestive tract of *C. elegans,* as revealed by CLSM. Bacteria are taken up by the worm and accumulate in the terminal bulb, where a chitin grinder disrupts the cells for processing through the digestive tract (Albertson and Thomson, 1976).

4.1.1.1 *C. elegans* as a model organism

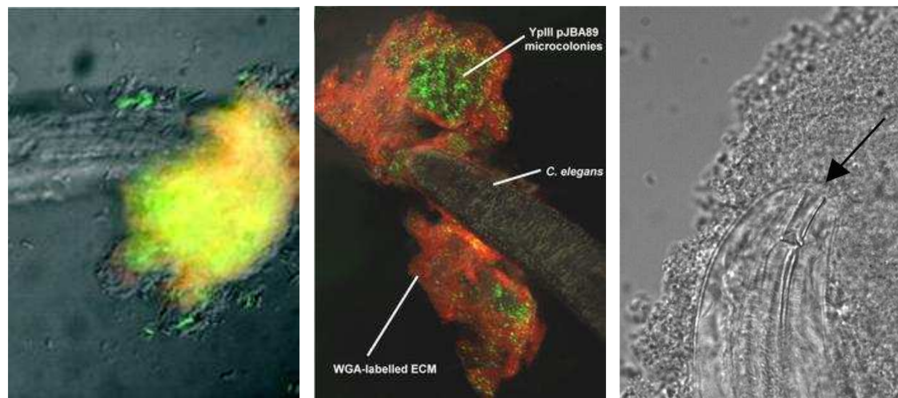
Despite the fact that little is known of the natural ecology of *C. elegans*, this organism has been adapted as a model system for genetics and developmental biology. *C. elegans* can be propagated rapidly in the lab, and the organism is translucent throughout its life-cycle, making it easy to observe by microscopy. In addition to its use in developmental biology and genetics, *C. elegans* has become a model organism for the study of pathogen-host interactions (Aballay and Ausubel, 2002, Sifri et al., 2005) for several bacterial species including *P. aeruginosa* (Tan et al., 1999), enteropathogenic *E. coli* (Anyanful et al., 2005), *S. typhimurium* (Labrousse et al., 2000) and *S. aureus* (Sifri et al., 2003). In addition to the modelling of mammalian pathogenesis, the study of microbe-nematode interactions may be especially important since nematodes are the most abundant metazoan organisms on Earth (Neher, 2001). Many nematode species feed on bacteria, and so this would make nematodes an important selective pressure for bacteria where they co-exist in the environment. It may not be surprising, therefore, to find that many bacterial species are pathogenic

towards nematodes, including *Agrobacterium tumefaciens*, *Bacillus megaterium*, *Erwinia* spp., *Aeromonas* spp., *Leucobacter chromiireducens* and *Yersinia* spp., which either kill the worm or possess mechanisms to prevent the worm feeding on the bacterial population (Couillault and Ewbank, 2002, Darby et al., 2002, Tan and Darby, 2004, Muir and Tan, 2008). Furthermore, the bacteria *Photorhabdus luminescens* and *Xenorhabdus nematophila*, which are usually found in symbiotic association with entomopathogenic nematodes of the families *Heterorhabditidae* and *Steinermatidae*, severely reduce the survival of *C. elegans* (Couillault and Ewbank, 2002). It is also noteworthy that as recently as the year 2000, new natural pathogens of *C. elegans* have been isolated, such as *Microbacterium nematophilium* (Hodgkin et al., 2000).

4.1.2 Biofilm-related infections in *C. elegans*

Biofilms are increasingly recognised as a mechanism by which bacteria use to prevent protozoan predation (Matz and Kjelleberg, 2005, Matz et al., 2004, Queck et al., 2006), and several species of bacteria form biofilms on larger metazoan predators such as *C. elegans*, including *Y. pestis*, *Y. pseudotuberculosis* and *X. nematophila* (Atkinson et al., 2011, Darby et al., 2005, Drace and Darby, 2008). Darby *et al*. (2002) revealed that the biofilms formed by *Y. pseudotuberculosis* on *C. elegans*, which accumulate around the mouthparts, were sufficient to form a blockage and prevent the worm feeding on bacteria (Darby et al., 2002), and since the biofilms formed by *Y. pseudotuberculosis, Y. pestis* and *X. nematophila* are morphologically similar (Figure 4.2), it is likely that they all function in this way. This blockage may be analogous to the biofilms formed on nematodes by the emerging human pathogen *Photorhabdus asymbiotica* which can be transferred to humans

(Waterfield et al., 2009), and also to the biofilms formed in the proventriculus of the flea vector during transmission of *Y. pestis* (Hinnebusch et al., 1996).



a) *X. nematophila* b) *Y. pseudotuberculosis* c) *Y. pestis*

Figure 4.2| The biofilms formed by *X. nematophila*, *Y. pseudotuberculosis* and *Y. pestis* on *C. elegans* are morphologically similar and accumulate at the anterior of the worm, around the mouthparts. For *X. nematophila* (a, adapted from Drace and Darby, 2008)) and *Y. pseudotuberculosis* (b, adapted from Atkinson et al., 2011)), bacterial cells are expressing *gfp* and appear green, biofilm exopolysaccharides are labelled red with the lectin wheat-germ agglutinin coupled to rhodamine. For *Y. pestis*, the arrow denotes the mouth of the worm (c, adapted from Darby et al., 2005).

Y. pestis, *Y. pseudotuberculosis, X. nematophila* and *S. epidermidis* all encode homologues of the *hmsHRFS* operon. In *S. epidermidis*, this locus, termed the *icaABCD* operon (for intercellular adhesin) produces partially deacetylated poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG), a biofilm matrix EPS important for adherence, biofilm formation, and biofilm-related infections in *C. elegans* (Vuong et al., 2004, Itoh et al., 2005, Begun et al., 2007). The *hmsHRFS* operons of *Yersinia* spp. and *X. nematophila* probably synthesise the same, or similar, partially deacetylated exopolysaccharide. This can be inferred from the homology of HmsR to IcaA and other glycosyltransferases such as *E. coli* PgaC, which are responsible for the production of PNAG, and the presence of the polysaccharide deaceylase gene *hmsF* (Darby, 2008). Additionally the biofilms

formed by *S. epidermidis*, *X. nematophila* and *Yersinia* spp. all share a similar affinity for wheat-germ agglutinin, a lectin which binds PNAG and when coupled to rhodamine (R-WGA), is a useful tool for visualising EPS by fluorescence microscopy (Darby, 2008, Drace and Darby, 2008, Atkinson et al., 2011).

4.1.3 Significance of the *Yersinia* / *C. elegans* relationship

Tan & Darby (2004) proposed that *Y. pseudotuberculosis* biofilms form on *C. elegans* in a snow-plough fashion, whereby movement of the worm through a bacterial lawn on agar causes the accumulation of biofilm at the anterior of the worm, around the head (Tan and Darby, 2004). Supporting this hypothesis, worms which cannot move accumulate a reduced biofilm (Tan and Darby, 2004). This could be seen to compromise the notion that such biofilms formed by *Y. pseudotuberculosis* are a natural adaptation to prevent predation by nematodes, and reduces the complexity of the *Y. pseudotuberculosis* / *C. elegans* relationship to an *in vitro* artefact of the interaction between the worm and a sticky bacterial lawn. However, other evidence indicates biofilm formation is an interactive process between *Y. pseudotuberculosis* and *C. elegans*, since *C. elegans* with mutations in *srf*-2, *srf*-3, *srf*-5 and *daf-1* and several surface proteins show resistance to *Y. pseudotuberculosis* biofilms, yet are motile and can track across agar seeded with *Y. pseudotuberculosis* normally (Joshua et al., 2003, Darby et al., 2007, Drace et al., 2009). In addition, male *C. elegans* worms are less prone to accumulate biofilms than are hermaphrodites, and dauer stage larva are resistant to biofilm accumulation (Darby et al., 2007). It has also been noted that during biofilm assays, some worms do not accumulate biofilms and appear to behave normally, in contrast to the aberrant movement usually associated with inoculation onto *Yersinia* lawns (Darby et al., 2007, Atkinson et al., 2011). Together, this suggests that there are many more factors

involved in the formation of biofilms by *Y. pseudotuberculosis* on the cuticle of *C. elegans* than can be explained by the snow-plough hypothesis.

Interestingly, many *Y. pseudotuberculosis* outbreaks are associated with agricultural activity (Nuorti et al., 2004, Laukkanen et al., 2008, Rimhanen-Finne et al., 2009). This correlates with the known natural habitats for *C. elegans*, which prefers to live in organic nutrient rich environments such as compost (Barrière and Félix, 2005, Félix and Braendle, 2010, Kiontke and Sudhaus, 2006). *Y. pestis* uses the flea *Xenopsylla cheopis* as an insect vector for transmission between one mammalian host and another (Perry and Fetherston, 1997), and so it is possible that *Y. pseudotuberculosis* uses nematodes such as *C. elegans* in a similar fashion. Clearly it is possible that the formation of biofilms by *Y. pseudotuberculosis* on *C. elegans* could represent bio-accumulation of the bacteria, which could result in transmission of the bacteria to predators of nematodes, and further up the food chain.

4.1.4 Chapter 4 aims

Chapter 3 demonstrated that LCBs are regulated by QS via a mechanism involving the T3SS. Another type of biofilm formed by *Y. pseudotuberculosis* occurs on the nematode worm *C. elegans*, where the biofilm grows on the worm cuticle, predominantly at the anterior and around the mouth, where it prevents the worm feeding on bacteria (Darby et al., 2002, Tan and Darby, 2004, Joshua et al., 2003). While these biofilms develop *in vitro*, when worms are placed on agar plates seeded with *Y. pseudotuberculosis*, work in this chapter shows that they can also develop in an environmental model, where nematodes are placed into compost seeded with *Y. pseudotuberculosis*. QS regulates the formation of these biofilms on *C. elegans* grown on agar surfaces (Atkinson et al., 2011), and

also affects the development of these biofilms in soil. The biofilms that develop on the cuticle of *C. elegans* are very similar in composition to LCBs, containing eDNA and EPS as part of the biofilm matrix, with eDNA forming an important structural component. Furthermore, as with LCBs, the Yop-Ysc T3SS impacts on the ability for *Y. pseudotuberculosis* to form biofilms on *C. elegans*- in this case the T3SS prevents *C. elegans* biofilms. This chapter presents work to show that when wild-type cells are conditioned to express the T3SS, they are incapable of forming biofilms on *C. elegans*. Additionally, the reduction in biofilm load observed following infection by the Δ*ypsI* Δ*ytbI* mutant, which has a de-repressed T3SS, can be restored to wild-type levels following the removal of the pYV or by mutation of *yscJ*, which encodes an essential structural component of the Ysc injectisome.

4.2 Results

4.2.1 QS regulates biofilm formation on *C. elegans*

The motility regulators *flhDC* and *fliA* are important for biofilm formation on *C. elegans* by *Y. pseudotuberculosis* (Atkinson et al., 2011), and since QS regulates the motility regulon (Atkinson et al., 2008), it was possible that QS also affected biofilm formation on *C. elegans*. To investigate this, the AHL negative Δ*ypsI* Δ*ytbI* mutant was used, since this strain cannot produce AHL signalling molecules, and is highly repressed for the expression of *flhDC* and *fliA* (Atkinson et al., 2008). When *C. elegans* is infected with the Δ*ypsI* Δ*ytbI* mutant, the biofilms which form are approximately three-fold smaller than those formed by the wild-type. Additionally, when supplied with functional copies of *ypsI* and *ytbI* (on pSA291), biofilm formation is partially restored (Figure 4.3).

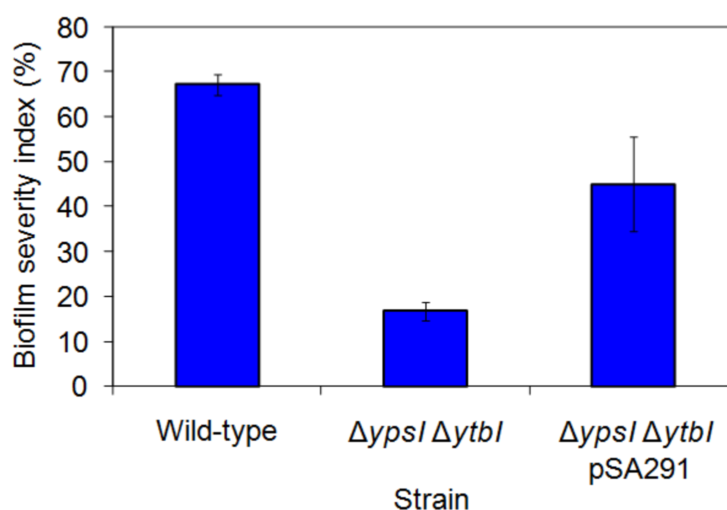


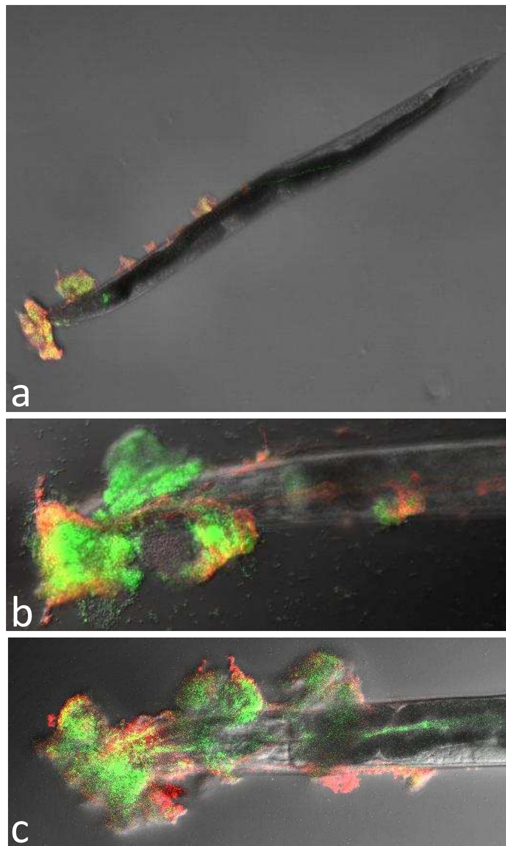
Figure 4.3| Biofilm severity index calculated for wild-type, Δ*ypsI* Δ*ytbI* and the complement strain Δ*ypsI* Δ*ytbI* pSA291. Biofilms are substantially reduced when *C. elegans* are infected with Δ*ypsI* Δ*ytbI* relative to wild-type, and restoration of the *ypsI* and *ytbI* genes as plasmid borne copies (on pSA291) restores the biofilm forming ability of *Y. pseudotuberculosis*. Bars represent the standard deviation of biofilm severity. Measurements are the mean of 60 worms from three plates per condition, 20 worms per plate.

To investigate the structure of the biofilms, the Δ*ypsI* Δ*ytbI* mutant and wild-type were used to infect *C. elegans* and biofilms that developed over 16 h were inspected by CLSM. Polysaccharides have previously been shown to be present in the ECM of *Y. pseudotuberculosis* biofilms on *C. elegans* (Tan and Darby, 2004), and were therefore highlighted using Rhodamine-coupled Wheat Germ Agglutinin (which has been used previously to label PNAG in *Y. pseudotuberculosis* biofilms on *C. elegans* and emits a red fluorescence (Atkinson et al., 2011)). To aid localisation of bacteria in the biofilm, all strains were expressing *gfp3* from pSB2020 (Figure 4.4).

The wild-type strain forms large biofilms that accumulate at the anterior end of *C. elegans*, particularly around the mouthparts (Figure 4.4 i a). These biofilms stain positively for EPS using R-WGA and appears red (Figure 4.4 i a & I b), which can also be observed in the worm digestive tract (Figure 4.4 i b). Biofilms formed by the Δ*ypsI* Δ*ytbI* mutant are substantially smaller than those formed by wild-type (compare Figure 4.4 i & ii), and the biofilm appears more diffuse across the surface of the worm rather than being localised to the worm head (Figure 4.4 ii a). R-WGA highlights that EPS is present in these biofilms, however GFP fluorescence suggests there to be less bacterial cells contained within the biofilm (compare Figure 4.4 ii b & ii c with Figure 4.4 i b & ii c). In some worms infected with the Δ*ypsI* Δ*ytbI* mutant, a large distended region of GFP fluorescence can be observed at the posterior end of the pharynx, probably in the terminal bulb (Figure 4.4 ii c), and may reflect the fact that *C. elegans* can graze on these bacteria, since this type of fluorescence is observed when *C. elegans* is fed its usual food source, *E. coli* OP50 (Darby et al., 2002).

I- Wild-type biofilms

II- Δ*ypsI* Δ*ytbI* biofilms



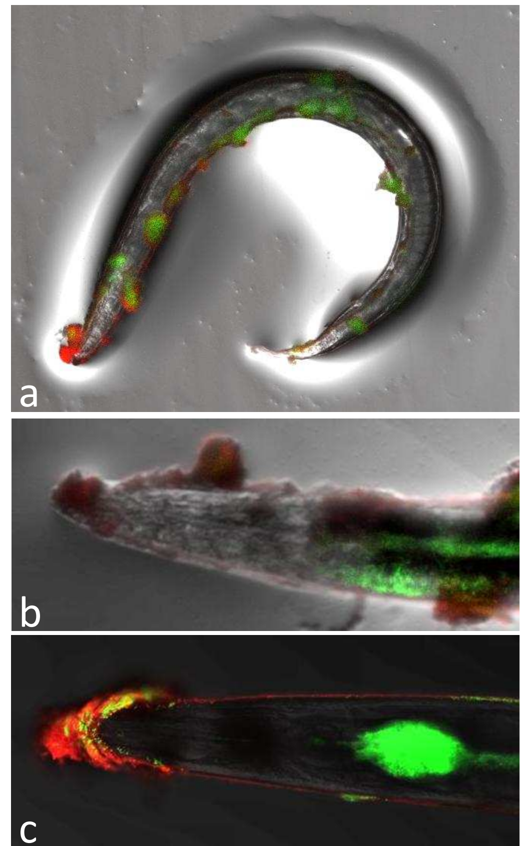


Figure 4.4| Biofilms formed on the cuticle of *C. elegans* by wild-type *Y. pseudotuberculosis* (i) and the AHL negative Δ*ypsI* Δ*ytbI* mutant (ii) as revealed by CLSM. Biofilms are labelled with R-WGA to highlight EPS, which appears red, while bacteria expressing *gfp3* appear green. Where bacteria and EPS co-localise, a yellow colour can be observed. The wild-type forms large biofilms that accumulate primarily at the anterior end of the worm, around the mouthparts (i a), with large micro-colonies imbedded in the matrix (i b & i c, arrows). Some R-WGA labelling within the worm digestive tract suggests the biofilm to extend inside the worm as well as outside (i b). The biofilms formed by the Δ*ypsI* Δ*ytbI* mutant are substantially smaller and appear to be more diffuse over the worm surface (ii a), with fewer bacterial cells and no clear micro-colonies within the matrix (ii b & ii c). *C. elegans* may be able to efficiently graze on Δ*ypsI* Δ*ytbI* cells, as shown by the distended region of GFP in the terminal bulb (II c & compare results presented by Darby *et. al.* (2002).

4.2.2 The biofilm ECM contains extracellular DNA

Chapter 3 reported that LCBs contain substantial amounts of eDNA as part of the biofilm matrix, similar to that described for several other species (Vilain et al., 2009, Whitchurch et al., 2002, Izano et al., 2008, Kreth et al., 2009, Böckelmann et al., 2006). To investigate the presence of eDNA in *Y. pseudotuberculosis* biofilms on *C. elegans*, worms infected with *Y. pseudotuberculosis* were labelled with DAPI, which has been previously used to label eDNA in bacterial biofilms (Vilain et al., 2009, Böckelmann et al., 2006).

Figure 4.5 reveals that eDNA is present throughout the biofilm matrix on the surface on *C. elegans* (Figure 4.5 a). When only DAPI fluorescence is visualised, this eDNA can be observed to extend throughout the worm digestive tract (Figure 4.5 b, arrow). The eDNA appears to be present as a network, since strands of eDNA can clearly be observed when the image is magnified (Figure 4.5 c arrows).

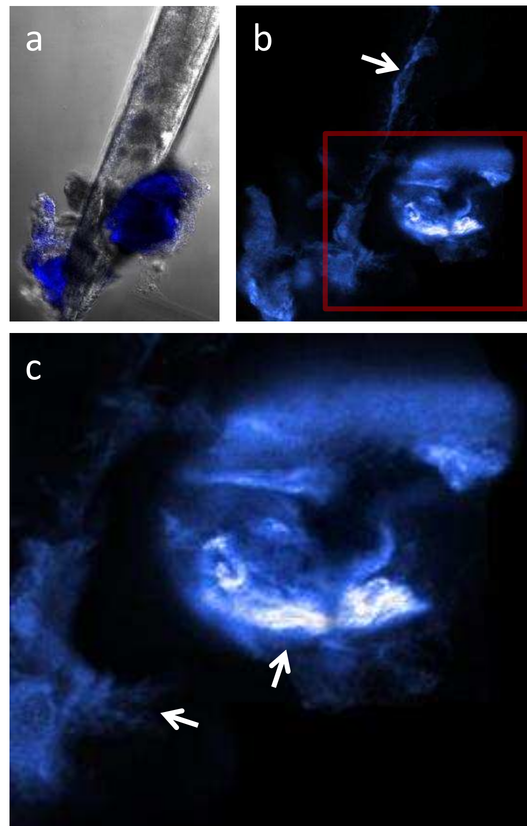


Figure 4.5| *Y. pseudotuberculosis* biofilms on *C. elegans* stain positively for eDNA using DAPI, which gives a blue fluorescence, as revealed by CLSM. Panels show eDNA labelled in *C. elegans* following infection with *Y. pseudotuberculosis* for 16 h (a) and a diagram showing only eDNA within the biofilm (b). Extracellular DNA can clearly be seen throughout the biofilm and extends into the worm digestive tract (b, arrow) The red square within panel b has been magnified (c) and shows the eDNA exists as a filamentous network in the biofilm, since strands can clearly be observed throughout and on the periphery of the biofilm (c, arrows).

Extracellular DNA represents an important structural component of the matrix for the biofilms of several species of bacteria, and can be disrupted by the addition of DNase I (Whitchurch et al., 2002, Izano et al., 2008). Chapter 3 presented data to show that LCBs formed by *Y. pseudotuberculosis* could be prevented by the addition of DNase I, and this raised the possibility that eDNA constitutes a structural component of *Y. pseudotuberculosis* biofilm on *C. elegans*. To investigate this, *C. elegans* were infected with a *Y. pseudotuberculosis* culture which had been treated with DNase I before being seeded onto worm- NGM

plates, in order to degrade eDNA. Figure 4.6 shows that when *C. elegans* is infected with wild-type *Y. pseudotuberculosis* in the presence of DNase I, the biofilms produced are reduced two-fold when compared with the untreated control.

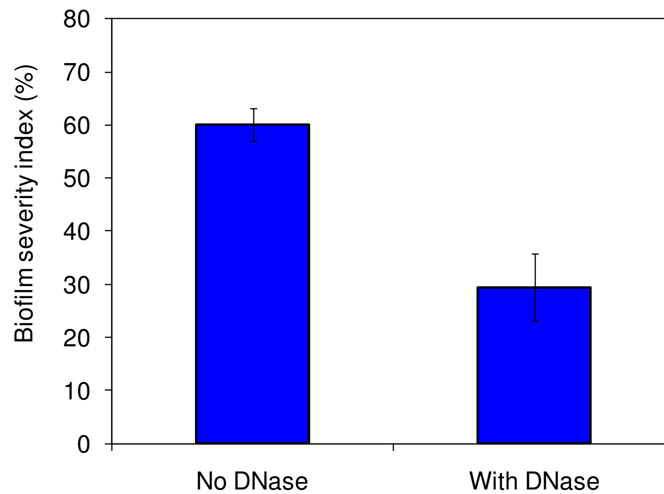


Figure 4.6| Biofilm severity index calculated for biofilms caused by *Y. pseudotuberculosis* on the surface of *C. elegans* when the bacterial culture is either treated with DNase I or untreated. Treatment of the bacterial culture prior with DNase I prior to seeding on NGM-plates reduces the severity of the biofilms that form on *C. elegans* by approximately 50 %. Error bars represent standard deviation of biofilm severity. Measurements are the mean of 60 worms per condition from three plates, 20 worms per plate.

Together, Figures 4.5 and 4.6 show that *Y. pseudotuberculosis* biofilms on *C. elegans* contain substantial amounts of eDNA that appears in a network through the biofilm, and that this eDNA is potentially important for the architecture of the biofilm.

4.2.3 *Y. pseudotuberculosis* colonises and forms biofilms on *C. elegans* in a compost model of infection

Biofilms are often considered to be an anti-predatory adaptation (Matz and Kjelleberg, 2005), however Tan *et al.* (2004) suggest that *Y. pseudotuberculosis* forms biofilms on the surface of *C. elegans* in a passive interaction (Tan and Darby, 2004), suggesting that these biofilms are an *in vitro* artefact. The possibility that *Y. pseudotuberculosis* uses biofilms on *C. elegans* as an anti-predatory mechanism relies on the ability for biofilms to develop in a natural environment which does not typically provide an ideal surface such as an agar plate for biofilm formation. To determine whether *Y. pseudotuberculosis* forms biofilms on *C. elegans* in an environment which more closely resembles the natural habitat of *C. elegans*, worms were introduced into sterilised commercial compost, seeded with *Y. pseudotuberculosis* and incubated for 24 to 48 h before being visualised by CLSM.

After 24 h, *Y. pseudotuberculosis* is found predominantly in the anterior region of the worm gut (Figure 4.7 a), whereas after 48 h, a large proportion of the worm is colonised by *Y. pseudotuberculosis*, with GFP labelled bacteria found in biofilm-like material which surrounds the mouth (Figure 4.7 b, white arrow), in the intestines (Figure 4.7 b, black arrow) and in the posterior digestive tract (Figure 4.7 b, red arrow). When worms are inoculated into compost not seeded with *Y. pseudotuberculosis*, no green florescence is observed (Figure 4.7 c).

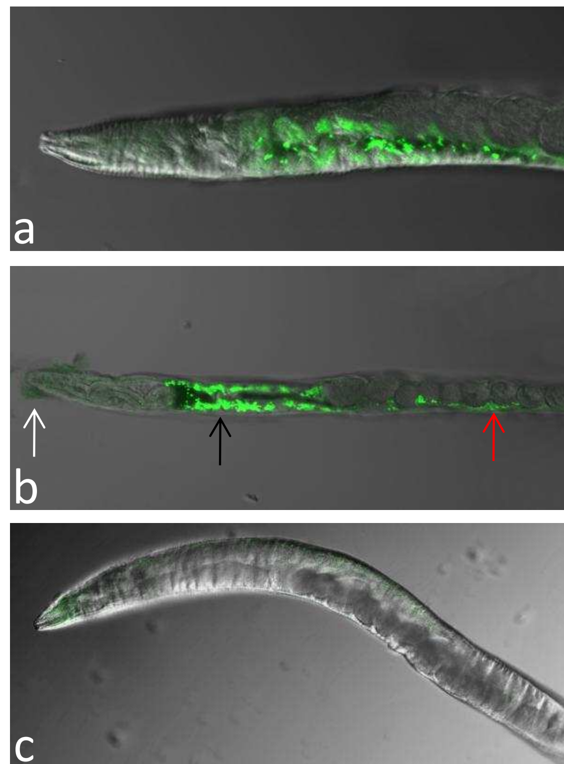


Figure 4.7| The colonisation of *C. elegans* by *Y. pseudotuberculosis* in compost, as revealed by CLSM. After 24 h, the bacteria are localised primarily in the anterior region of the worm digestive tract (a), after 48 h, the bacteria can be found throughout the worm digestive tract (red arrow) and in the intestines (black arrow). The material surrounding the worm mouth also shows some GFP fluorescence (white arrow). When *C. elegans* is inoculated into soil without *Y. pseudotuberculosis*, no GFP fluorescence can be observed (c).

To investigate the possibility that the colonisation of *C. elegans* by *Y. pseudotuberculosis* after 48 h in compost is a consequence of a biofilm-related infection, the worms were labelled with R-WGA to highlight EPS (Figure 4.8 i a, b & d) and DAPI to label eDNA (Figure 4.8 i c & d), before being inspected by CLSM. Figure 4.8 shows that the worms appear to contain biofilm material throughout the gut. EPS is found blocking the mouthparts of the worm (Figure 4.8 i a & b) and throughout the gut of worms infected with *Y. pseudotuberculosis* (Figure 4.8 i a & b white arrows). Extracellular DNA is also found in these biofilms, which appears in a network like structure surrounding the mouthparts

(Figure 4.8 i c), and blue DAPI fluorescence co-localises with R-WGA in the biofilm blocking the mouthparts of the worm (Figure 4.8 i d).

To investigate if QS was also involved in the formation of biofilms on *C. elegans* in compost, as in agar, the Δ*ypsI* Δ*ytbI* mutant was inoculated into compost and used to infect *C. elegans*. The biofilms that grew in this assay were labelled with R-WGA and DAPI to label EPS and eDNA respectively, and visualised by CLSM. In comparison to wild-type biofilms, biofilms formed by the Δ*ypsI* Δ*ytbI* mutant cannot be clearly observed on the worm (Figure 4.8 ii a & b). GFP fluorescence from the bacteria indicates that the Δ*ypsI* Δ*ytbI* mutant is less able to proliferate inside the worm, and does not show the marked dissemination to the intestines observed with the wild type (compare Figure 4.8 i a & b with ii a & b). When only the DAPI and R-WGA channels are visualised, very small biofilms can be seen in the mouth of the worm (Figure 4.8 ii c & d), with a R-WGA labelled 'plug' and DAPI labelling the lining of the buccal cavity, although these biofilms are substantially smaller than those formed by the wild-type (compare Figure 4.8 ii d with i c). When worms are placed into compost inoculated only with OP50, and are labelled with R-WGA and DAPI, no red or blue fluorescence can be observed, and only low levels of green fluorescence can be seen (figure 4.8 iii a & b).

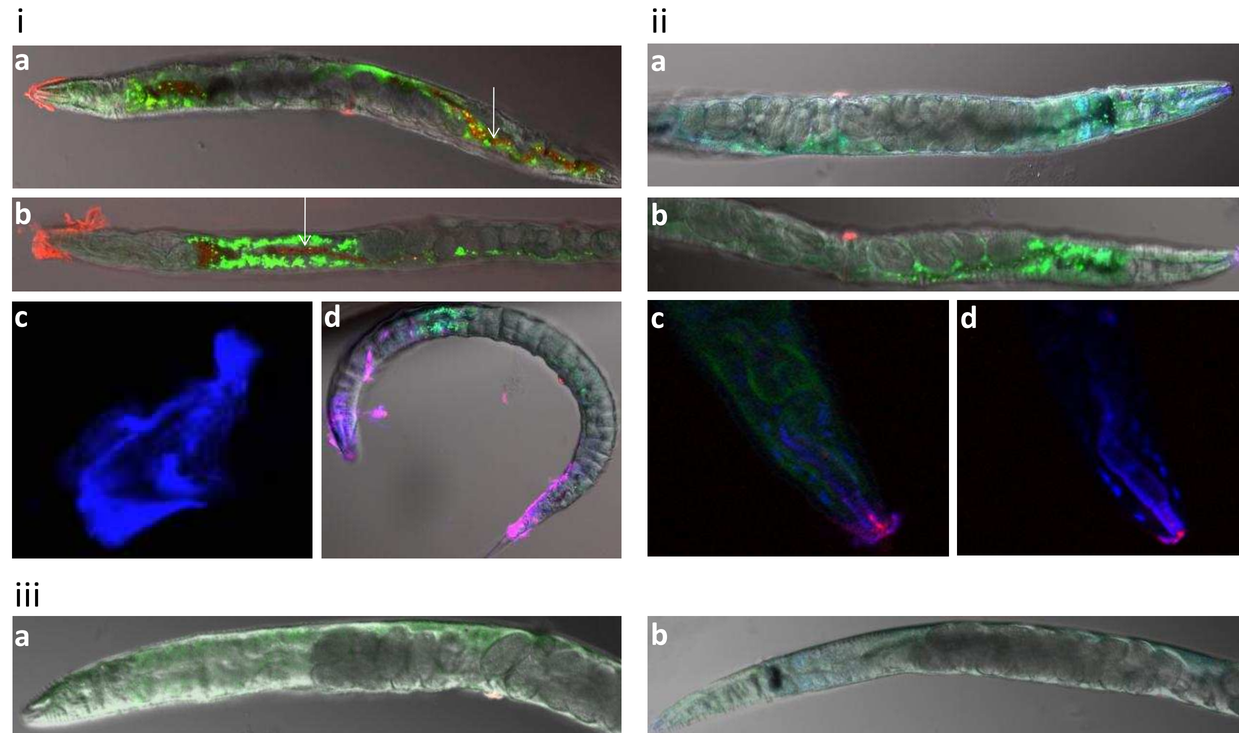


Figure 4.8| QS is important for *Y. pseudotuberculosis* to form biofilms on *C. elegans* in compost, as revealed by CLSM. When R-WGA is used to label wild-type biofilms, a large EPS cap can be observed to cover the worm mouthparts (i a & b), and EPS also extends into the worm digestive tract, where bacteria in the intestines also co-localise with R-WGA and appear green / yellow (i a & b white arrows). DAPI labelling reveals a network of eDNA that can surround the mouthparts of the worm (i c) and largely co-localises with EPS (i d). When the Δ*ypsI* Δ*ytbI* mutant is used to infect *C. elegans* in compost, practically no biofilm can be seen on the worm (ii a & b). When only DAPI and R-WGA are visualised, a small amount of R-WGA can be seen in the worm mouthparts, and DAPI labels the inside of the buccal cavity, however in these images, the contrast on DAPI and R-WGA has been increased to aid visualisation. When worms are fed OP50, very little background fluorescence can be seen, suggesting that the material labelled by R-WGA and DAPI are derived from *Y. pseudotuberculosis*, which are expressing *gfp3* and appear green.

4.2.4 QS regulated biofilms involve the pYV plasmid

Chapter 3 presented data to show that LCBs are modulated by QS in *Y. pseudotuberculosis* through control of the T3SS. To investigate the possibility that the ability for the Δ*ypsI* Δ*ytbI* mutant to form biofilms on the surface of *C. elegans* was linked to the virulence plasmid, pYV cured derivatives of both wild-type and the Δ*ypsI* Δ*ytbI* (pYV^-) were evaluated in *C. elegans* agar plate biofilm experiments.

Figure 4.9 reveals that the presence or absence of the pYV plasmid does not affect the ability of the wild-type to form biofilms on *C. elegans*, consistent with the results described by Joshua *et al*. (2003). However, when the pYV plasmid is cured from the Δ*ypsI* Δ*ytbI* mutant, biofilm formation is restored to that of the wild-type. This indicates that QS normally represses the expression of a pYV-associated factor which would otherwise prevent biofilm formation on *C. elegans*.

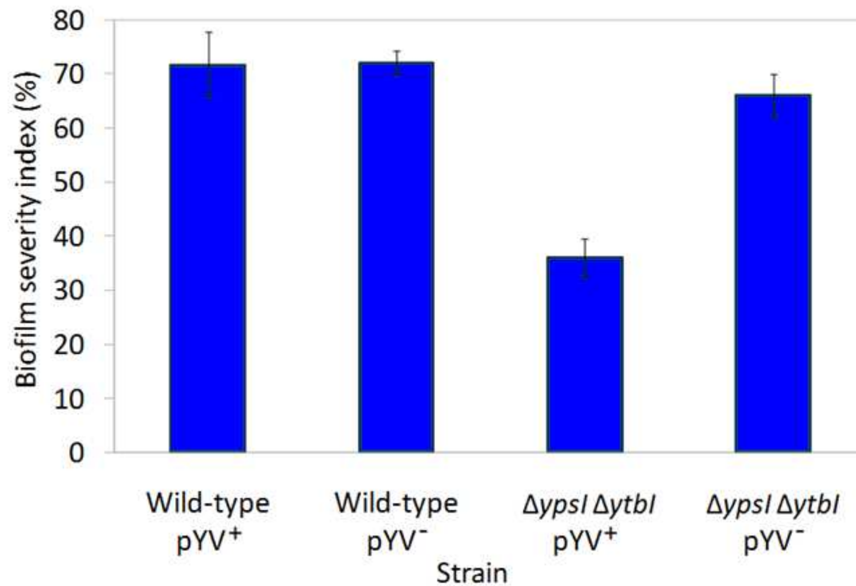


Figure 4.9| The pYV plasmid determines the reduction in biofilm load in a Δ*ypsI* Δ*ytbI* mutant. Biofilm formation on the surface of *C. elegans* is unaffected by the presence or absence of pYV in the wild-type. However, the Δ*ypsI* Δ*ytbI* pYV$^-$ mutant forms biofilms with similar severity to the wild-type, in contrast to the Δ*ypsI* Δ*ytbI* pYV$^+$ mutant, in which biofilm formation is reduced approximately two-fold. Measurements are the mean of 60 worms per condition from three plates, 20 worms per plate.

4.2.5 QS affects biofilms through deregulation of the T3SS

Figure 4.9 supports the data presented in Chapter 3, which showed that formation of LCBs by the Δ*ypsI* Δ*ytbI* mutant involved the pYV*,* and suggests that the pYV plasmid is important for biofilm formation on *C. elegans*. For LCBs, this was related to the Yop-Ysc T3SS, since the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant could not form LCBs. To investigate whether the Yop-Ysc system affected biofilm formation on *C. elegans*, the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant described in Chapter 3 was used in *C. elegans* biofilm assays. When *yscJ* is deleted in the Δ*ypsI* Δ*ytbI* mutant background to produce the triple Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant, biofilm severity was restored to wild-type levels. Complementation of the Δ*ypsI* Δ*ytbI*

Δ*yscJ* triple mutant with *yscJ* on pHG*yscJ* reduced biofilm formation three-fold, to levels similar to the Δ*ypsI* Δ*ytbI* mutant (Figure 4.10).

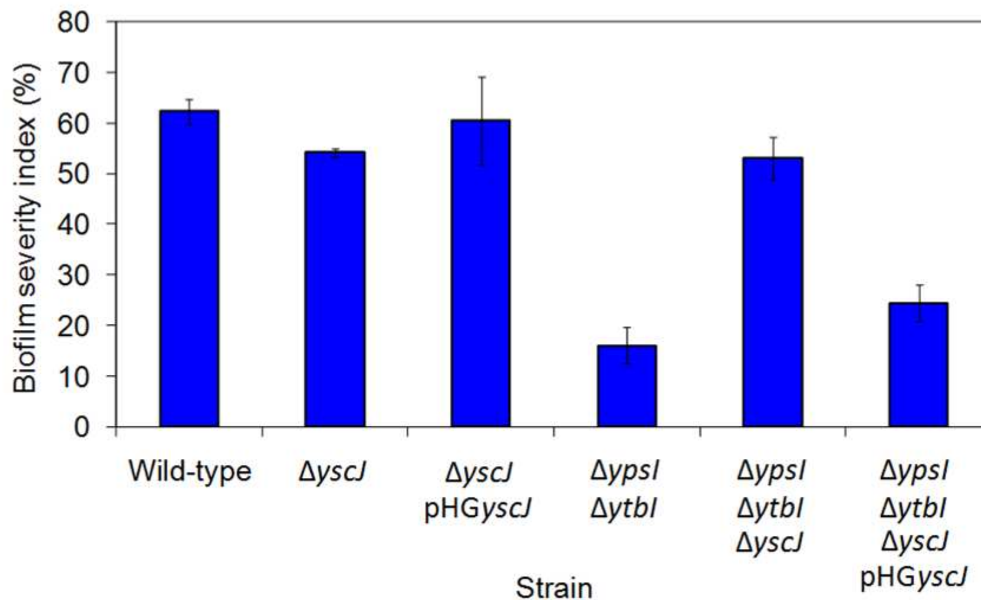


Figure 4.10| The attenuation of biofilm formation in the Δ*ypsI* Δ*ytbI* mutant is reversed by mutation of *yscJ* (Δ*ypsI* Δ*ytbI* Δ*yscJ*). In the wild-type background, there does not appear to be an effect of the *yscJ* mutation on the formation of biofilms on the cuticle of C. elegans (compare wild-type and Δ*yscJ*). Complementation of the *yscJ* mutation in Δ*ypsI* Δ*ytbI* (Δ*ypsI* Δ*ytbI* Δ*yscJ* pHG*::yscJ*) restores the reduction in biofilm severity to levels similar to the Δ*ypsI* Δ*ytbI* strain. Measurements are the mean of 60 worms per condition from three plates, 20 worms per plate.

The reduction in biofilm severity exhibited by Δ*ypsI* Δ*ytbI* compared with the wild-type is, therefore, dependent upon the presence of the pYV, and biofilms formed by the Δ*ypsI* Δ*ytbI* mutant can be restored to levels comparable with the wild-type by deletion of *yscJ*. Additionally, the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant biofilms can be restored to Δ*ypsI* Δ*ytbI* mutant levels by providing *yscJ* encoded on pHG*yscJ*. These results are consistent with a role for the type III injectisome in preventing biofilm formation on *C. elegans*, and suggest that the injectisome, the secreted Yop effectors, or perhaps both, prevent biofilm formation on *C. elegans*.

4.2.6 The injectisome is responsible for repressing biofilms

Chapter 3 presented data to show that LCBs are as a consequence of secretion of proteins through the T3SS. Since the biofilm load on *C. elegans* can be restored to wild-type levels in the Δ*ypsI* Δ*ytbI* mutant by deletion of *yscJ*, this could suggest that a similar process underlies the QS-dependent regulation of biofilm formation on *C. elegans*. Alternatively, it is possible that the Yop-Ysc injectisome structure represents a physical barrier, and prevents the association between the bacterial cell and the nematode surface. To attempt to differentiate between these possibilities, wild-type *Y. pseudotuberculosis* was grown at 37°C in the presence of calcium to permit the production of the T3SS injectisome, but not the release of Yop effectors (Forsberg et al., 1991, Michiels et al., 1990). *Y. pseudotuberculosis* was seeded onto NGM agar supplemented with calcium alongside a wild-type control grown at 30°C.

When *Y. pseudotuberculosis* is grown at 37°C in LB (conditions to express the T3SS injectisome, but not to secrete Yop effectors), biofilms are not produced on *C. elegans* (Figure 4.11). This suggests that the presence of the needle rather than secretion through the injectisome is responsible for the QS-dependent repression of biofilm formation on *C. elegans*.

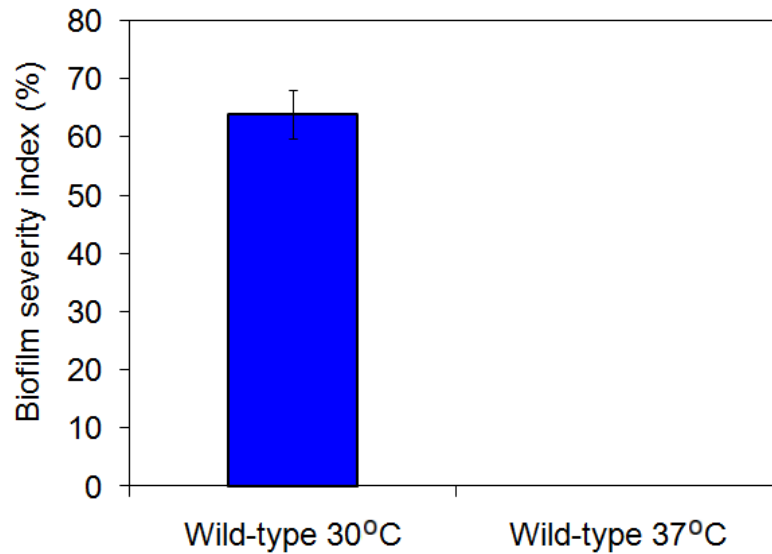


Figure 4.11| *Y. pseudotuberculosis* grown at 37°C does not form biofilms on *C. elegans*. When *Y. pseudotuberculosis* is conditioned to express the injectisome needle by growth at 37°C in medium containing calcium, the formation of biofilms is abolished, in contrast to cultures grown at 30°C which develop substantial biofilms on *C. elegans*. Measurements are the mean of 60 worms per condition from three plates, 20 worms per plate.

Tan *et al.* (2004) showed that the biofilm matrix may not be produced during growth at 37°C (Tan and Darby, 2004). To ensure that the presence of the injectisome on the cell surface, rather than the lack of matrix components, was responsible for the abrogation of biofilm formation, *Y. pseudotuberculosis* conditioned at 37°C was re-suspended in supernatant from *Y. pseudotuberculosis* grown at 30°C. In this case, even though biofilm matrix components had been supplied to the bacteria, they were still incapable of forming biofilms when compared with *Y. pseudotuberculosis* grown at 30°C (data not shown), emphasising the fact that the structural components of the T3SS are intrinsically important for preventing biofilm formation on *C. elegans*.

4.3 Discussion

4.3.1 The significance of QS regulated biofilm formation

Y. pseudotuberculosis forms biofilms on *C. elegans*, which are positively regulated by QS since the Δ*ypsI* Δ*ytbI* mutant, which cannot produce AHLs, is substantially reduced in its ability to form biofilms compared with the wild-type strain. Darby *et al*. (2002) previously showed that the biofilms formed by *Y. pseudotuberculosis* on *C. elegans* were sufficient to prevent the worm feeding on the bacteria, and in that study, when *C. elegans* was fed with *E. coli* OP50 a bolus of bacteria could be observed in the posterior pharynx, probably in the terminal bulb (Darby et al., 2002). Worms infected with the Δ*ypsI* Δ*ytbI* mutant often show a distended area of GFP fluorescence in the terminal bulb, which may reflect that these bacteria are being actively grazed upon by *C*. *elegans*. Comparatively, the absence of this distended area of green fluorescence in worms infected with the wild-type, and also the fact that bacteria through the intestines co-localise with matrix components suggests that the wild-type resists grazing by *C. elegans* in a way that is QS-dependent.

4.3.2 Extracellular DNA in the biofilm matrix

By using low concentrations of DAPI, eDNA can be labelled and not chromosomal DNA (Vilain et al., 2009). This has shown that large quantities of eDNA can be observed in the biofilms caused by *Y. pseudotuberculosis*. The fact that biofilms are reduced in the presence of DNase I further suggests that eDNA is an important matrix component of *Y. pseudotuberculosis* biofilms, while several

other bacteria also use eDNA as part of the matrix, including *P. aeruginosa*, *Bacillus cereus*, *Staphylococcus* spp., and *Streptococcus mutans* (Izano et al., 2008, Whitchurch et al., 2002, Vilain et al., 2009, Perry et al., 2009). Other studies have found that *Y. pseudotuberculosis* biofilms on *C. elegans* could be dispersed using M9 buffer raised to pH 10, which has been attributed to the breakdown of polysaccharides under high pH (Tan and Darby, 2004). However, DNA also dissociates under basic conditions, and given the importance of DNA in *Y. pseudotuberculosis* biofilms it is possible that polysaccharides and / or DNA were affected in that study, and in other bacteria, DNA and polysaccharides may both be necessary to maintain biofilm architecture (Izano et al., 2008). The eDNA appears to be organised in a network throughout the biofilm, as has been described for other bacteria, (Allesen-Holm et al., 2006, Böckelmann et al., 2006), and it is tempting to speculate that *Y. pseudotuberculosis* may use this DNA network as nanowires to facilitate movement within the biofilms. This has been suggested for other bacteria (Flemming et al., 2007), although any movement of *Y. pseudotuberculosis* in this way must be independent of flagella, since a non-motile Δ*fliC* mutant can still form biofilms (Atkinson et al., 2011).

There also appears to be substantially less eDNA in the biofilms formed by the Δ*ypsI* Δ*ytbI* mutant than those caused by wild-type. In other bacteria such as *P. aeruginosa* and *Streptococcus* spp., QS affects the release of eDNA, probably resulting from the controlled lysis of a sub-population of cells (Allesen-Holm et al., 2006, Spoering and Gilmore, 2006). However, AHL-dependent QS could not be demonstrated to directly affect eDNA production (data not shown). It is possible however that QS affects biofilm specific eDNA release, although it is more likely that the reduction of eDNA in biofilms formed by the Δ*ypsI* Δ*ytbI* mutant is a consequence of the reduced size of the biofilm or increased ability for the worm to graze on the mutant, rather than the reduced size of the biofilm being a consequence of reduced eDNA release. This notwithstanding, the

discovery that eDNA is present in and important for *Y. pseudotuberculosis* biofilms on *C. elegans* may prove to be important for the future bio-control of this and related organisms in the event of disease outbreaks.

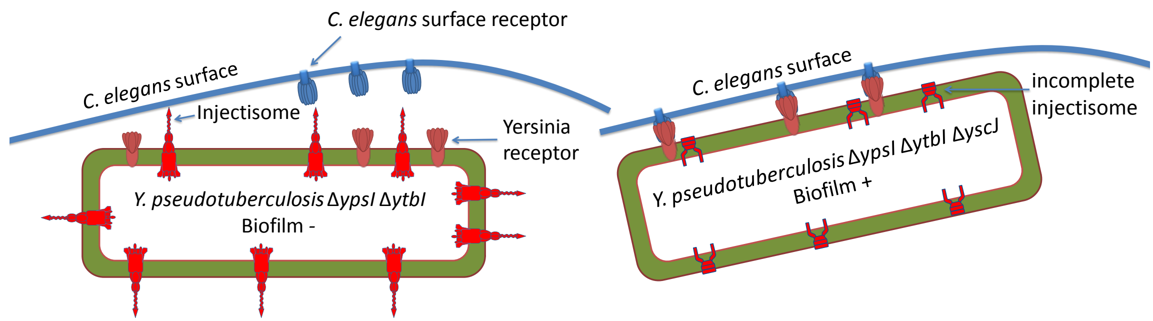
4.3.3 The relationship between QS, T3S and biofilms

In light of the relationship between QS, T3S and LCBs at 37°C, and since LCBs and *C. elegans* biofilms share similar matrix compositions, biofilms formed on *C. elegans* were investigated using plasmid cured derivatives of wild-type and the Δ*ypsI* Δ*ytbI* mutant alongside their pYV harbouring counterparts. This showed that in the absence of the pYV plasmid, the reduced biofilms formed by the Δ*ypsI* Δ*ytbI* mutant were restored to wild-type levels. This suggested that biofilm load on *C. elegans* was modulated by QS-dependent regulation of the pYV plasmid. To exclude factors on the pYV plasmid other than the T3SS, targeted deletion of *yscJ* in the Δ*ypsI* Δ*ytbI* mutant background (Δ*ypsI* Δ*ytbI* Δ*yscJ*) also restores biofilm load to wild-type levels, suggesting that the T3SS modulates biofilms on the cuticle of *C. elegans*.

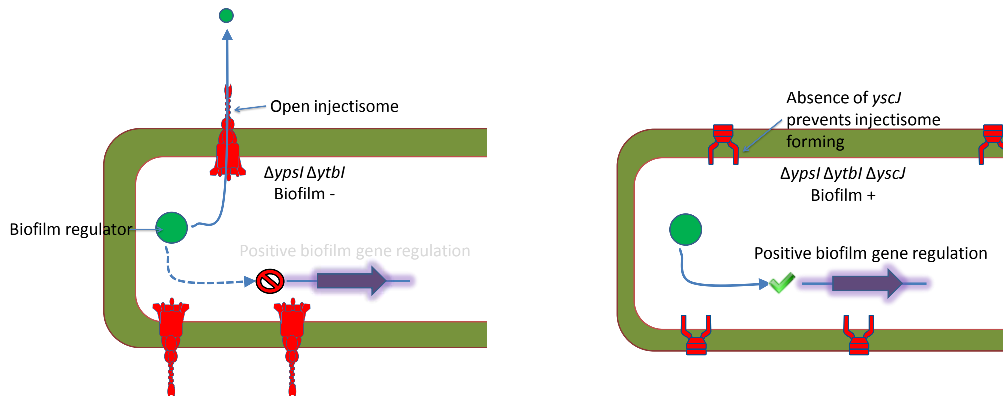
In the case of LCBs, T3S appears to release a factor into the supernatant which induces LCB formation, and so perhaps a similar process underlines the effect of T3S on *C. elegans* biofilms, albeit oppositely, whereby this factor would repress biofilm formation on *C. elegans*. Alternatively, it is possible that the Yop-Ysc injectisome structure represents a physical or regulatory barrier to the association between the bacterial cell and the nematode surface. To distinguish between these two possibilities, wild-type *Y. pseudotuberculosis* was conditioned by overnight growth at 37°C in calcium containing (i.e. not calcium chelated) media. This permits the production of the T3SS injectisome, but not the release of Yop effectors (Forsberg et al., 1991, Michiels et al., 1990), and allows for the

dissection of the role of secretion versus the injectisome structure in the control of biofilm formation on *C. elegans*. These results revealed that the presence of the Yop injectisome alone was sufficient to prevent biofilm development and, unlike LCBs, precluded a requirement for T3S *per se* in the regulation of biofilms on *C. elegans*.

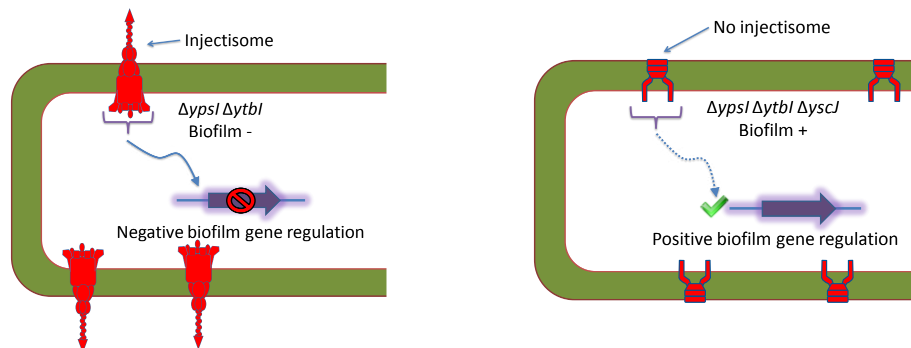
It is possible that the presence of the injectisome acts as a barrier to occlude the normal binding between *Y. pseudotuberculosis* cell surface proteins and components on the nematode surface (Figure 5.12 a). This would be consistent with the loss of biofilm formation which occurs following mutation of several *C. elegans* surface-determinants (Drace et al., 2009, Darby et al., 2007). However, it is also possible that the injectisome is able to feed back information to the cell which causes the prevention of biofilm formation- perhaps a constitutively open injectisome (which might occur in the Δ*ypsI* Δ*ytbI* mutant) releases a 'factor' which, when intracellular, fulfils a role in the induction of biofilms, and when the export of this factor is prevented, either by removal of the pYV or by deletion of *yscJ*, biofilm formation is restored to wild-type levels (Figure 4.12 b). The T3SS may do this in a way analogous to the FlgM / FliA relationship in flagella (Cornelis and Wolf-Watz, 1997a), and involves the Cpx two-component system and an extra-cytoplasmic function sigma factor (Carlsson et al., 2007a). However, this would not explain why when conditioning the wild-type at 37°C, where the injectisome is present but secretion should not occur, the strain does not produce biofilms, since calcium within the worm NGM should also continue to suppress T3S. It is also possible that the structure of the injectisome itself somehow signals gene regulation in the cell (Figure 4.12 c). It would be interesting to investigate if other processes are also affected in this way, and to dissect the nature of this regulation.



a) Injectisome physically prevents association



b) Injectisome secretes positive biofilm regulator



c) Structure of injectisome negatively affects biofilm gene regulation

Figure 4.12| Three possible mechanisms for biofilm repression by the Ysc injectisome in the Δ*ypsI* Δ*ytbI* mutant. The injectisome could prevent close association between the bacterial cell and the nematode surface, thus preventing attachment and biofilm formation (a). The injectisome may secrete a 'factor' which fulfils and intracellular role in activating biofilm formation (b), or the injectisome itself may cause changes in gene regulation that down-regulate biofilm formation when the injectisome is present (c).

Feedback may also involve other regulatory systems, especially those which reciprocally regulate the T3SS and biofilm formation. In *P. aeruginosa* the RetS and LadS sensor proteins accomplish this- RetS is required for expression of the T3SS genes and for repression of the *pel* and *psl* matrix polysaccharide genes, while LadS has the opposite effect on T3SS gene expression and biofilm formation (Goodman et al., 2004, Laskowski et al., 2004, Zolfaghar et al., 2005, Ventre et al., 2006). The SadARS three-component system also functions in this way, regulating both biofilm maturation and T3S, and provides a link between sensor-response systems in regulating biofilm formation versus T3S (Kuchma et al., 2005). Indeed in *P. aeruginosa*, the ExoS toxin of the T3SS, is regulated by the RhlI/R QS system and is down-regulated in biofilms (Hogardt et al., 2004). Mutants in T3SS also sometimes show altered biofilm formation in other bacteria, such as in *P. aeruginosa* where transposon mutants in the T3SS have been shown to exhibit more attachment to plastic, indicating that the T3SS can influence biofilm formation (Kuchma et al., 2005). These data provide evidence that T3SSs are more than just conduits for the delivery of toxins into eukaryotic cells, and are tightly linked into the lifestyles of bacteria, and that pleiotropic behaviours such as attachment and biofilm formation are affected depending on the ability for the bacteria to engage in T3S.

4.3.4 Biofilm formation on agar and in compost

Bacteriovorous nematodes such as *C. elegans* are found almost exclusively in organic nutrient rich soils (Barrière and Félix, 2005, Félix and Braendle, 2010, Kiontke and Sudhaus, 2006), and many *Y. pseudotuberculosis* outbreaks are associated with agricultural activity (Nuorti et al., 2004, Laukkanen et al., 2008, Rimhanen-Finne et al., 2009), indicating these organisms may share a natural habitat. In *Y. pseudotuberculosis* and other bacteria, biofilms aid resistance to

predation (Matz and Kjelleberg, 2005, Pickup et al., 2007, Drace and Darby, 2008); however Tan and Darby (2003) suggest that *Y. pseudotuberculosis* accumulates on *C. elegans* in a 'snow-plough' fashion (Tan and Darby, 2004), which may be difficult to visualise in soil environments which lack a lawn of bacteria for a 'snow-plough' to move through, since smooth agar plates are ideal for the lateral movement of worms through a viscous bacterial lawn, and on agar plates biofilms grow quickly (Tan and Darby, 2004, Joshua et al., 2003), while the compost model however presents a coarse, 3-dimensional environment. In spite of this, *Y. pseudotuberculosis* biofilms grow on *C. elegans* in compost, which shares the same profile of matrix components as those grown in the agar model, showing an extensive network of eDNA and EPS. The R-WGA labelled EPS is probably poly β-1,6-*N*-acetyl-D-glucosamine, the product of the *hms* locus (Bobrov et al., 2008). Although R-WGA can label other molecules, including peptidoglycan, which contains *N*-acetyl glucosamine as the sugar backbone (Sizemore et al., 1990), it is probably not labelling peptidoglycan in these biofilms, since R-WGA does not solely co-localise with bacterial cells expressing *gfp3*. Both *Y. pseudotuberculosis* and *X. nematophila* require the *hms* genes to form biofilms on *C. elegans* on agar (Drace and Darby, 2008), and these data suggest it is also involved in biofilms in compost.

Importantly, DAPI and R-WGA labelling shows the majority of the biofilm in the compost model appears to be throughout the worm digestive tract, so it is tempting to speculate that the biofilms originate inside the worm before protruding from the mouthparts. This would contradict the 'snow-plough' hypothesis, which would be difficult to use to explain the formation of these internal biofilms. This is interesting as biofilm matrix can also be visualised in the digestive tract of worms infected with *Y. pseudotuberculosis* in the agar model- when biofilms are labelled with DAPI and R-WGA, some eDNA and EPS can be observed in the *C. elegans* digestive tract. However, the density of the

biofilm around the worm head may largely occlude DAPI and R-WGA and prevent most of the ECM in the internal biofilm from being labelled. For example, when R-WGA is used on 48 h biofilms grown in the agar model, even the external biofilm mass is impenetrable to the label (Atkinson et al., 2011). Additionally, although biofilms that grow in the agar model can sometimes be multi-focal, the majority of the biofilm, both in soil and on agar, appears to emerge from the worm mouthparts. The presence of biofilm material in the worm digestive tract could suggest that these biofilms are regurgitated by the worm and accumulate outside the mouth; this would be analogous both to how *Y. pestis* is transferred from the flea vector to the mammal, and how *Photorhabdus spp.* are transferred from the digestive tract of entomopathogenic nematodes to the insect host (Ciche and Ensign, 2003) and raises the possibility that *Y. pseudotuberculosis* biofilms accumulation on nematodes may also help spread disease. This may be especially important since nematodes are the most abundant metazoan on Earth (Neher, 2001).

Both on agar and in soil, biofilm load appears substantially reduced in the Δ*ypsI* Δ*ytbI* mutant compared with the wild-type. The finding that mutation of *ypsI* and *ytbI* dramatically affects biofilm formation in soil and on agar presents evidence that QS is involved in regulating natural anti-predatory biofilms in *Y. pseudotuberculosis*. This requirement for QS in anti-predatory biofilms is becoming increasingly recognised (Matz et al., 2004, Queck et al., 2006), providing evidence that the extrapolation of a role for QS within *in vitro* biofilms to those in the environment is appropriate.

4.3.5 Conclusions

The work presented in this chapter suggests that *Y. pseudotuberculosis* biofilms which form on *C. elegans* are possibly a natural adaptation to resist predation by *C. elegans*, since biofilms that develop on *C. elegans* when incubated on a lawn of *Y. pseudotuberculosis* seeded onto an agar plate are also observed when worms are allowed to graze on *Y. pseudotuberculosis* inoculated into sterile compost. Biofilms that develop on agar and in soil are very similar in composition, staining positively for matrix components eDNA and polysaccharides. These biofilms contain substantial amounts of extracellular DNA, which is an important structural component of the biofilm since biofilms in the agar model can be reduced in the presence of DNase I. QS is important in the regulation of these biofilms, and influences the growth of biofilms in a way that is related to the repressive effect of QS on the *Y. pseudotuberculosis* T3SS. The precise mechanism by which the T3SS affects biofilm formation is still unclear, but clearly requires an intact injectisome rather than secretion through the injectisome *per se*. The injectisome may present a physical barrier between the bacterial cell and worm surface, preventing the close association required for attachment. However, it is equally possible that a pleiotropic effect of the injectisome is responsible for blocking biofilm formation, since injectisomes are known to be able to feed-back information such as the progress of injectisome assembly and competence for secretion, and it is perhaps one of these mechanisms which repress biofilm formation. While presently it is not possible to distinguish between these possibilities, evidence favours a pleiotropic role for the injectisome in regulating behaviour in *Y. pseudotuberculosis*, which will be the subject of the following Chapter.

Chapter 5 |

Reciprocal control of the motility regulon and the type 3 secretion system involves quorum sensing

5.1 Introduction

5.1.1 The biological link between flagella and the T3SS

Flagella are macromolecular bacterial motors attached to a long, thin, external propeller-like filament, which facilitate bacterial movement (Macnab, 1999, Macnab, 2003). Conversely, bacterial type 3 secretion systems are macromolecular organelles which penetrate eukaryotic cell membranes and deliver anti-host effector proteins into the cytoplasm of eukaryotic cells (Galán and Collmer, 1999, Cornelis, 2006). Flagella are important for invasion and virulence in several bacterial species (Josenhans and Suerbaum, 2002), while T3SSs modulate the physiology of the host cell to co-opt host cells to facilitate bacterial growth, for example by suppressing phagocytosis or inducing apoptosis (Galán and Collmer, 1999, Mota and Cornelis, 2005).

Motility and virulence are intricately linked (Ottemann and Miller, 1997, Josenhans and Suerbaum, 2002), and these organelles have a remarkably similar architecture and have evolved from a common ancestor (Blocker et al., 2003, Saier, 2004, Erhardt et al., 2010, Gophna et al., 2003) (Figure 5.1). Underlining the structural homology between the two systems, there is clear conservation in the proteins required to build these structures, with approximately half the proteins required for building flagella being homologous to their injectisome counterparts (Erhardt et al., 2010, Cornelis, 2006).

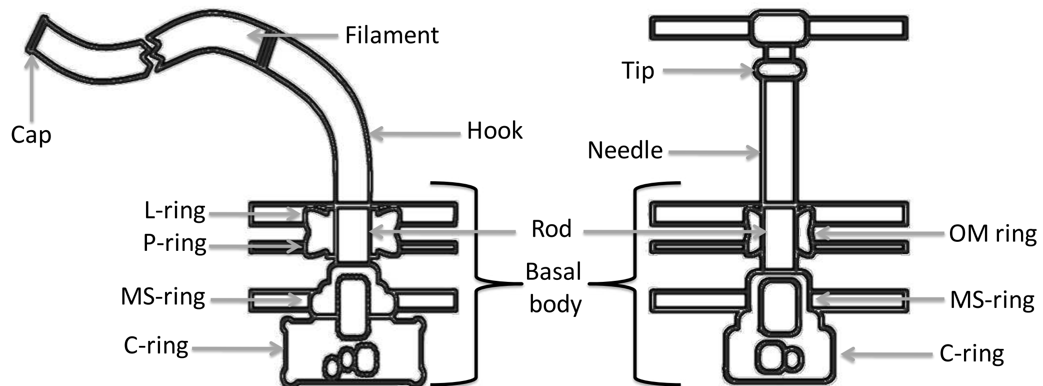


Figure 5.1| Comparison of the structure of the flagella and injectisome. The bacterial flagella and type 3 secretion injectisome are structurally similar, both comprising a cytoplasmic C-ring like structure, an inner membrane MS-ring which connects to an inner rod, attached to the outer membrane OM-ring (comprised of the P- and L-ring in flagella) (Cornelis and Van Gijsegem, 2000, Cornelis, 2006). In flagella, a hook connects the basal body to the filament (Homma et al., 1990), while in the injectisome the needle emerges from the basal body (Cornelis, 2006). Both structures possess a cap (known as the tip in the injectisome) which covers the most distal end of the nanomachine. Figure adapted from Erhardt et al., 2010.

5.1.2 The dichotomy of Flagella and the T3SS in the *Yersinia*

In *Y. pseudotuberculosis* and *Y. enterocolitica t*here is a natural dichotomy between the expression of the flagella and Yop-Ysc T3SS, whereby flagella are only expressed at ambient temperatures (<28°C) and repressed at 37°C (Kapatral and Minnich, 1995), while the T3SS is only produced during growth at 37°C (Cornelis, 2002b). The down regulation of flagella at 37°C is co-incident with the transcriptional down-regulation of the flagella master regulator *flhDC* and the flagella specific sigma factor *fliA* (Kapatral and Minnich, 1995, Atkinson

et al., 2008) due to the action of global modulators including Csr (Wei et al., 2001, Heroven et al., 2008), and H-NS (Bertin et al., 1994). For the Yop-Ysc system, expression of the T3SS regulator, *virF*, is modulated by DNA topology changes (Rohde et al., 1994, Rohde et al., 1999) so that it becomes active only at 37°C. However, several other systems also regulate *virF* expression and the T3SS. These include YmoA (Cornelis et al., 1991, de Rouvroit et al., 1992), FlhDC (Bleves et al., 2002) and FliA (Horne and Prüß, 2006), which repress the T3SS and modulate the environmental control of Yop production, such that mutants in these genes do not require growth at 37°C in order to produce Yop proteins, and can also secrete Yop effectors into the growth supernatant at lower temperatures, regardless of calcium concentration (Bleves et al., 2002, Horne and Prüß, 2006, Cornelis et al., 1991). Several systems influence both motility and the T3SS, particularly DNA-methylation by the Dam adenine methyltransferase (Julio et al., 2002, Falker et al., 2006, Falker et al., 2007), O-antigen status of *Yersinia* lipopolysaccharide, which is also regulated by Dam (Falker et al., 2007, Bengoechea et al., 2004, Perez-Gutierrez et al., 2007), the RNA chaperone Hfq (Schiano et al., 2010) and nucleosome-associated proteins H-NS and YmoA (Cornelis et al., 1991, Ellison and Miller, 2006). This suggests that DNA topology changes alone do not explain the thermoregulation of T3S, and instead indicates that a complex regulatory circuit underpins the regulatory relationship between flagella and the Yop-Ysc T3SS. However, while it has been shown that the flagella system can regulate the Yop-Ysc T3SS, it has not been shown that this regulation can operate reciprocally.

5.1.3 Chapter 5 aims

The data presented in Chapter 3 demonstrated that QS and the Yop-Ysc T3SS were linked, and that the Ysc injectisome played a role in the formation of QS-mediated LCBs in *Y. pseudotuberculosis*, probably as a result of T3S-dependent protein secretion. The data presented in Chapter 4 demonstrated that the formation of biofilms by *Y. pseudotuberculosis* on the nematode *C. elegans* is regulated by QS via a mechanism involving the Ysc injectisome. The finding that the injectisome could influence biofilm formation in liquid culture and on the *C. elegans* cuticle is interesting as it suggests that the injectisome can play a role in regulating pleiotropic behaviours, and sets a precedent for the involvement of the T3SS in QS-associated traits. Alongside clumping, motility was the first phenotype to be linked to QS-dependent regulation in *Y. pseudotuberculosis* (Atkinson et al., 1999, Atkinson et al., 2008). The flagella regulon also regulates biofilm formation, both in liquid culture (Chapter 3) and on the surface of *C. elegans* (Atkinson et al., 2011), while in *Y. enterocolitica* biofilm formation and T3S are also linked to the flagella regulon (Bleves et al., 2002, Horne and Prüß, 2006, Kim et al., 2008). This prompted an investigation into the role that the Ysc injectisome played in regulating flagella-mediated motility. A Δ*yscJ* mutant, unable to produce injectisomes (Diepold et al., 2010) was constructed in an attempt to investigate the role of the injectisome itself, since the underlying regulatory elements of the T3SS remain genetically intact in this mutant.

The data presented in this chapter demonstrates that motility is negatively regulated by some component or components of the Ysc injectisome, since a Δ*yscJ* mutant, which cannot build the injectisome, shows a hyper-motile phenotype during growth at 22°C. This is supported by the observation that expression of the flagella regulators *flhDC* and *fliA* are de-repressed in this

mutant. Hyper-motility and up-regulation of *flhDC* is dependent on QS, since deletion of the AHL synthase genes *ypsI* and *ytbI* results in the down-regulation of *flhDC* expression and motility. Furthermore, the data reveals that the relationship between T3S, QS and flagella may involve an effect of the Ysc injectisome on the expression of the QS genes, since in the Δ*yscJ* mutant, the expression of *ypsI* and *ytbR* is down-regulated compared with the wild-type, whereas the expression of *ypsR* and *ytbI* is up-regulated. In addition, and further to the results presented in Chapter 3, the results in this chapter show that the regulation of Yop secretion by FlhDC, FliA and FlhA is under-lined by de-repression of the expression of the major Yop-Ysc regulator, VirF, and up-regulated *virF* expression is also observed in several QS mutants which secrete Yop-related proteins into the growth supernatant during growth at 30°C.

5.2 Results

5.2.1 Mutation of *yscJ* results in hyper-motility and over-production of flagellin

To investigate whether any regulation was exerted by structural elements of the Yop-Ysc system on flagella, a *Y. pseudotuberculosis yscJ* mutant was constructed. This mutant then represents an injectisome-minus strain, and can indicate if structural elements of the T3SS injectisome could play a role in regulating flagella, since the underlying regulatory elements of the T3SS are genetically intact.

When viewed by light microscopy, cultures of the Δ*yscJ* mutant grown overnight at 22°C could be seen to be motile, similar to the Δ*ypsI* and Δ*ypsR* mutants, but unlike the wild-type, which can take up to 72 h to become motile (Atkinson et al., 1999, Atkinson et al., 2008). To investigate this further, motility plate assays were carried out by inoculating overnight cultures of *Y. pseudotuberculosis* wild-type, the Δ*yscJ* mutant, the complemented strain Δ*yscJ* pHG*yscJ*, where Δ*yscJ* is provided with a functional copy of *yscJ* on pHG*yscJ*, into swimming motility agar as previously described (Atkinson et al., 1999). These plates were incubated at 22°C and 37°C for 48 h, and the degree of motility measured as the radial distance of the swim-colony from the point of inoculation (Figure 5.2).

These results show that at 22°C and as has been reported previously (Atkinson et al., 1999, Atkinson et al., 2008), no motility could be observed in the wild-type under these conditions, (Figure 5.2 i a). However, the Δ*yscJ* mutant shows

substantial motility (Figure 5.2 i b), reaching a radial diameter of 40 mm. In contrast, swimming is reduced when functional *yscJ* is restored to the Δ*yscJ* mutant on pHG*yscJ* (Figure 5.2 i c). At 37°C, similarly to that seen in liquid culture, none of the strains appeared to swim (Figure 5.2 ii).

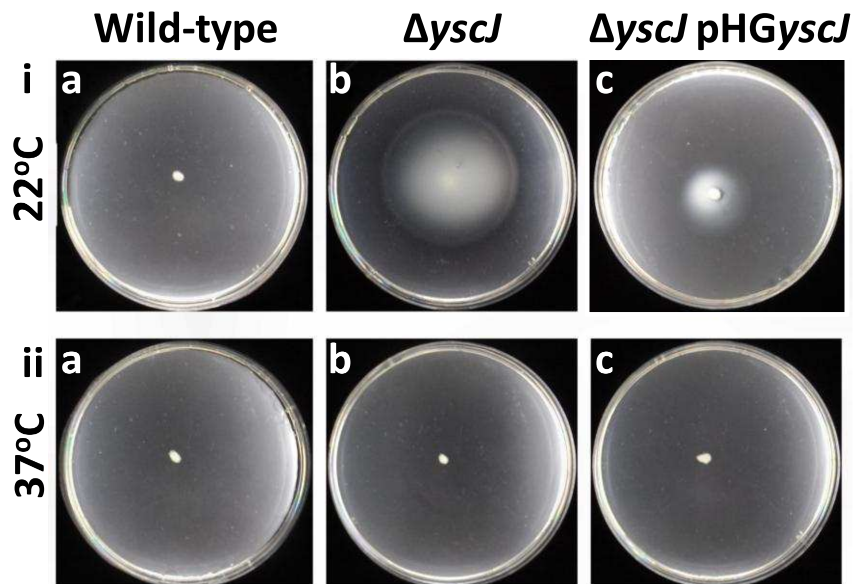


Figure 5.2| Mutation of *yscJ* results in hyper-motility during growth at 22°C. In the absence of the gene encoding the T3SS structural lipoprotein YscJ, *Y. pseudotuberculosis* is hyper-motile in agar during incubation at 22°C (i b), and displays a radial distance of approximately 40 mm, which is not observed in the wild-type control (i a). Complementation with functional *yscJ* on pHG*yscJ* however reduces Δ*yscJ*-dependent swimming to levels much more similar to the wild-type (i c). During incubation at 37°C however, none of the strains were motile in agar (ii).

The increased motility of the Δ*yscJ* mutant strongly suggested that it may over-produce flagella. By examining Δ*yscJ* mutant cultures that have been stained with a modified Leifson stain to visualise flagella by microscopy (Clark, 1976), long filamentous structures emanating from the cell surface were visible which were not apparent in the wild-type (data not shown). To confirm the increase in

flagella production in the Δ*yscJ* mutant, flagella were purified from cultures grown for 24 h at 22°C using the cannular method previously described (Atkinson et al., 1999), and following analysis by SDS-PAGE revealed a dense band (~46 kDa) in the Δ*yscJ* mutant preparations that is substantially reduced in the wild-type or the Δ*yscJ* pHG*yscJ* strains. This band probably corresponds to flagellin (FliC: 45 kDa) and demonstrates that flagella are over-produced in the Δ*yscJ* mutant when compared with the wild-type (Figure 5.3).

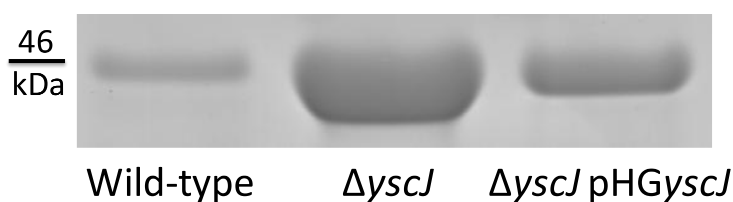


Figure 5.3| The Δ*yscJ* mutant over-produces flagellin during growth at 22°C. By purifying flagella from bacterial cultures via the cannular method, a dense band at near the 46 kDa marker can be observed in the Δ*yscJ* mutant which is substantially reduced in wild-type and the complement Δ*yscJ* pHG*yscJ* strain. This band probably corresponds to the flagellin protein (FliC: 45 kDa). This dense ~45 kDa protein band is not seen in the strains following growth at 37°C (data not shown).

Flagella, alongside pili, are also involved in swarming motility (Kohler et al., 2000) To investigate the possibility that de-repression of *flhDC* and flagella mediated swimming also affected the ability for *Y. pseudotuberculosis* to engage in this type of surface translocation, which has not previously been reported in *Y. pseudotuberculosis* (Atkinson et al., 1999), the wild-type, Δ*yscJ* mutant and the complemented strain Δ*yscJ* pHG327 were inoculated on to swarm agar plates, and incubated at either 22°C or 37°C for 72 h. However, none of the strains were observed to form swarm colonies (data not shown).

5.2.2 The Δ*yscJ* mutant over-expresses flhDC and fliA

To understand the mechanism of the over-production of flagella and hyper-motility in the Δ*yscJ* mutant, a series of reporter constructs were used, which link luciferase expression and light production to the activity of a chosen promoter. The promoter fusion P_{flhDC}::*lux*, which reports expression of the flagella master regulator *flhDC* (Atkinson et al., 2008) was used to measure the expression of *flhDC* in the Δ*yscJ* mutant and the complemented strain Δ*yscJ* pHG*yscJ* during growth at 22°C or 37°C (Figure 5.4). At both temperatures, *flhDC* is up-regulated in the Δ*yscJ* mutant, and is approximately 4-fold higher at 22°C, and 8-fold higher during growth at 37°C when compared with the wild-type. During growth at 22°C, wild-type expresses *flhDC* approximately 3 fold higher than during growth at 37°C, similar to the difference described previously (Atkinson et al., 2008). At both 22°C and 37°C, when the Δ*yscJ* mutant is complemented with *yscJ* on pHG*yscJ*, expression of *flhDC* is restored to levels similar to the wild-type.

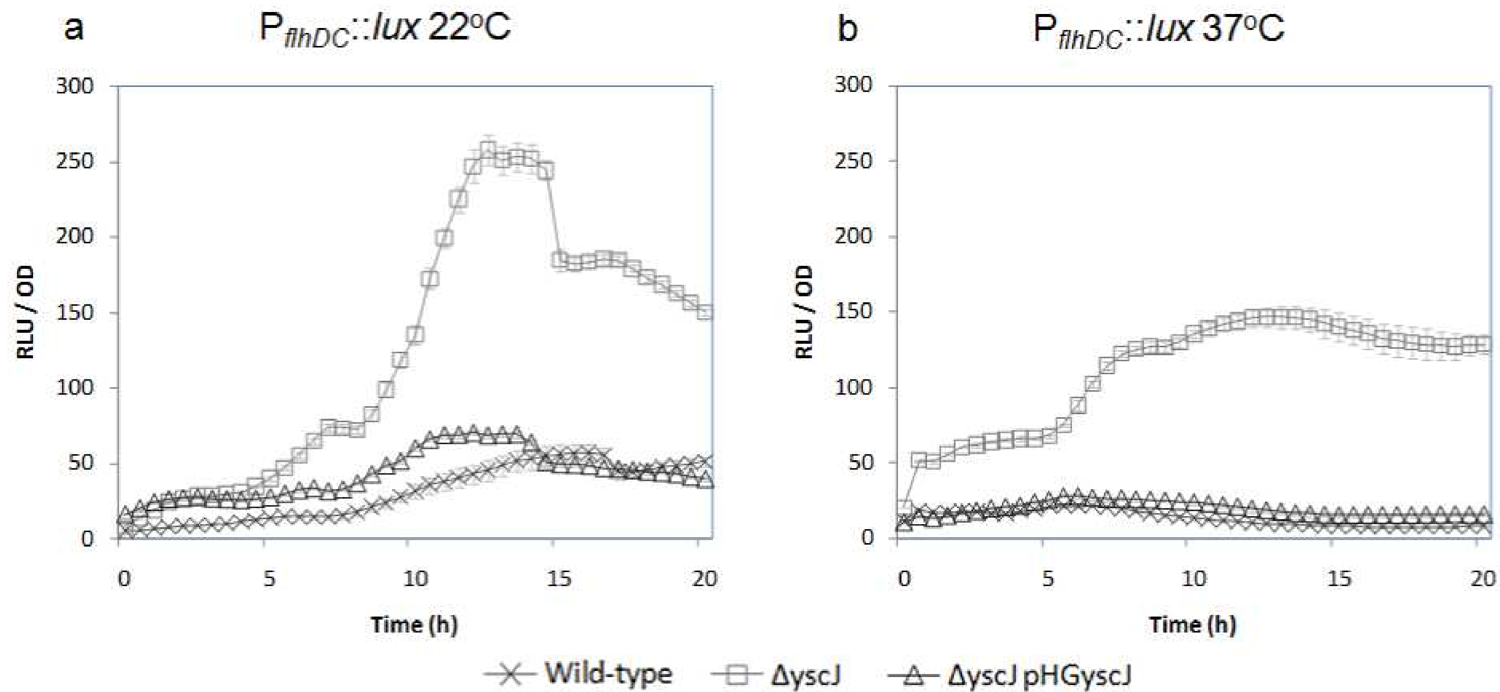


Figure 5.4| The Δ*yscJ* mutant is up-regulated for *flhDC* expression. Measurement of the expression of *flhDC* in the wild-type, the Δ*yscJ* mutant and Δ*yscJ* pHG*yscJ* strains during growth at 22°C and 37°C (b) reveals that at 22°C *flhDC* expression in the Δ*yscJ* mutant is approximately 4 fold higher than the wild-type (a), while at 37°C *flhDC* expression is and 8 fold up-regulated in Δ*yscJ* compared with the wild-type (b). At both temperatures, when functional *yscJ* is provided to the Δ*yscJ* mutant on pHG*yscJ* expression of *flhDC* is restored to levels similar to the wild-type. Error bars represent standard deviation.

Flagella are also regulated by the flagella sigma factor FliA (Liu and Matsumura, 1995, Atkinson et al., 2008), and so the expression of *fliA* was measured in wild-type, the Δ*yscJ* mutant and the complemented strain Δ*yscJ* pHG*yscJ* over the growth phase at 22°C and 37°C using a P_{fliA}::*lux* promoter fusion (Atkinson et al., 2008). Figure 5.5 shows that, similar to *flhDC* expression, *fliA* expression is increased in the Δ*yscJ* mutant during growth at 22°C (Figure 5.5 a) or 37°C (Figure 5.5 b) when compared with the wild-type. In agreement with previous studies (Atkinson et al., 2008), expression of *fliA* is repressed in the wild-type during growth at 37°C when compared with growth at 22°C, with expression at 22°C approximately 10-fold higher than that at 37°C. In the Δ*yscJ* mutant, during growth at 22°C, *fliA* expression is roughly 5-fold higher than in the wild-type, while during growth at 37°C *fliA* expression in Δ*yscJ* is roughly 25-fold higher than in the wild-type.

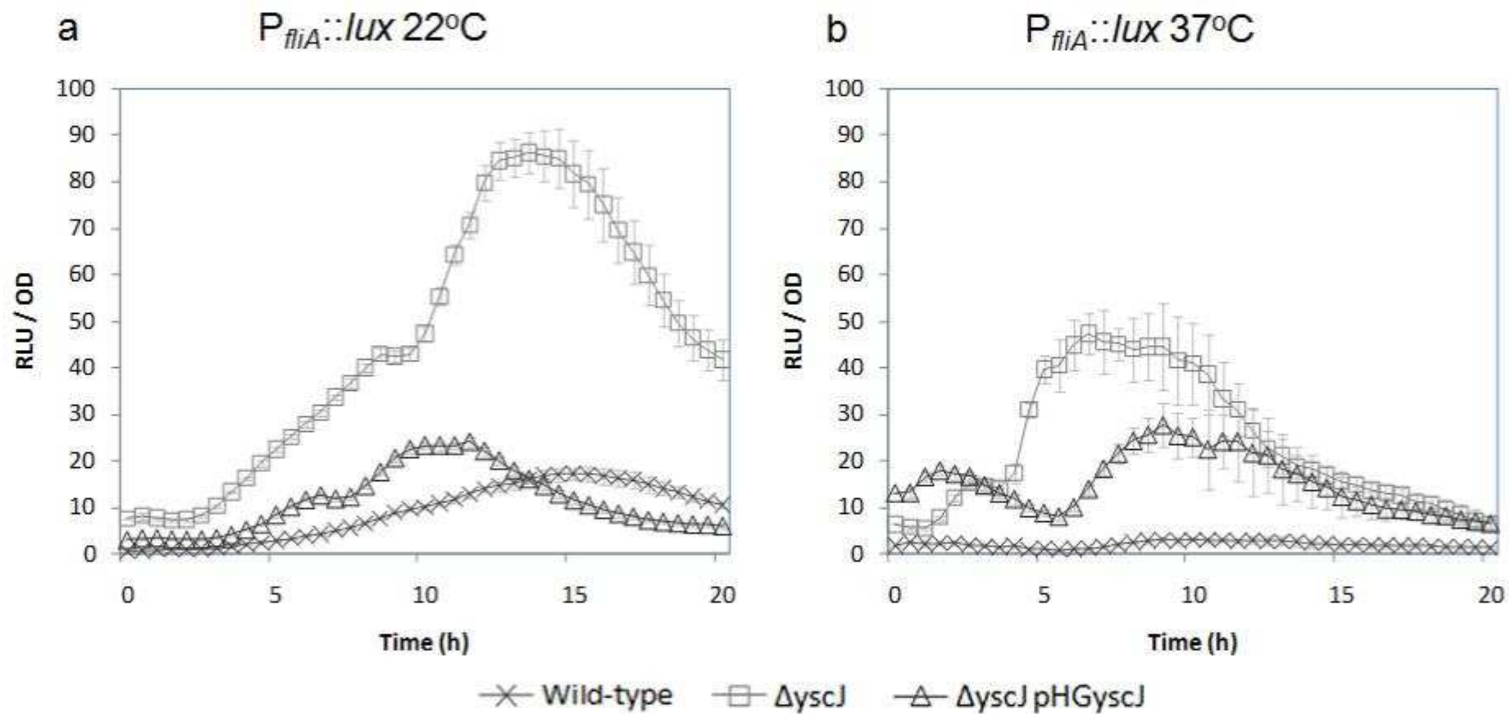


Figure 5.5| The Δ*yscJ* mutant is up-regulated for *fliA* expression. Measuring the expression of *fliA* in wild-type, the Δ*yscJ* mutant and Δ*yscJ* pHG*yscJ* during growth at 22°C or 37°C reveals that at 22°C the Δ*yscJ* mutant expresses *fliA* approximately 10 fold more than does the wild-type (a), while at 37°C the Δ*yscJ* mutant expresses *fliA* 25 fold higher than the wild-type (b). Supplying functional *yscJ* to the Δ*yscJ* mutant on pHG*yscJ* restores *fliA* expression to levels much more similar to the wild-type during growth at 22°C (a), while at 37°C complementation reduces *fliA* expression approximately 2 fold (b). Error bars represent standard deviation.

Together, these results show that the up-regulation of flagella production and hyper-motility in the Δ*yscJ* mutant during growth at 22°C is mirrored by an up-regulation of *flhDC* and *fliA* expression.

5.2.3 Hyper-motility and up-regulation of *flhDC* in the Δ*yscJ* mutant requires YpsI and / or YtbI

It has previously been reported that the two *Y. pseudotuberculosis* QS systems (YpsR/I and YtbR/I) regulate motility via *flhDC* and *fliA* in an AHL-dependent manner (Atkinson et al., 1999, Atkinson et al., 2008, Atkinson et al., 2011). To investigate whether QS plays a role in Δ*yscJ*-dependent hyper-motility, the AHL-negative, injectisome-minus Δ*ypsI* Δ*ytbI* Δ*yscJ* triple mutant was inoculated into motility agar alongside the Δ*yscJ* mutant, the Δ*ypsI* Δ*ytbI* mutant, and the wild-type, and incubated for 48 h at 22°C (Figure 5.6). These results show that, in contrast to the hyper-motility observed in the Δ*yscJ* mutant, the AHL-negative Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant, like the Δ*ypsI* Δ*ytbI* mutant and the wild-type, is non-motile. These results suggest a link between QS-mediated motility and the presence of functional YscJ. No motility was seen in any of the strains during incubation at 37°C (data not shown).

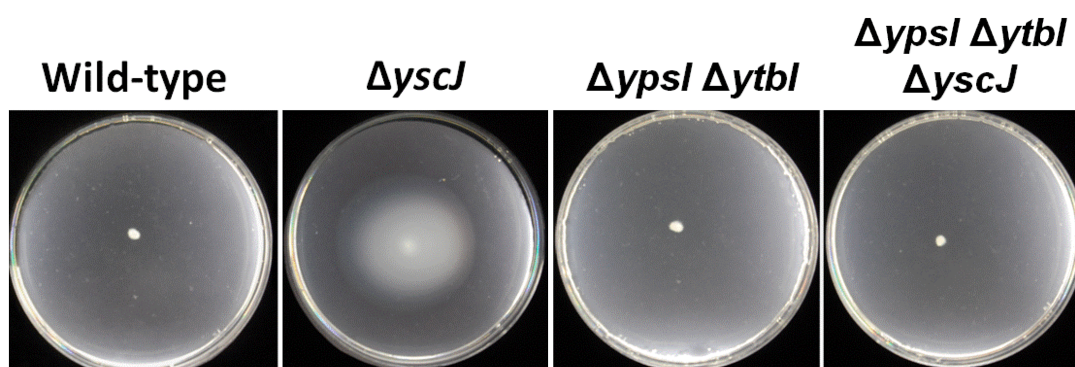


Figure 5.6| YpsI and YtbI are necessary for the Δ*yscJ* mutant to exhibit hyper-motility. The wild-type and AHL-negative Δ*ypsI* Δ*ytbI* mutant do not show motility after 48 h in swimming agar. The Δ*yscJ* mutant however, shows hyper-motility at this temperature, while the AHL-negative Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant does not swim under these conditions.

The expression of *flhDC* is known to be repressed in the Δ*ypsI* Δ*ytbI* mutant (Atkinson et al., 2008). In order to determine if the absence of motility in the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant (compared with Δ*yscJ*) was due to down-regulation of *flhDC*, P_{flhDC}*::lux* was introduced into the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant and the complement Δ*ypsI* Δ*ytbI* Δ*yscJ* pHG*yscJ* strain, and light output measured over 20 h of growth at both 22°C and 37°C.

Figure 5.7 shows that, in contrast to the Δ*yscJ* mutant, which is up-regulated for *flhDC* expression during growth at 22°C (Figure 5.7 a) or 37°C (Figure 5.7 b), the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant expresses *flhDC* at levels lower than that observed in the wild-type, although during growth at 22°C the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant express *flhDC* slightly higher than does the Δ*ypsI* Δ*ytbI* mutant. Taken together, these results confirm that QS, in conjunction with YscJ, influence the expression of *flhDC* and swimming motility, although *yscJ* appears to have a small, QS-independent effect on *flhDC* expression during growth at 22°C.

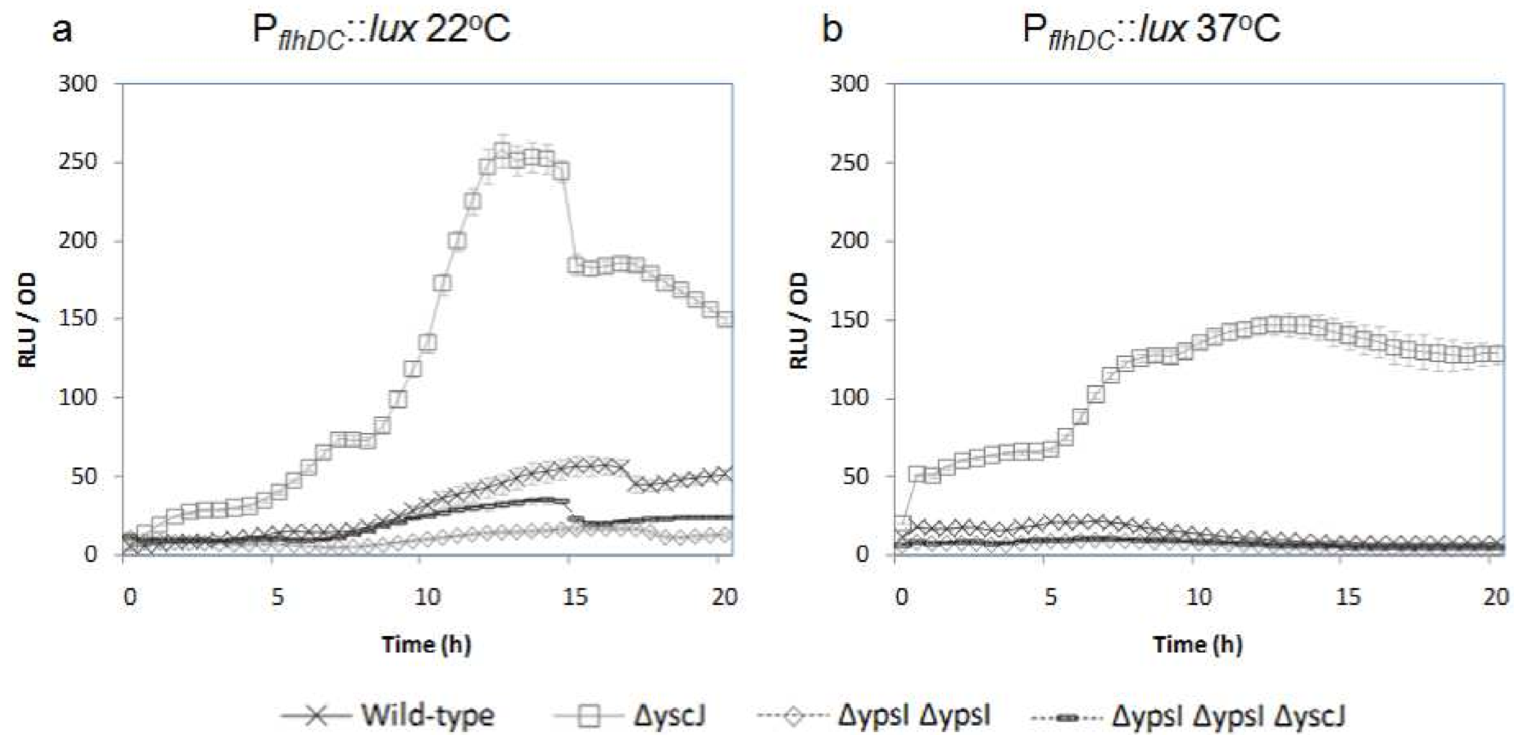


Figure 5.7| YpsI and YtbI are necessary for up-regulation of *flhDC* expression in Δ*yscJ*. The high expression of flhDC observed in Δ*yscJ* is reduced approximately 10-fold following subsequent mutation of *ypsI* and *ytbI* (Δ*ypsI* Δ*ytbI* Δ*yscJ*) during growth at 22°C (a) and approximately 12-fold during growth at 37°C (b) to levels lower than that observed in the wild-type. Error bars represent standard deviation.

5.2.4 The expression of QS is modulated by functional YscJ

On the basis of the unexpected reciprocal relationship between the T3SS and motility that required QS, the observation that QS regulated the T3SS led to the hypothesis that the T3SS might also reciprocally regulate QS. Previously, YpsI has been suggested to repress motility in agar, whereas YtbI probably promotes this phenotype (Atkinson et al., 2008). Since hyper-motility in Δ*yscJ* was abrogated following deletion of *ypsI* and *ytbI*, it is possible that hyper-motility in Δ*yscJ* is the result from either repression of *ypsI* or increased expression of *ytbI* in the mutant. To investigate this, lux-based promoter fusions (P_{ypsI}::*lux* and P_{ytbI}::*lux*) which report the expression of *ypsI* and *ytbI* (Atkinson et al., 2008) were introduced into the Δ*yscJ* mutant and Δ*yscJ* pHG*yscJ* to investigate the expression of the AHL synthase genes over the growth phase (Figure 5.8). These results show that, over the growth phase, the expression of *ypsI* is highly repressed in the Δ*yscJ* mutant when compared with the wild-type. Complementation of Δ*yscJ* by providing *yscJ* on pHG*yscJ* partially restores *ypsI* expression to wild-type levels (Figure 5.8 a). Conversely, the expression of *ytbI* is increased approximately 5-fold in the Δ*yscJ* mutant compared with the wild-type. When *yscJ* is provided on pHG*yscJ*, expression is reduced to wild-type levels (Figure 5.8 b).

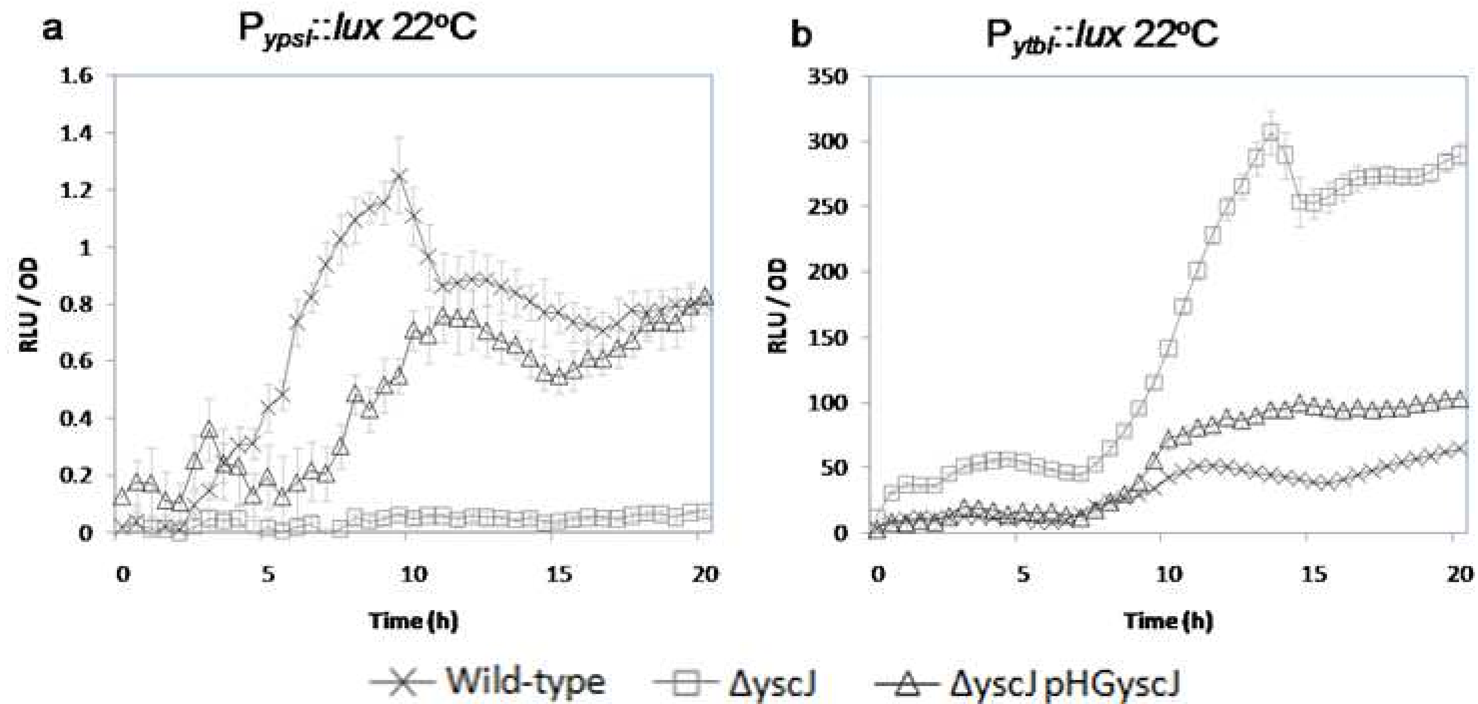


Figure 5.8| The expression of the AHL synthase genes *ypsI* and *ytbI* at 22°C is altered in the Δ*yscJ* mutant. Analysis of the expression of *ypsI* and *ytbI* as measured by P_{ypsI}*::lux* or P_{ytbI}*::lux* reveals that expression of *ypsI* is completely abolished in the Δ*yscJ* mutant, while complementation with pHG*yscJ* restores expression to levels more similar to the wild-type (a). However, the Δ*yscJ* mutant expresses *ytbI* approximately 5 fold higher than does the wild-type, which is restored to levels similar to the wild-type following complementation with pHG*yscJ* (b).

The correlation between the expression of the AHL synthase enzymes and the hyper-motile phenotype of the Δ*yscJ* mutant illustrates a very clear relationship; expression of the motility repressor, YpsI, is itself repressed in the Δ*yscJ* mutant compared with the wild-type, and the motility activator, YtbI, is over-expressed. To examine the relationship between the AHL receptor proteins and Δ*yscJ*-associated motility, the expression of *ypsR* and *ytbR* was measured over the growth phase at 22°C (Figure 5.9). These results show that *ypsR* expression is approximately 3-fold higher in the Δ*yscJ* mutant compared with the wild-type (Figure 5.9 a), while expression of *ytbR* is completely abolished in the Δ*yscJ* mutant (Figure 5.9 b). Restoration of functional *yscJ* to the Δ*yscJ* mutant on pHGyscJ reduces expression of *ypsR* to wild-type levels (Figure 5.9 a); however, complementation results in only a small, but consistent, increase in expression of *ytbR* when compared with the Δ*yscJ* mutant, although the expression of *ytbR* in the wild-type is approximately 8-fold higher than the Δ*yscJ* pHGyscJ strain (Figure 5.9 b).

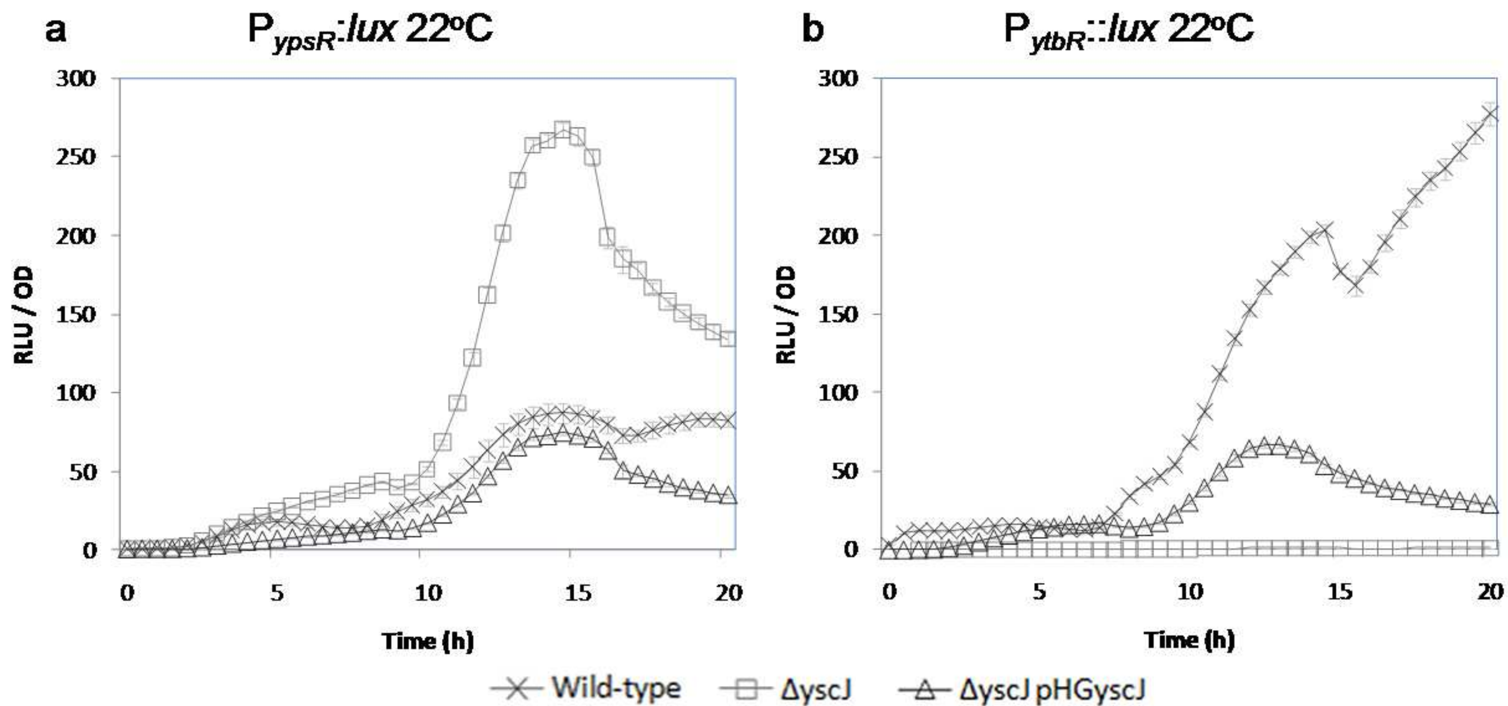


Figure 5.9| The expression of the AHL receptor genes *ypsR* and *ytbR* at 22°C is altered in the Δ*yscJ* mutant. Measurement of *ypsR* and *ytbR* expression reveals that the Δ*yscJ* mutant expresses *ypsR* approximately 4-fold higher than the wild-type, which is reduced to wild-type levels following complementation with functional *yscJ* on pHG*yscJ* (a). Conversely, *ytbR* is highly expressed in the wild-type, whereas expression is practically abolished in the Δ*yscJ* mutant. Complementation with pHG*yscJ* partially restores the expression of *ytbR*; however there is still an approximate 8-fold difference in the expression of *ytbR* between the wild-type and the Δ*yscJ* pHG*yscJ* strain (b).

These data show that, like the AHL synthase genes, expression of the receptors YpsR and YtbR is differentially regulated by either YscJ itself, or some down-stream component of the injectisome which requires YscJ for its function. However unlike the AHL synthases, where *ypsI* appears activated by YscJ and *ytbI* appears repressed by YscJ, the expression of *ypsR* is up-regulated in the Δ*yscJ* mutant while expression of *ytbR* is effectively abolished in the mutant compared with the wild-type. This suggests that the presence of functional YscJ- either due to YscJ itself or a downstream element of the injectisome- acts repressively towards *ypsR* and activates *ytbR*.

5.2.5 The flagella regulon reciprocally regulates the T3SS via an effect on the expression of *virF*

The up-regulation of swimming in the Δ*yscJ* mutant is interesting as it shows a component of the Ysc-injectisome can influence motility, which is strikingly similar to the results in Chapter 3 which revealed that the flagella structural component FlhA could influence the production of Yop-related proteins during culture at 37°C or 30°C. The up-regulation of *flhDC* and *fliA* expression in the Δ*yscJ* mutant is also significant as it shows that, at least for Δ*yscJ*-associated motility, this relationship is at the level of the transcriptional control of key regulators, and suggests that structural components of the injectisome can behave as a checkpoint in the transcriptional regulation of flagella. This raised the possibility that the up-regulation of Yop-related proteins in the Δ*flhDC*, Δ*fliA* and Δ*flhA* mutants shown in Chapter 3 involved over-expression of the main transcriptional regulator of the Yop-Ysc system- VirF (Cornelis et al., 1989), particularly at 30°C, since at temperatures below 37°C *virF* is considered to be down-regulated (de Rouvroit et al., 1992, Hoe and Goguen, 1993). To

investigate this, a *virF* promoter fusion (P_{virF}::*lux*) was constructed and used to measure expression of *virF* over the growth phase at 30°C, and in the presence of calcium, in the wild-type and the Δ*flhDC*, Δ*fliA*, Δ*flhA* and Δ*fliC* mutants, and the Δ*flhDC* mutant complement strain, Δ*flhDC* pGem*::flhDC*, where functional *flhDC* is provided to on pGem::*flhDC* (Atkinson et al., 2008).

Figure 5.10 shows the Δ*fliA*, Δ*flhA* (Figure 5.10 a) and Δ*flhDC* (Figure 5.10 b) mutants express *virF* in a way that increases in over the growth phase to levels approximately 4 to 6-fold higher than that observed in the wild-type, which appears to express *virF* constitutively at low levels over the growth phase. The Δ*fliC* mutant, conversely, displays no difference in the expression of *virF* when compared with the wild-type (Figure 5.10 a), in line with the lack of Yop production observed in this strain during growth at 30°C (Atkinson et al., 2011). Complementation of the Δ*flhDC* mutant with pGem::*flhDC* also reduces expression of *virF* to levels similar to the wild-type (Figure 5.10 b). The data from these two panels are taken from the same experiment, and have been plotted separately for clarity of presentation.

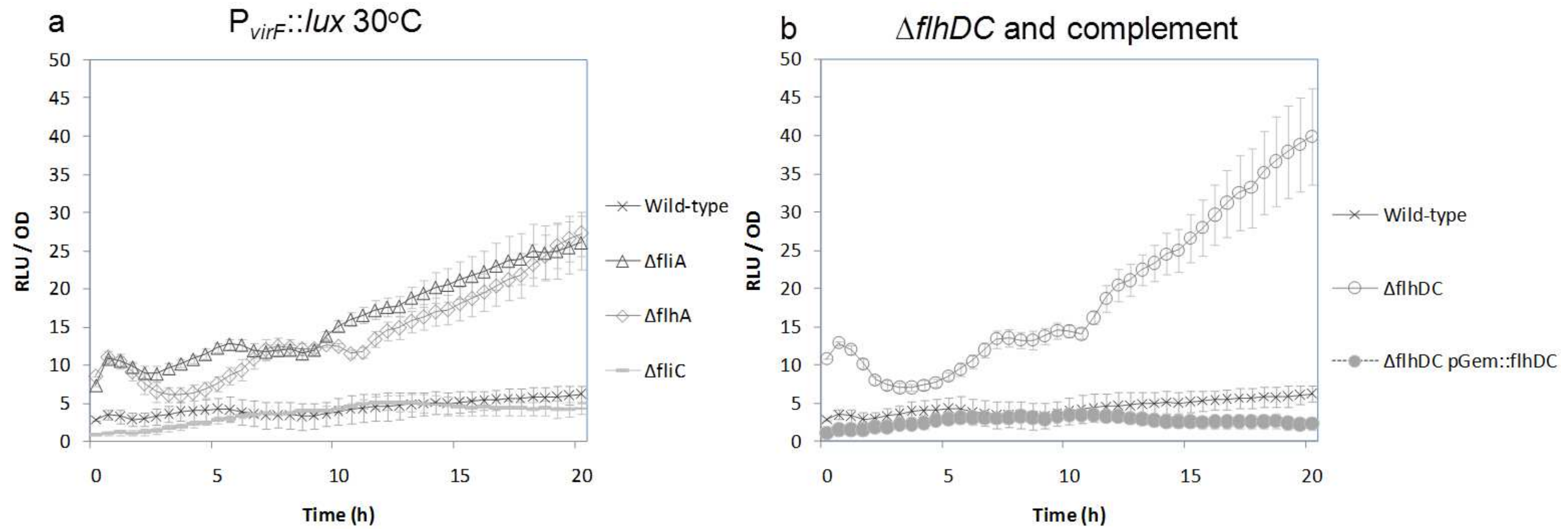


Figure 5.10| Mutants in *flhDC*, *fliA* and *flhA* but not *fliC* are up-regulated for *virF* expression at 30°C. Measurement of *virF* expression in the flagella mutants reveals that *virF* is over-expressed in the Δ*fliA*, Δ*flhA* (a) and Δ*flhDC* (b) mutants approximately 6 – 8 fold, when compared with the wild-type, which expresses *virF* constitutively at low levels. However, expression of *virF* in the Δ*fliC* mutant is similar to the wild-type (a), and expression of *virF* is restored to wild-type levels when functional *flhDC* is restored to the Δ*flhDC* mutant on pGem::*flhDC* (b). All data presented was gathered from the same experiment. Error bars represent standard deviation.

These results suggest that the up-regulation of Yop-production observed in the Δ*flhDC* Δ*fliA* and Δ*flhA* mutants during growth at 30°C (Chapter 3) is under-lined by up-regulation of *virF* expression, which indicates that the flagella regulon represses *virF* at 30°C.

5.2.6 QS represses T3S at non-inductive temperatures via an effect on *virF* expression

The repressive effect of the flagella system towards *virF* expression and the Yop regulon is interesting, since QS is known to regulate flagella and *flhDC* expression (Atkinson et al., 2008, Atkinson et al., 1999), and, as Chapter 3 revealed, several QS mutants secrete Yop-related proteins into the culture supernatant during growth at 30°C. Similar to the flagella mutants, it was likely that the up-regulation of Yop production in the QS mutants was due to over-expression of *virF*. To confirm this, P_{virF}::*lux* was introduced into the Δ*ypsR*, Δ*ypsI*, Δ*ytbR*, Δ*ytbI*, Δ*ypsI* Δ*ytbI* mutants and the complement Δ*ypsI* Δ*ytbI* pSA291 strain, and the expression of *virF* determined over the growth phase at 30°C. Figure 5.11 (a) shows that the Δ*ypsR*, Δ*ytbI* and Δ*ytbR* mutants all over-express *virF* at 30°C, whereas only low constitutive expression of *virF* is observed in the wild-type or Δ*ypsI*. Figure 5.11 (b) shows that *virF* expression is also up-regulated in the Δ*ypsI* Δ*ytbI* mutant, whereas this is reduced to wild-type levels following complementation with pHG291. Similar over-expression of *virF* was observed in the Δ*ypsR* Δ*ytbR* mutant and when the strains were grown at 37°C (data not shown). These results suggest that *virF* is repressed by QS at 30°C.

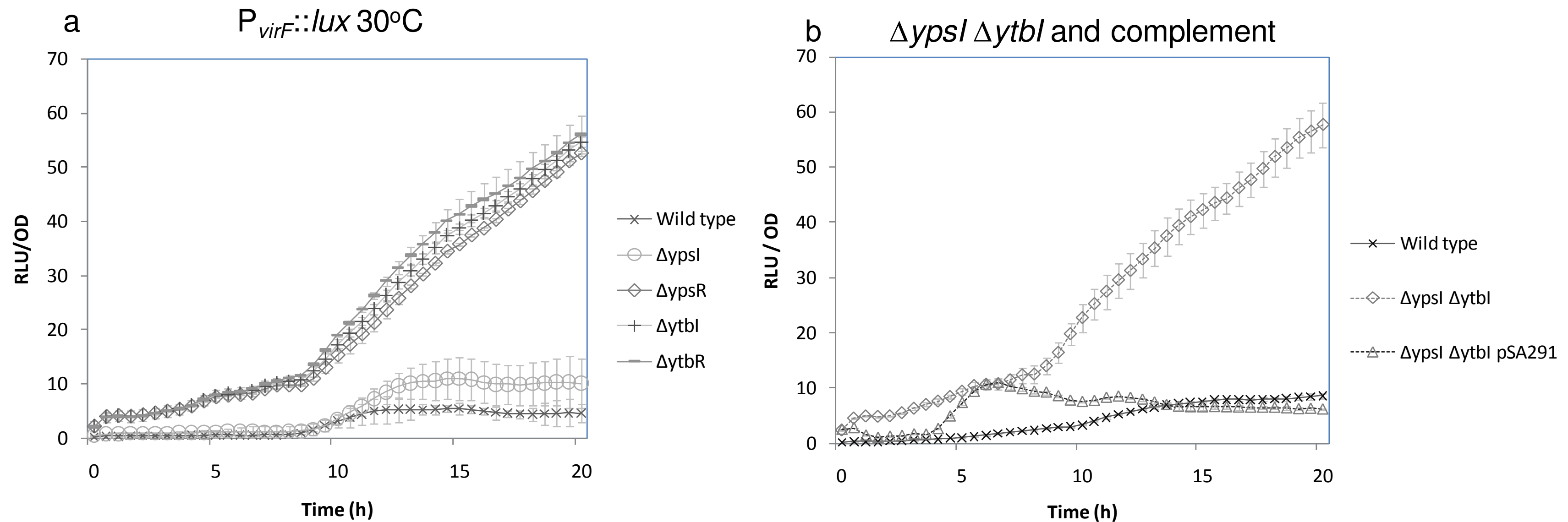


Figure 5.11| The QS genes *ypsR*, *ytbI* and *ytbR*, but not *ypsI*; repress the expression of *virF* during growth at 30°C. Measurement of *virF* expression in the QS mutants reveals that *virF* is approximately 6 fold over-expressed in the Δ*ypsR*, Δ*ytbI* Δ*ytbR* (a), and, Δ*ypsI* Δ*ytbI* (b) mutants when compared with the wild-type during growth. However, up-regulation of *virF* is not observed in the Δ*ypsI* mutant, which expresses *virF* at levels similar to the wild-type (a). When functional *ypsI* and *ytbI* are restored to the Δ*ypsI* Δ*ytbI* mutant on pSA291, expression of *virF* is reduced to wild-type levels (b). Error bars represent standard deviation.

Together, these data show that QS represses the expression of *virF* at 30°C in a way similar the repression caused by the flagella regulon.

5.3 Discussion

5.3.1 The regulation of motility by YscJ and QS

The work described in this chapter has revealed that the presence or absence of YscJ affects the regulation of flagella-mediated swimming motility. Mutation of *yscJ* results in the de-repression of the flagella master regulator *flhDC* and the flagella specific sigma factor *fliA* and causes a hyper-motile phenotype, suggesting that either YscJ itself, or an as yet unidentified element that senses the lack of the injectisome, causes the induction of motility during growth at 22°C. Mutation of *yscJ* also results in increased *flhDC* and *fliA* expression at 37°C, however the level of expression of *flhDC* and *fliA* in Δ*yscJ* during growth at 37°C is only half that observed during growth at 22°C, and no motility is observed during growth at 37°C. This could suggest either that the expression levels of *flhDC* and *fliA* at 37°C are not sufficient to induce motility, or that additional regulators negatively control motility during growth at 37°C. Indeed in *Y. enterocolitica* it has been suggested that FlhDC does not regulate flagella genes during growth at 37°C (Kapatral et al., 2004), which could provide a reason why YscJ-associated modulation of *flhDC* expression does not affect motility during growth at this temperature.

The induction of hyper-motility in the Δ*yscJ* mutant is abolished in the Δ*ypsI* Δ*ytbI* Δ*yscJ* triple mutant, indicating that YscJ-associated repression of motility is QS dependent. The expression of *flhDC* is also repressed in the Δ*ypsI* Δ*ytbI*

Δ*yscJ* mutant when compared with the wild-type or the single Δ*yscJ* mutant, suggesting that QS is specifically required for the activation of the Class I flagella regulator, which then leads to Δ*yscJ*-dependent motility. However, an intact YscJ also appears to partially drive the repression of *flhDC* independently of QS, since the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant expresses *flhDC* at slightly higher levels than does the Δ*ypsI* Δ*ytbI* mutant. This notwithstanding, the up-regulation of motility observed in the Δ*yscJ* mutant, and the dependence of this phenotype on YpsI and YtbI is probably related to the fact that YscJ exerts control over the YpsR/I and YtbR/I QS systems, and acts differentially to enhance the expression of *ypsI* and *ytbR* and repress *ypsR* and *ytbI.*

5.3.2 A checkpoint possibly controls the reciprocal regulation between motility and T3S

The observation that the presence or absence of the Yop-Ysc structural lipoprotein YscJ could affect motility is reminiscent of the results presented in Chapter 3, which revealed that the flagella secretion component, FlhA, is involved in repression of the Yop-Ysc system and LCB formation. In a manner analogous to FlhA, it is possible that YscJ plays a regulatory role in addition to its function as a structural component of the IM ring of the T3SS, and previously, *Y. pseudotuberculosis yscJ* mutants have been shown to be calcium-blind with respect to Yop expression at 37°C (Holmstrom et al., 1995), suggesting that YscJ can function as part of a regulatory checkpoint controlling gene expression. However, it is more likely that there is a sensory mechanism, whereby perhaps protein-protein interactions sense the presence or absence of YscJ, and this acts as a checkpoint in measuring the state of production of theT3SS. This checkpoint may be responsible for driving both the regulation of Yop gene

expression and the reciprocal regulation between the T3SS and flagella. The Yop effectors are also known to be auto-regulated by negative feedback, whereby the intracellular accumulation of Yops when the needle is closed (or absent) leads to down-regulation of their expression (Allaoui et al., 1995b, Plano and Straley, 1993, Plano and Straley, 1995). This may be analogous to FlgM/FliA controlling flagella biosynthesis (Cornelis and Wolf-Watz, 1997a), and involves the Cpx two-component system and an extracytoplasmic function sigma factor (Carlsson et al., 2007a). It is possible that this sigma factor driven negative feedback mechanism could form the basis of the checkpoint which controls the expression of the QS genes, which, in turn, may influence the expression of *flhDC* and motility.

For example, in flagella biosynthesis, FliC is produced subsequent to FliA activation by FlgM export (Kutsukake and Iino, 1994, Ding et al., 2009), whereas the production of FlhA only requires FlhDC, and not FliA (Liu and Matsumura, 1994). In some species, FlhA influences the activation of FliA by interacting with FlhM (Rust et al., 2009) and affects the production of FliC (Yang et al., 2009), suggesting that FlhA acts in a checkpoint to control the activation of FliA. Since the production of FliC occurs after FliA has been activated, whereas FlhA influences the activation of FliA, and since FliA is important for the regulation of Yop production, this may go some way to explain why *flhA*, but not *fliC*, is important for *virF* expression. It would therefore be interesting to investigate the effect of a *yscF* mutation, since YscF may, like FliC, be exported only after the proposed T3SS sigma factor (Carlsson et al., 2007a) has been activated. This mutant may then be considered to be analogous to the Δ*fliC* mutant and, if this strain displays only wild-type expression of QS, *flhDC* and motility (analogous to how the Δ*fliC* mutant displays wild-type Yop secretion and *virF* expression), it would provide further support for the existence of such a switch controlling the reciprocal regulation of flagella and T3S via QS.

However, it is not clear why the presence of FlhA results in the repression of secretion and *virF* expression at 30°C or 37°C, nor why the presence of YscJ should influence the repression of motility at 22°C since these temperatures are not normally associated with their respective functions. It is interesting to note that neither hyper-motility in the Δ*yscJ* mutant, nor Yop secretion in the Δ*flhA* mutant occurs during growth at 37°C or 22°C, respectively, suggesting that the absolute temperature regulation of these processes is still in place.

5.3.3 Transcriptional control of QS by the T3SS and consequences for motility

The abrogation of motility in the Δ*yscJ* mutant following the loss of AHL production could be due to a requirement of QS for the absolute induction of motility, and not involve a direct relationship between the T3SS, QS and motility. However, it is likely that hyper-motility and up-regulation of *flhDC* expression in the Δ*yscJ* mutant is a consequence of altered expression of QS genes in the Δ*yscJ* mutant. Expression of both *ypsI* and *ytbR* is abolished in the Δ*yscJ* mutant, indicating they are activated by the presence of functional YscJ or by a sensory mechanism that involves YscJ or a downstream component of the injectisome that requires YscJ for its localisation. Conversely, *ypsR* and *ytbI* expression is up-regulated in the Δ*yscJ* mutant, implying that an element sensing the presence or absence of YscJ acts as a checkpoint to repress the expression of these genes.

Atkinson *et al*. (2008) previously suggested that the AHL synthase gene YtbI acts as an inducer of motility in agar, while YpsI represses this phenotype

(Atkinson et al., 2008). The data in this chapter may support this, since the deletion of *ytbI* (alongside *ypsI*) from the hyper-motile Δ*yscJ* mutant abolishes motility. The loss of motility in the Δ*ypsI* Δ*ytbI* Δ*yscJ* mutant compared with the Δ*yscJ* mutant is probably a result of the loss of *ytbI*, since the single Δ*ypsI* mutant, like the Δ*yscJ* mutant, is hyper-motile, but this hyper-motility is lost following subsequent mutation of *ytbI* (Atkinson et al., 2008). In addition, the expression of *ytbI* is increased in Δ*yscJ* relative to wild-type, where it is restored to wild-type levels following the addition of functional *yscJ* on pHG*yscJ,* coincident with the reversion of motility to levels more similar to the wild-type. Atkinson *et al*. (2008) also suggested that YpsI represses motility, since a single *ypsI* mutant is hyper-motile (Atkinson et al., 2008). This is supported by the results in this chapter which show that *ypsI* expression is almost completely abolished in the Δ*yscJ* mutant compared with the wild-type. In this way, the Δ*yscJ* mutant simulates the effect of the *ypsI* mutation, abolishing the production of YpsI, and it is therefore not surprising that the Δ*yscJ* mutant shares the hyper-motile phenotype with the Δ*ypsI* mutant.

The relationship between the AHL receptor proteins YpsR and YtbR, YscJ and motility is less obvious. Firstly, although a Δ*ypsR* mutant displays hyper-motility, it is also repressed for the expression of *flhDC*, suggesting that YpsR is a positive regulator of *flhDC* but a negative regulator of motility, perhaps in conjunction with YtbR and via an effect on the expression of the sigma factor *fliA* (Atkinson et al., 2008, Atkinson et al., 1999). However, in this chapter, the results show that *ypsR* expression is up-regulated in the Δ*yscJ* mutant, which is inconsistent with the role of YpsR as a repressor of motility, since in spite of the up-regulation of *ypsR*, the Δ*yscJ* mutant is hyper-motile. Secondly and in contrast, mutation of *ytbR* does not substantially change the expression of *flhDC* when compared with the wild-type, but when *ytbR* is mutated in the hyper-motile Δ*ypsR* mutant background, the resulting Δ*ypsR* Δ*ytbR* double mutant is

not motile in swimming agar. This suggests YtbR positively regulates swimming motility (Atkinson et al., 2008). However, the expression of *flhDC* in the Δ*ypsR* Δ*ytbR* mutant, although lower than in the wild-type, is higher than in the single *ypsR* mutant, suggesting that at least in the absence of YpsR, YtbR can repress *flhDC* expression and, by inference, motility. To support the hypothesis that YtbR represses motility, it has been reported that the Δ*ytbR* Δ*ytbI* double mutant also displays a hyper-motile phenotype (H. Patrick, unpublished results), even though the single *ytbR* or *ytbI* mutants are non-motile (Atkinson et al., 2008). Here, the results indicate that the expression of *ytbR* is almost entirely abolished in the Δ*yscJ* mutant, which is inconsistent with the role of YtbR as an activator, but may further suggest that YtbR acts as a repressor of motility.

5.3.4 Potential links between FlhDC, QS and the T3SS

The repression of *flhDC* expression in the Δ*ypsI* Δ*ytbI* mutant compared with the wild-type correlates with the up-regulation of *virF* observed in the Δ*ypsI* Δ*ytbI* mutant, and since FlhDC also represses *virF* and the T3SS it is likely that the regulation of *virF* by YpsI and YtbI occurs through FlhDC (Bleves et al., 2002). It is unclear how QS and FlhDC influence *virF* expression; however it may involve an effect on nucleosome-associated proteins such as YmoA or H-NS. YmoA is thought to modulate the expression of the Yop virulon, and a *Y. enterocolitica ymoA* mutant is up-regulated for Yop production at 28°C (Cornelis et al., 1991), similar to that seen in this chapter in the *Y. pseudotuberculosis* flagella and QS mutants. The nucleosome-associated protein H-NS regulates motility in several species (Bengoechea et al., 2004, Bertin et al., 1994), and may interact with YmoA, which would provide a link between both these phenotypes and nucleosome-associated proteins (Cornelis et al., 1991, Ellison and Miller, 2006, Marceau, 2005, Nieto et al., 2002). In *Y. enterocolitica*, H-NS over-expression

leads to enhanced *flhDC* expression and motility (Bengoechea et al., 2004), similar to that observed here in the Δ*yscJ* mutant. It is possible that mutation of *yscJ*, *ypsI* or *ypsR* also leads to H-NS over-expression, while the up-regulation of *virF* expression and Yop production in the flagella or Δ*ypsI* Δ*ytbI* mutants could be due to down-regulation of YmoA. It is interesting to find that H-NS may bind to the *ypsR* promoter (S. Atkinson, unpublished results), which could suggest that H-NS and YpsR regulate one another. The fact that the Δ*yscJ* mutant, along with the Δ*ypsI* and Δ*ypsR* mutant is only hyper-motile at low temperatures and not at 37°C, and that the flagella and QS mutants over-produce Yop effectors during growth at 30°C, but not at 22°C, also suggests that the environmental regulation of these systems is relaxed, but not abolished. This could imply that the target for flagella-, T3SS- and QS-dependent regulation of motility is a modulator rather than a strict activator / inactivator, and indeed both H-NS and YmoA have previously been described as such (de Rouvroit et al., 1992, Marceau, 2005, Atlung and Ingmer, 1997).

Other potential candidate targets for QS regulation must also include the several regulators and systems that affect both motility and the Yop-Ysc T3SS. Both these systems are affected by Dam-dependent DNA methylation, whereby a Dam over-producing strain (Dam^{OP}) of *Y. pseudotuberculosis* is relaxed for the temperature, but not calcium dependency of Yop secretion (Julio et al., 2002), while Dam^{OP} in *Y. enterocolitica* is relaxed for the calcium, but not temperature regulation of Yop secretion (Falker et al., 2006). In *Y. enterocolitica*, Dam^{OP} also exhibits increased motility compared with the wild-type (Falker et al., 2007). Interestingly, Dam also affects the O-antigen status of *Yersinia* lipopolysaccharide, increasing the amount of 'rough' LPS lacking O-antigen side chains (Falker et al., 2007); O-antigen status is also involved in controlling motility and the Yop-Ysc T3SS, probably via an effect on H-NS (Bengoechea et al., 2004, Perez-Gutierrez et al., 2007). Recently it has also been shown that the

RNA chaperone Hfq regulates both motility and the Yop-Ysc T3SS; a *Y. pseudotuberculosis hfq* mutant displays hyper-motility and is reduced for the synthesis and secretion of Yop effector proteins (Schiano et al., 2010). Two component regulator systems such as EnvZ / OmpR and CpxA / CpxR may also be involved since the former positively regulates flagella via-regulation of *flhDC* expression in *Y. pseudotuberculosis* and *Y. enterocolitica* (Hu et al., 2009, Raczkowska et al., 2011), while the latter regulates the production and translocation of Yop effectors through the injectisome via the activity of an extracytoplasmic function sigma-factor (Carlsson et al., 2007a). Both systems converge in regulating invasin production, probably through modulation of the global regulator RovA or RovM (Carlsson et al., 2007b, Brzostek et al., 2007, Heroven and Dersch, 2006), although neither RovA or RovM affect the Yop-Ysc T3SS (Heroven and Dersch, 2006). Due to the reciprocal regulation of motility and the T3SS, any and all of these regulators are potentially involved in QS-mediated bi-directional control of flagella and the T3SS.

5.3.5 Conclusions

This chapter has shown that the T3SS, QS and motility are intricately linked in *Y. pseudotuberculosis*- although the precise mechanism which underlies the relationship is unknown. It is not clear why the hyper-motile Δ*yscJ* mutant over-expresses *ypsR*, but is completely repressed for *ytbR* expression, when YpsR is thought to negatively regulate motility, and YtbR to positively regulate motility. It is possible that there are nuances by which the AHL receptors can regulate motility both as enhancers or repressors, perhaps depending on the cellular availability of YpsR and YtbR which could potentially form hetero- as well as homo-dimers, and may fulfil different functions depending on the composition of the active complex. The range of AHLs produced by YpsI and YtbI could also act

to differentially activate or inhibit the receptor proteins; the AHL profile of *Y. pseudotuberculosis* cultures is known to change according to growth temperature (Ortori et al., 2007), and since expression of the AHL synthase genes are altered following mutation of the receptors (Atkinson et al., 2008), this could suggest that the phenotyes of the Δ*ypsR* and Δ*ytbR* mutants could be due to a modified AHL profile. However, it is also possible that previous investigations into the relationship between QS and motility have over-looked the potential involvement of the pYV plasmid, which might be absent from some of the strains from which promoter fusion data has been gathered (Atkinson et al., 2008). Several flagella and QS mutants may lose the pYV plasmid more rapidly than the wild-type (S. Atkinson, unpublished results), and this might affect the expression of QS and flagella genes, therefore calling into question the accuracy of the model built upon that data. In light of this, it is also interesting to note that a pYV negative derivative of YPIII has not been reported to display the hyper-motile phenotype of the Δ*yscJ* mutant. Since *yscJ* is also encoded on pYV, this suggests that the de-repressive effect of *yscJ* mutation on *flhDC* expression and motility requires other pYV encoded loci to induce *flhDC* and *fliA* expression, and to stimulate motility.

Chapter 6|

Conclusions

and future directions

6.1 Conclusions

The work in this thesis has sought to build on the emerging model of how QS regulates lifestyle choices in *Y. pseudotuberculosis*. Previously, it has been shown that QS regulates several behaviours in this bacterium, including motility and clumping(Atkinson et al., 1999), and that QS is, itself, sensitive to thermoregulation (Atkinson et al., 2008), which is prevalent in modulating the biology and pathogenesis of *Y. pseudotuberculosis* (Straley and Perry, 1995). Considering that the two major targets of QS regulation, for example FlhDC and FliA, have been implicated in controlling biofilm formation (Kim et al., 2008) and virulence (Bleves et al., 2002, Horne and Prüß, 2006) in *Y. enterocolitica*, these previous results strongly suggest that QS could be involved in regulating these behaviours in *Y. pseudotuberculosis*. These behaviours, including virulence, motility and biofilm formation, can be regarded as lifestyle choices for *Y. pseudotuberculosis* and are used under very different ecological conditions. For example, virulence, by way of T3S, is utilised to inject toxic proteins into eukaryotic cells under the conditions prevalent in infection (for example at 37°C and in close contact with host cells). Conversely, flagellum-mediated motility is down-regulated at 37°C, possibly because flagella are incredibly immunogenic (Honko et al., 2006) and would quickly alert the mammalian immune system to the invading bacteria. Instead motility is considered to be utilised at temperatures prevalent in the environment (for example 22°C – 30°C) and in liquid or semi-solid medium (Atkinson et al., 1999). Biofilms, however, form following the contact of *Y. pseudotuberculosis* with the surface of *C. elegans* at environmental temperatures, in a specific interaction with the nematode surface, since *Y. pseudotuberculosis* biofilms are not observed to form on plastics (Joshua et al., 2003). However, the possibility that *Y. pseudotuberculosis* may form

biofilms on other surfaces in the environment, both abiotic and biotic, cannot be discounted.

Despite the very different ecological niches required for these behaviours, previous studies on *Y. enterocolitica* and *Y. pseudotuberculosis* have suggested that they are linked by the involvement of the flagella regulators FlhDC and FliA (Bleves et al., 2002, Horne and Prüß, 2006), and that by mutating these regulators, the normal regulation of these lifestyle choices is affected- for example, in a *flhDC* mutant, no motility occurs (Bleves et al., 2002, Atkinson et al., 2008), T3S is de-repressed under normally non-inducing conditions (Bleves et al., 2002), and the ability for the bacterium to form biofilms is reduced (Kim et al., 2008, Atkinson et al., 2011). In *Y. pseudotuberculosis*, QS also regulates *flhDC* and *fliA* expression and flagella mediated motility (Atkinson et al., 2008), indicating that QS is embedded in the regulatory network that governs the expression of these traits. This prompted the investigation of the role of QS in regulating lifestyle choices in *Y. pseudotuberculosis*.

From the results presented in this thesis, a clear narrative emerges, describing an intricate and inter-dependent relationship between QS, motility and T3S in modulating each other to dictate lifestyle choices such as biofilm formation versus the planktonic mode of growth. Initially, the key finding in Chapter 3, revealing that the formation of cell clumps during liquid culture of certain *Y. pseudotuberculosis* QS mutants, first described by Atkinson *et al.* (1999) (Atkinson et al., 1999), is correlated with the ectopic secretion of Yop-related proteins into the culture supernatant and depends on the presence of the T3SS and a functional *yscJ* gene. Along with results in Chapter 6, which show the virulence regulator *virF* is over-expressed in the QS mutants- this strongly suggests that QS represses Yop production. The consequences of the repression of T3S by QS was further explored in Chapter 5, where key findings revealed

that the hyper-activity of the T3SS was probably responsible for mediating the down-regulation of biofilm formation on the surface of *C. elegans* infected with the *Y. pseudotuberculosis* QS mutant strains. Although it is not clear how a hyper-active T3SS contributes to the repression of *C. elegans* biofilm formation, results in Chapter 6 reveal that the T3SS is intricately embedded in a regulatory network, alongside QS, in regulating the expression of *flhDC*, *fliA*, and flagella-mediated motility. Indeed, the involvement of the motility regulon is a theme which pervades throughout the work presented here, being involved in regulating- in addition to motility- the production of the T3SS, LCB formation, the growth of biofilms on the surface of *C. elegans*, and potentially acts down-stream in the same pathway as QS. A simplified diagram of this relationship is presented in Figure 6.1.

Surrounding this general narrative are results which contribute to our understanding of the process by which QS and the flagella system repress T3S, and how T3S reciprocally regulates flagella. Both QS and the flagella system repress the transcription of the virulence regulator *virF*, and the up-regulation of *virF* in the Yop over-producing QS and flagella mutants probably contributes to the appearance of Yop proteins in the culture supernatant during growth at 30°C. It is likely that, at least for QS, this depends on the AHLs produced by YtbI- since the single Δ*ypsI* mutant does not show up-regulation of *virF*, nor does it secrete Yop proteins under non-inducing conditions, whereas the single Δ*ytbI* mutant is up-regulated for *virF* expression and Yop production, similar to the Δ*ypsI* Δ*ytbI* mutant. Using bioreporters, Atkinson *et al*. (1999) showed that during growth at 37°C, mutation of *ypsI* does not largely affect the AHL profile produced by *Y. pseudotuberculosis* (Atkinson et al., 1999), while specific measurement of AHL concentrations using liquid chromatography coupled to hybrid quadrupole–linear ion trap mass spectrometry has shown that during growth at 30°C, YtbI is principally responsible for the production of 3-oxo-C_7-

HSL, and solely responsible for the production of C_8-HSL, 3-oxo-C_8-HSL, and long chain AHLs including 3-oxo-C_{10}-HSL (Ortori et al., 2007). It is therefore tempting to speculate that the high concentration or the timing of the AHLs produced by YtbI dictates the repression of *virF* and T3S.

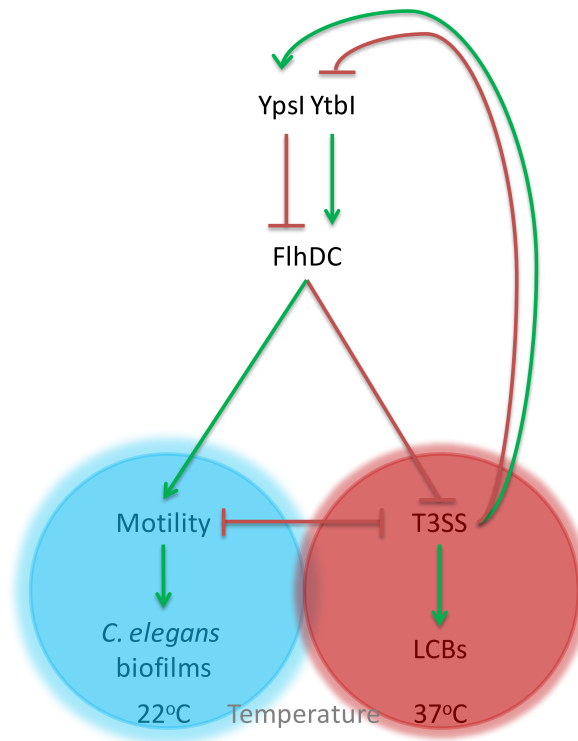


Figure 6.1 | A simplified model for the relationship between QS and FlhDC in regulating temperature modulated behavioural decisions in *Y. pseudotuberculosis*. QS regulates *flhDC* expression (Atkinson et al., 2008), and while both QS and FlhDC negatively regulate the T3SS at 37°C, they positively influence motility at 22°C. The motility system and the T3SS reciprocally regulate one another, and it can be proposed that this reciprocal regulation causes the induction of LCBs in the flagella mutants. The T3SS reciprocally and differentially regulates QS, and this may underlie the effect of *yscJ* deletion on QS-regulated *C. elegans*.

QS regulates *flhDC, fliA* and flagella mediated motility in *Y. pseudotuberculosis* (Atkinson et al., 2008), and since FlhDC and FliA regulate T3S in *Y. enterocolitica* (Bleves et al., 2002, Horne and Prüß, 2006), it perhaps not surprising that FlhDC and FliA affect *virF* expression and T3S in *Y. pseudotuberculosis*. The fact that *flhDC* expression is down-regulated in Δ*ypsI* Δ*ytbI*, and that the Δ*flhDC* mutant shares many phenotypes with the Δ*ypsI* Δ*ytbI* mutant (i.e. over-expresses *virF*, secretes Yops, forms LCBs and is attenuated for biofilm formation on *C. elegans* (Atkinson et al., 2011)) strongly suggests that the flagella system operates down-stream of QS in regulating T3S. It is especially interesting however that the Δ*flhA* mutant shares these phenotypes with the Δ*ypsI* Δ*ytbI*, and Δ*flhDC* mutants, and suggests that the regulatory activity of the flagella extends beyond the levels of FlhDC and FliA. It would be interesting to investigate *flhDC* expression in the Δ*flhA* mutant; it could be expected that *flhDC* may be down-regulated in the Δ*flhA* mutant, suggesting feedback between the structure of the flagella and the flagella regulatory cascade.

However, although QS and the flagella system clearly impact on the expression of *virF*, the relaxation of the calcium block for T3S suggests other genes in addition to *virF* are modulated by QS, specifically those required for the low-calcium response. Several proteins form a gate complex at the injectisome, preventing the secretion of Yop proteins until LCR conditions are encountered, including LcrQ, (Rimpilainen et al., 1992, Pettersson et al., 1996), LcrG (Wulff-Strobel et al., 2002), YopN (Cheng et al., 2001, Day et al., 2003) and TyeA (Iriarte et al., 1998, Day et al., 2003), and deletion of any of these allows the secretion of Yop proteins into the culture supernatant in the presence of calcium. This could suggest that QS positively regulates these proteins, leading to their absence in the Δ*ypsI* Δ*ytbI* mutant and concomitant secretion of Yop proteins. However it is unlikely that YopN is positively regulated by QS / flagella, since this

protein is present in abundance in culture supernatants of the Yop over-producing mutants.

The theory that the flagella system operates down-stream of QS in regulating T3S is paralleled, though reversed, by the idea that QS operates down-stream of the T3SS in regulating motility. It is interesting, given the proposition that AHLs produced by YtbI are principally involved in the down-regulation of T3S, that in the case of motility, the Δ*yscJ* mutant should behave similarly to the Δ*ypsI* mutant. The fact that *ypsI* expression in the Δ*yscJ* mutant is also practically abolished supports the notion that the AHLs produced by YpsI, previously suggested to be repressive towards motility (Atkinson et al., 1999), acts down-stream of YscJ in repressing motility. Additionally however, the up-regulation of *ytbI* expression in the Δ*yscJ* mutant, and the fact that Δ*yscJ*-dependent motility appears to be dependent on the presence of functional YtbI suggests that the AHLs produced via the second AHL synthase also act down-stream of YscJ in the regulatory cascade leading to modulation of flagella-mediated motility. Given this relationship, it is possible that YpsI plays an as yet unidentified role in the regulation of T3S, perhaps acting as an activator, rather than a repressor, of the system.

Motility and T3S are behaviours carried out by *Y. pseudotuberculosis*, which suggests that QS occupies a central position in the regulatory system that determines which behaviour is most adaptable for a particular environment. A third behaviour for *Y. pseudotuberculosis* is the formation of biofilms, either in liquid culture or on the surface of *C. elegans*. Although these biofilms manifest in very different ecological niches, QS, the flagella system and T3S converge to regulate both these types of biofilm. However, the direction of this regulation appears to be opposite, with QS and the flagella system repressing LCBs but enhancing biofilm formation on *C. elegans*. In both biofilms, the effect of QS

(and probably the flagella system) depends on the T3SS; however, again the manner of this association is reversed, where LCBs are positively associated with T3S, while biofilm formation on *C. elegans* is negatively associated with T3S. Indeed, the mechanism by which the T3SS affects LCBs and *C. elegans* may also differ, since it is likely that the structure of the injectisome is important for inhibiting biofilms on *C. elegans*, while secretion through the injectisome appears to be a key component for LCB formation.

Although the significance of the QS / T3SS relationship in affecting biofilms is unclear, it may reflect the ability for *Y. pseudotuberculosis* to finely regulate lifestyle choices when confronted with specific conditions. For example, the Δ*ypsI* Δ*ytbI* mutant, in being AHL negative, cannot communicate the density of the population, and perhaps it is more adaptable for *Y.* pseudotuberculosis, in low cell density, to suppress biofilm formation in favour of T3S. In this way it would be interesting to investigate if the Yop effector proteins have a toxic effect towards *C. elegans*. Conversely, by removing the ability for the bacteria to engage in T3S biofilms can be restored, perhaps since the 'adaptive' response has been prevented, the bacteria revert to what could be considered 'plan A'- that is to form biofilms. It would be very interesting therefore to investigate if a low cell density *Y. pseudotuberculosis* population that was prevented from engaging in this proposed 'adaptive' behaviour suffered a fitness cost in *C. elegans*, when compared to those bacteria able to carry out T3S.

6.2 Future directions

Emerging from this work are clear avenues of research to explore. For example, a recurrent pattern in these results is the fact that environmental regulation of lifestyle choices is relaxed, rather than abolished, when the QS or motility systems are mutated. For example, QS mutants do not form LCBs and do not produce Yops at 22°C, while both these behaviours occur at 30°C and above, while Δ*yscJ*-dependent hyper-motility does not occur at 37°C. This suggests that the inter-relationship between QS, the flagella system and the T3SS acts to modulate the existing thermoregulation of these behaviours rather than strictly act as activators / repressors. It is very likely, therefore, that QS, the flagella system and the T3SS interact with other modulators such as YmoA or H-NS. Indeed, preliminary results suggest that H-NS may bind directly to the promoter of *ypsR*, while deletion of *ymoA* may affect the expression of the QS genes (S. Atkinson, unpublished data). Further work may reveal the involvement of these histone-like proteins, as they play a central role in determining the response of *Y. pseudotuberculosis* to the environment (Cornelis et al., 1991, Banos et al., 2008).

QS is increasingly being recognised to play a central role in regulating lifestyle choices in bacteria, including biofilm formation (Parsek and Greenberg, 2005), T3S (Bleves et al., 2005, Henke and Bassler, 2004, Liu et al., 2008) and motility (Kim et al., 2007, Atkinson et al., 2006, Atkinson et al., 2008, Hussain et al., 2008). It is clear that bacteria, particularly *Y. pseudotuberculosis*, have evolved to strike a balance in regulating these processes to occur under different environmental conditions, and while QS appears to be poised as an integral part of the switch between these behaviours, the feedback from these to the expression of QS also seems to be critical. While results in this thesis reveal that

feedback does exist between the T3SS and QS, the mechanism of this feedback is unclear, and deserves full characterisation, as it is clearly a key part in the regulation of lifestyle choices in *Y. pseudotuberculosis*. It is likely that the regulation of the flagella system by QS is also reciprocal, and future experiments may possibly show the expression of QS to be affected by mutation of the flagella system genes. It is, however, difficult to predict the direction of this regulation, since the individual QS genes appear to differentially affect the behaviour of *Y. pseudotuberculosis*, and discovering which genes are up-regulated and which are down-regulated following the mutation of the flagella system may help to clarify the contribution of each QS gene to the behaviours of *Y. pseudotuberculosis*. Indeed, it is unclear why, for example, the single Δ*ytbI* or Δ*ytbR* mutants over-produce Yop proteins, while the double Δ*ytbI* Δ*ytbR* mutant is similar to the wild-type and does not over-produce Yops (data not shown). Similarly, this dichotomy between the single *ytbI*/*R* system mutants and the double mutant is seen with respect to flagella mediated motility, whereby the single mutants are non-motile whereas the double mutant is hyper-motile in the same way as is the Δ*ypsI* or Δ*ypsR* mutant (H. Patrick, University of Nottingham thesis 2010). It will be important to characterise the down-stream elements of this regulatory pathway, which will be invaluable in order to develop a global model for the role for QS in regulating the lifestyle switch of *Y. pseudotuberculosis*.

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