NOVEL INSIGHTS INTO THE QUORUM SENSING SYSTEM OF *YERSINIA ENTEROCOLITICA* 8081

Yen-Kuan Ng, B.Sc. M.Sc.

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

2010

Declaration

No part of the work in this report is the same as any that I have summated for a degree, diploma, or any qualification at the University of Nottingham or any other institute.

The work described in this report is the result of my own effort, unless otherwise stated. It is also of my own composition.

Yen-Kuan Ng

January 2010

Table of contents

Abstract

Y. enterocolitica possesses an *N*-acyl-homoserine lactone (AHL)-dependent quorum sensing (QS) system which consists of the *luxRI* homologues, *yenRI*. The objective of this project was to increase our understanding of the QS system(s) in *Y. enterocolitica* by characterising the AHL-dependent system on a genus wide basis. Species-wide analysis of an *Y. enterocolitica* multi-strain genome database revealed an additional *luxR* homologue, we termed *ycoR*, which is present in the fully sequenced, highly virulent *Y. enterocolitica* 8081 strain and other USA strains but absent in UK strains. This indicates genetic differences in the QS systems of 'New World' (North America) and 'Old World' (Europe and Japan) strains of *Y. enterocolitica*. *ycoR* is an 'orphan' *luxR* homologue which is not associated with an adjacent *luxI* homologue. Consequent work described in this study focused on the *Y. enterocolitica* 8081 strain.

LC-mass spectrometry of spent culture supernatants from *Y. enterocolitica* 8081 grown at 30°C identified 16 different AHLs, mainly *N*-(3-oxohexanoyl)-L-homoserine lactone (62.4%) and *N*-hexanoyl-L-homoserine lactone (27.4%). 11 of the 16 AHLs had not previously been documented for this bacterium, including *N*-(3-oxoheptanoyl)-L-homoserine lactone (with an odd number of carbons in the acyl chain) which constituted 5.1% and has rarely been documented previously.

The λ red recombinase method of mutagenesis was used for the rapid generation of QS mutants in *Y. enterocolitica* 8081. Seven QS mutants were generated, three were single mutants: Δ*yenI* Δ*yenR* and Δ*ycoR*, three were double mutants: Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenRycoR*, and one was a triple mutant: Δ*yenIyenRycoR*. Analysis of the QS mutants revealed that the level of AHLs synthesised at 30°C by the R mutants: Δ*yenR*, Δ*ycoR* and Δ*yenRycoR*, were similar to the wildtype while for AHL synthase mutants: Δ*yenI*, Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenIyenRycoR*, levels of AHL were greatly reduced compared with the wildtype but were not entirely abolished.

Transcriptomic analysis of *Y. enterocolitica* 8081 wildtype and its QS mutants using microarray technology revealed many possible QS-related regulatory networks including motility, type III secretion system and High Pathogenicity Island. Microarray data, together with RQ-PCR, phenotypic studies and promoter fusion studies showed

that QS is correlated with virulence regulation (expression of *virF* and *tyeA*), virulence factors (expression of *yadA* and *invA*) and maintenance of the pYVe plasmid (expression of *repA* and *spyA*), which have not been previously documented.

In conclusion, this study revealed that *Y. enterocolitica* 8081 has a sophisticated QS system which consists of the AHL synthase, YenI and two LuxR-type regulators, YenR and YcoR working in tandem and sharing overlapping regulon. This system maybe linked to the hypervirulence in the 8081 strain but further work is needed to confirm this.

Acknowledgments

It has been 4 years since I embarked on this journey and it has been quite a journey of self-discovery. It has been difficult at times with blood, sweat and tears, finally culminating in this thesis completed on time! This journey was made fruitful and even enjoyable at times because I was very fortunate to have a network of good people supporting me.

I would like to thank my supervisors, Prof. Paul Williams, Prof. Miguel Cámara and Dr. Steve Atkinson for their guidance, advice and great patience. My gratitude to you is boundless. Special thanks to Dr. Catherine Ortori (Centre for Analytical Bioscience, University of Nottingham) for the mass spectrometry work and Ms. Victoria Wright (Genomics Lab, Centre of Biomolecular Sciences) for the tremendous help with the microarray and RQ-PCR work. To my colleagues in Lab B39/40, especially Stephan, Steve D., Jenny, Sarah, Hannah, Catherine, Andy, Davy, Christian and Rob, many thanks for their help, advice, and company.

I would also like to thank my husband, Joe for his love and support, without which I am sure I would not have made it through. Last but not least, I would like to thank my family back home in Malaysia for their never waning support and love, and for putting up with me being so far away for so long. I hope I have made you all proud.

List of Figures

List of Tables

Abbreviations

°C	degrees Celsius
%	percentage
% (w/v)	percentage weight per volume
% (v/v)	percentage volume per volume
C4-HSL	*N*-butanoyl-L-homoserine lactone
C6-HSL	*N*-hexanoyl-L-homoserine lactone
C7-HSL	*N*-heptanoyl-L-homoserine lactone
C8-HSL	*N*-octanoyl-L-homoserine lactone
C12-HSL	*N*-dodecanoyl-L-homoserine lactone
C14-HSL	*N*-tetradecanoyl-L-homoserine lactone
3-oxo-C6-HSL	*N*-(3-oxohexanoyl)-L-homoserine lactone
3-oxo-C8-HSL	*N*-(3-oxooctanoyl)-L-homoserine lactone
3-oxo-C7-HSL	*N*-(3-oxoheptanoyl)-L-homoserine lactone
3-oxo-C8-HSL	*N*-(3-oxooctanoyl)-L-homoserine lactone
3-oxo-C10-HSL	*N*-(3-oxodecanoyl)-L-homoserine lactone
3-oxo-C12-HSL	*N*-(3-oxododecanoyl)-L-homoserine lactone
3-oxo-C14-HSL	*N*-(3-oxotetradecanoyl)-L-homoserine lactone
3-OH-C4-HSL	*N*-(3-hydroxybutanoyl)-L-homoserine lactone
3-OH-C6-HSL	*N*-(3-hydroxyhexanoyl)-L-homoserine lactone
3-OH-C8-HSL	*N*-(3-hydroxyoctanoyl)-L-homoserine lactone
3-OH-C12-HSL	*N*-(3-hydroxydodecanoyl)-L-homoserine lactone
3-OH-C14-HSL	*N*-(3-hydroxytetradecanoyl)-L-homoserine lactone
ACP	acyl carrier protein
Ag	autoagglutination
AHL	*N*-acylhomoserine lactone
AI	autoinducer
AIP	autoinducing polypeptide
AHQ	2-alkyl-3-hydroxy-4-quinolone
Amp	ampicillin
Å	Ångstrom
APS	ammonium persulphate
ATCC	American type culture collection
ATP	adenosine triphosphate
BHI	brain heart infusion
BLAST	basic local alignment search tool
bp	basepair
Caco-2	human colonic adenocarcinoma cell line
cDNA	complimentary DNA
CDSs	coding sequences
CG	control gene
CIAP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
CO_2	carbon dioxide
CRMOX	Congo Red-magnesium oxalate agar
CSP	competence stimulating peptide
C_T	cycle threshold
Cya	adenylate cyclase

DHFR	mouse dihydrofolate reductase
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic *E. coli*
EtBr	ethidium bromide
EtOH	ethanol
FMN	flavin mononucleotide
g	gram
GC	gas chromatography
GFP	green fluorescence protein
GOI	gene of interest
h	hour
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
H_2O	water
HCl	hydrochloric acid
HGT	horizontal gene transfer
HPLC	high performance liquid chromatography
HPI	high pathogenicity island
IPTG	isopropyl-1-thio-(-D-galactopyranoside)
IS	insertion sequence
IL-8	Interleukin 8
Inc	incompatibility
kb	kilobase
kDa	kiloDalton
Km	kanamycin
kV	kilovolt
L	litre
LB	Luria Bertani
LCMS	liquid chromatography-mass spectrometry
LC-QqQLIT	liquid chromatography coupled to hybrid quadrupole–linear ion trap
LCR	low calcium response
LEE	loci of enterocyte effacement
LOS	loss of type III targeting specificity
µF	microfarad
µg	microgram
µm	micrometre
µM	micromolar concentration
M	molar concentration
mA	milliampere
MeOH	methanol
mg	milligram
min	minute
ml	millilitre
mM	milimolar concentration
MEM	minimum essential medium

MOI	multiplicity of infection
MOPS	3-(*N*-morpholino)-propanesulfonic acid
MS	mass spectrometry
MS-MS	high-resolution tandem mass spectrometry
NaCl	sodium chloride
NaOH	sodium hydroxide
Nal	nalidixic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nM	nanomolar concentration
nm	nanometre
OD	optical density
Ω	omega
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
psi	pounds per square inch pressure
QS	quorum sensing
RCHO	fatty-aldehyde
Rf	retention factor
RLU	relative luminescence unit
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RQ-PCR	relative quantification polymerase chain reaction
s	second
SAM	*S*-adenosylmethionine
Sarkosyl	sodium *N*-lauroyl sarcosinate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp.	species
Sm	streptomycin
Syc	specific Yop chaperone
T3SS	type III secretion system
TAE	tris-acetate-EDTA buffer
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	*N,N,N',N'*-tetramethylehylenediamine
Tm	melting temperature
TNF-α	tumor necrosis factor-alpha
TLC	thin layer chromatography
Tp	trimethoprim
U	enzyme unit
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt
W	watt
Yops	*Yersinia* outer proteins
Ysc	Yops secretion

CHAPTER 1

INTRODUCTION

In a letter written to the Royal Society (London) in 1683, Antoni van Leeuwenhoek described and illustrated different kinds of bacteria present in his own mouth: these can readily be identified as a mobile rod-like bacterium, *Selenomonas sputigerum*, *Pseudomonas* and *Leptothrix buccalis* (Schierbeek, 1959). Hailed as 'the Father of Microbiology', he opened up an entire world of microscopic life to the awareness of scientists. His legacy is ongoing, as new discoveries are still being made about these fascinating 'animalcules'.

1.1 The genus *Yersinia*

The genus *Yersinia* was named after Alexandre Yersin (1863-1943), a Swiss physician who discovered the plague bacillus in 1894 while visiting Hong Kong during an epidemic of bubonic plague. Yersin described Gram-negative bipolar bacilli in the buboes and blood of patients who had died of plague. He cultured the bacteria and inoculated mice and guinea pigs with them. After the animals died, he found bacilli in their tissues. He then returned to Paris with the pure cultures and published his results. The genus *Yersinia* was named posthumously in the 1960s when other genera were renamed. *Yersinia pestis*, the plague bacillus had been called *Pasteurella pestis*, *Yersinia enterocolitica* had been called *Bacterium enterocoliticum*, and *Yersinia pseudotuberculosis* had been called *Shigella pseudotuberculosis* (Butler, 1994).

The genus *Yersinia*, a member of the family *Enterobacteriaceae*, consists of eleven species of which two enteropathogenic species, *Y. pseudotuberculosis*, and *Y. enterocolitica,* and the plague bacillus *Y. pestis*, are considered to be principal pathogens of mammals (Bottone, 1997; Perry, 1997). Figure 1.1 shows the transmission routes of the pathogenic *Yersiniae* in humans. The others are environmental species (*Y. aldovae*, *Y. bercovieri*, *Y. frederiksenni*, *Y. intermedia*. *Y. kristensenni*, *Y. mollaretii*, *Y. rohdei*) and a primary fish pathogen (*Y. ruckeri*).

Y. enterocolitica and *Y. pseudotuberculosis* are enteropathogens which infect humans usually *via* the faecal-oral route and they present similar but distinct clinical features. Both have a biphasic lifestyle alternating between the food/water environment and the mammalian gastrointestinal tract. Yersiniosis from *Y. pseudotuberculosis* is contracted *via* infected animals or their excrement or by eating contaminated food. After ingestion, enterocolitis is the presenting feature in most cases and causes a relatively mild, self-limiting lymphadenitis that often mimics appendicitis. The

incidence of disease is greatest between November and February, the same seasonality observed in animals (Perry, 2004). Infection can also result in a number of complications such as adenitis and/or terminal ileitis, reactive arthritis and septicaemia (Butler, 1995). Although a septicaemia that is often fatal can occur from inflammation of the mesenteric lymph nodes, the majority of such patients have predisposing conditions such as liver disease, hemochromatosis, or diabetes (Perry, 2004).

Y. enterocolitica is widely distributed in nature in aquatic and animal reservoirs, with pigs as a common reservoir of serotypes pathogenic to humans. Infections in humans, normally after ingestion or through direct inoculation following a blood transfusion, cause a range of diseases like diarrhoea, enterocolitis, mesenteric lymphadenitis and terminal ileitis (Bottone, 1997; Bottone, 1999; Perry, 2004). *Y. enterocolitica* is a psychrotroph which is able to grow at refrigeration temperatures, resulting in food-borne epidemics and infection from transfusion of contaminated blood. *Y. enterocolitica* multiply in the intestinal lumen and invade gut-associated lymphoid tissues, where they cause ulcers in the terminal ileum and abscesses on Peyer's patches. Disseminated yersiniosis (sepsis with infection of the liver and spleen) is rare but can occur in elderly patients and those with predisposing conditions (reviewed by Perry, 2004).

Y. enterocolitica was first recognised as a human pathogen in 1939, but its significance as a pathogen was not greatly appreciated until the mid 1970s. As a result of greater awareness of the organism and more laboratories testing for its presence in enteric specimens, there has been a dramatic increase the number of reported cases of *Y. enterocolitica* infection throughout the world (Doyle, 1985). Also, unlike most other enteric pathogens of which the greatest incidences of infections occur during the summer months, infections due to *Y. enterocolitica* are most prevalent during the autumn and winter months. In parts of Europe, *Y. enterocolitica* rivals *Salmonellae* as a cause of gastrointestinal disease (Doyle, 1985; Bottone, 1997; Bottone, 1999; Howard *et al.*, 2006). Food-borne infections of *Y. enterocolitica* in humans are thought to occur primarily from pigs to humans through consumption of contaminated, uncooked/undercooked pork products such as chitterlings or tongue. *Y. enterocolitica* can also be isolated from other food products such as seafood and dairy products. A recent abattoir survey in the United Kingdom (1999 and 2000) revealed the faecal carriage rates of *Y. enterocolitica* in cattle, sheep, and pigs sent for slaughter as 6.3, 10.7, and 26.1%, respectively (McNally *et al.*, 2004). The surprisingly high carriage

rate of *Y. enterocolitica* in livestock was unexpected and suggests that livestock may contribute to human yersiniosis (Howard *et al.*, 2006).

Y. pestis is primarily a disease of rodents or other wild mammals that is usually transmitted by fleas and is often fatal. Human disease is rare and usually associated with contact with rodents and their fleas. The proventricular valve in the foregut of the flea, *Xenopsylla cheopis*, is blocked physically by a mass of the bacteria. This ensures efficient transmission, which occurs when a completely or partially blocked flea bites repeatedly in futile attempts to feed and regurgitates infected blood back into the bite site, effectively injecting the bacteria into the new host (Hinnebusch, 1998; Hinnebusch *et al.*, 2002; Wren, 2003; Jarrett, 2004). Symptoms of bubonic plague include fever, chills, headache, rapidly progressive weakness, as well as painful swollen regional lymph nodes (buboes). If the infection then progresses to the lungs, pneumonic plague develops, which is highly infectious and rapidly fatal (Butler, 1995; Wren, 2003).

Unlike *Y. pseudotuberculosis* and *Y. enterocolitica*, *Y. pestis* is not considered to be free-living but is entirely dependent on its flea and mammalian hosts. Infection of a flea *via* a contaminated blood meal completes the life cycle of this obligate parasite and humans are accidental dead-end hosts for bubonic plague (Perry & Fetherston 1997; Perry, 2004). *Y. pestis* is infamous for three plague pandemics. The first pandemic (Justinian's plague, 541 to 767 AD) is thought to have been imported from East or Central Africa and spread from Egypt to countries surrounding the Mediterranean. The second pandemic (the Black Death and subsequent epidemics from 1346 to the early 19th century) spread from the Caspian Sea to all of Europe and may have been imported from central Asia. The third pandemic began in the mid-19th century in the Yunnan region of China and spread globally from Hong Kong in 1894, the same year that *Y. pestis* was first described by Alexandre Yersin (reviewed by Achtman *et al.*, 1999). The outbreak of plague in Surat, western India (September to October of 1994) and in the rural Ziketan in Qinghai Province of north-western China (ongoing since July 2009) demonstrated that plague is not an eradicated disease and remains one of the most feared of the infectious diseases. This zoonotic disease, with reservoirs on nearly every major continent, exhibits an impressive ability to overcome mammalian host defenses (Perry & Fetherston, 1997; BBC News, 2009).

The first *Yersinia* strain to be sequenced was *Y. pestis* CO92 biovar Orientalis (Parkhill *et al.*, 2001). Subsequently, a second *Y. pestis*, strain KIM10 (biovar Mediaevalis), was sequenced (Deng *et al.*, 2002). Since then, the genomes of multiple

strains of *Yersinia* spp. have been completed, including *Y. enterocolitica* 8081 (Thomson *et al.*, 2006). Many more strains, including environmental strains, are near to completion (http://www.ncbi.nlm.nih.gov/sites/entrez). Table 1.1 shows a list of *Yersinia* spp. strains and their genome sequencing status. It has emerged that, genetically, *Y. pestis* is a recently emerged clone of the *Y. pseudotuberculosis* which arose between 1,500 to 20,000 years ago, shortly before the first known pandemics of human plague (Achtman *et al.*, 1999; Achtman *et al.*, 2004). Genome-sequence data confirm that *Y. pestis* and the *Y. pseudotuberculosis* share gene homology of nearly 97 % and largely co-linear gene organization (Wren, 2003). *Y. pestis* is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b (Skurnik *et al.*, 2000). Despite their close genetic relationship, they differ radically in their pathogenicity and transmission. A recent analysis by Chain *et al.* (2004) revealed 32 *Y. pestis* chromosomal genes and two *Y. pestis* specific plasmids, represent the only new genetic material in *Y. pestis* acquired since the divergence from *Y. pseudotuberculosis*. Interestingly, 149 other pseudogenes and 317 genes absent from *Y. pestis* were detected, indicating that as many as 13 % of *Y. pseudotuberculosis* genes no longer function in *Y. pestis*. Insertion sequence-mediated genome rearrangements and reductive gene loss, resulting in elimination and modification of pre-existing gene expression pathways, appear to be more important than acquisition of genes in the evolution of *Y. pestis*. These results provide an example of how a highly virulent epidemic clone can suddenly emerge from a less virulent, closely related progenitor (Chain *et al.*, 2004).

In contrast, *Y. enterocolitica* is more distantly related, sharing only 70 % sequence similarity with *Y. pseudotuberculosis* and *Y. pestis* (Joshua *et al.*, 2003). Thomson and Parkhill (2002) estimate that *Y. enterocolitica* is as evolutionally distant from *Y. pseudotuberculosis* and *Y. pestis* as *E. coli* is from *Salmonella* species (Wren, 2003).

Table 1.1. A list of *Yersinia* spp. strains and their genome sequencing status. Source: http://www.ncbi.nlm.nih.gov/sites/entrez

Strain	Note	Status
Y. enterocolitica subsp. *enterocolitica* 8081	Food and water-borne pathogen that causes gastroenteritis	Completed
Y. pestis Antiqua	Strain of the causative agent of plague isolated from a soil sample from the Republic of Congo	Completed
Y. pestis CO92	Extremely virulent organism that causes plague	Completed
Y. pestis KIM	Extremely virulent organism that causes plague	Completed
Y. pestis Nepal 516	Strain of the causative agent of plague isolated from a soil sample from Nepal	Completed
Y. pestis Pestoides F	This strain lacks a plasminogen activator and is virulent by the aerosol route	Completed
Y. pestis biovar Microtus str. 91001	Extremely virulent organism that causes plague	Completed
Y. pseudotuberculosis IP 31758	Serotype 1b strain isolated from a patient in Russia	Completed
Y. pseudotuberculosis IP 32953	Pathogen that causes a tuberculosis-like infection of the lung	Completed
Y. pseudotuberculosis PB1/+	Serotype IB strain	Completed
Y. pseudotuberculosis YPIII	Serotype O3 strain	Completed
Y. aldovae ATCC 35236	Isolated from water in the Czech republic	In progress
Y. bercovieri ATCC 43970	Type strain for this organism	Draft assembly
Y. frederiksenii 7175	Pig isolate	In progress
Y. frederiksenii 867	Human isolate from Belgium	In progress
Y. frederiksenii ATCC 33641	Type strain for this organism	Draft assembly
Y. frederiksenii CNY 867	Strain for comparative analysis	In progress
Y. frederiksenii WA 933	German isolate	In progress
Y. frederiksenii WA 935	Isolated in Germany	In progress
Y. frederiksenii WE 83/02	Human isolate from Belgium	In progress
Y. intermedia ATCC 29909	Type strain for this organism	Draft assembly
Y. kristensenii 105	Isolate from Denmark	In progress
Y. kristensenii 490	Isolated from a hare in France	In progress
Y. kristensenii ATCC 33638	Isolated from human urine	In progress
Y. kristensenii WA 948	Human isolate from Finland	In progress
Y. kristensenii WE 180/98	Human isolate from Belgium	In progress
Y. mollaretii ATCC 43969	Type strain for this organism	Draft assembly
Y. pestis CA88-4125	Causes plague	Draft assembly
Y. pestis FV-1	This strain is being sequenced for comparative genome analysis	Draft assembly
Y. pestis Nepal516	Strain of the causative agent of plague isolated from a soil sample from Nepal	In progress
Y. pestis Pestoides A	An atypical enzootic variant	In progress
Y. pestis biovar Antiqua str. B42003004	Antiqua biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Antiqua str. E1979001	Antiqua biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Antiqua str. UG05-0454	Antiqua biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Mediaevalis str. K1973002	Mediaevalis biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Medievalis str. Harbin 35	Virulent human isolate	In progress
Y. pestis biovar Orientalis str. F1991016	Orientalis biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Orientalis str. IP275	Orientalis subtype isolated in Madagascar that causes plague	Draft assembly
Y. pestis biovar Orientalis str. India 195	Strain from India	In progress
Y. pestis biovar Orientalis str. MG05-1020	Orientalis biovar of the causative agent of plague	Draft assembly
Y. pestis biovar Orientalis str. PEXU2	Biovar Orientalis strain for comparative analysis	In progress
Y. rohdei ATCC 43380	Isolated from dog feces	In progress
Y. ruckeri ATCC 29473	Rainbow trout isolate	In progress

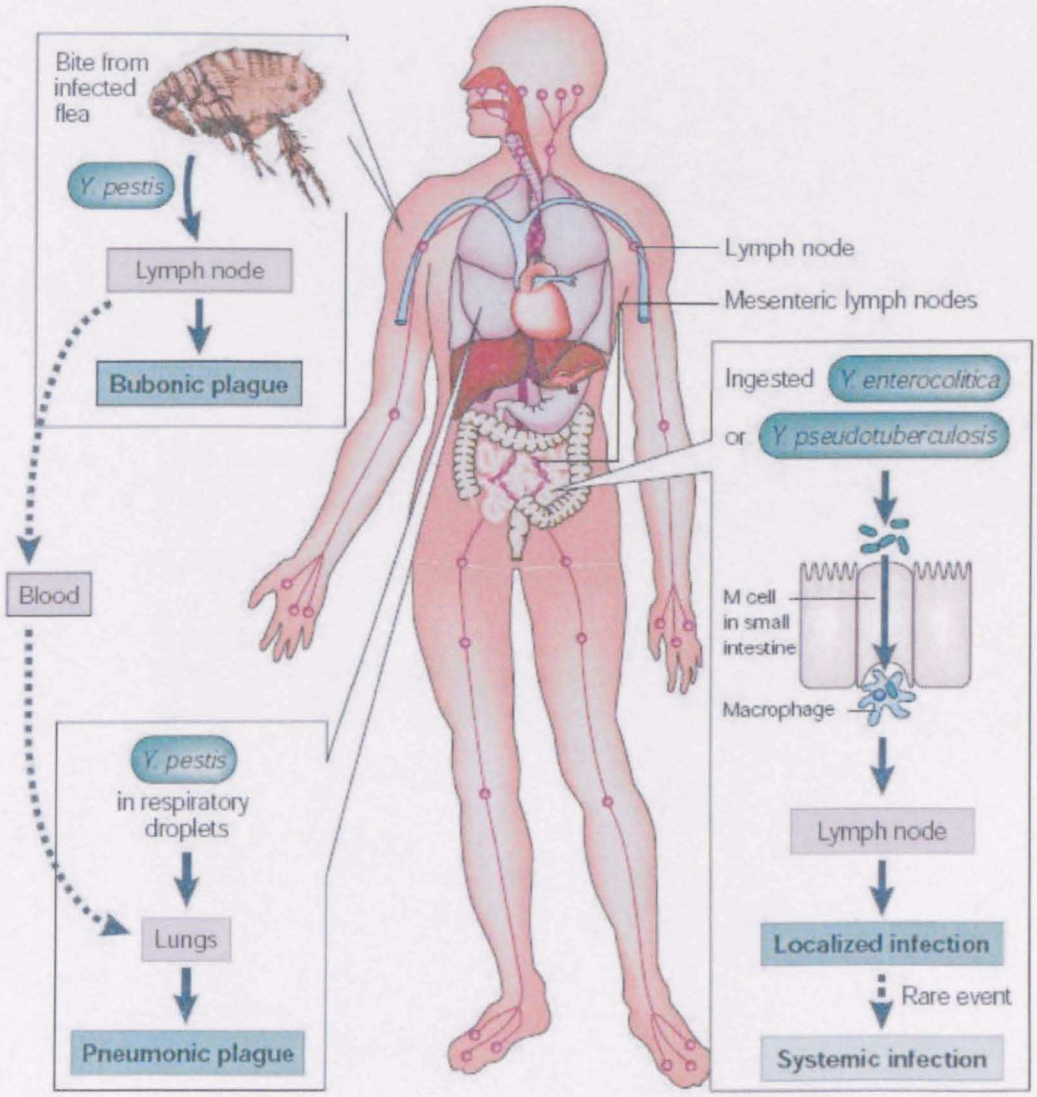


Figure 1.1. Steps in the transmission of the pathogenic *Yersiniae* in humans. *Y. pestis* has a rodent reservoir. The rodent's fleas acquire *Y. pestis* from a meal of infected blood, and transmit the bacterium primarily to other rodents, or to humans. Human-to-human transmission can occur through human fleas. Pneumonic plague is transmitted from human to human through respiratory droplets, or possibly by artificially generated *Y. pestis* aerosols. *Y. enterocolitica* and *Y. pseudotuberculosis* are ingested, and in contrast to *Y. pestis*, enter the lymphatic system through the M cells of the small intestine (adapted from Wren, 2003).

1.2 Subgroups and pathogenicity in *Y. enterocolitica*

1.2.1 Subgroups in Y. enterocolitica

Y. enterocolitica comprises a biochemically and genetically heterogeneous collection of organisms that has been divided into six biogroups, known as 1A, 1B, 2, 3, 4 and 5, that can be differentiated by biochemical tests. These can be placed into three lineages according to their lethality in a murine infection model which closely resembles the naturally acquired human infection: a non-pathogenic group which lacks the *Yersinia* virulence plasmid pYVe (biogroup 1A); a weakly pathogenic group that is unable to kill mice (biogroups 2 to 5); and a highly pathogenic, mouse-lethal group (biogroup 1B). Biotypes with low pathogenicity are generally isolated in Europe and Japan and are termed Old World strains. By contrast, highly pathogenic strains are most commonly isolated in North America and usually referred to as New World strains. These biogroups are further divided into serogroups (Bottone, 1997; Wren, 2003). Table 1.2 shows the virulence of *Y. enterocolitica* correlated with biogroup and serogroup and Table 1.3 shows the biochemical tests used to group *Y. enterocolitica* strains.

Table 1.2. Virulence of *Y. enterocolitica* correlated with biogroup, serogroup and ecologic and geographic distribution (Bottone, 1997).

Associated with human infections	Biogroup	Serogroup(s)	Ecologic distribution
Yes	1B	O:8, O:4, O:13a,13b, O:18, O:20, O:21	Environment, pigs (O:8), mainly in the United States
Yes	2	O:9, O:5,27	Pigs, Europe (O:9), United States (O:5,27), Japan (O:5,27)
Yes	3	O:1,2,3, O:5,27	Chinchilla, pigs (O:5,27)
Yes	4	O:3	Pigs, Europe, United States
Yes	5	O:2,3	Hare
No[a]	1A	O:5, O:6,30, O:7,8, O:18, O:46, nontypeable	Environment, pigs, food, water animal and human feces, United States

[a] *Y. enterocolitica* isolates comprising biogroup 1A may be opportunistic pathogens in patients with underlying disorders.

Table 1.3. Biochemical tests used to group *Y. enterocolitica* strains (Modified from Bottone, 1997).

Test	Biogroup reaction [a]					
	1A	1B[b]	2	3	4	5
Lipase activity	+	+	0	0	0	0
Salicin (acid production in 24 h)	+	0	0	0	0	0
Esculin hydrolysis (24 h)	+/0	0	0	0	0	0
Xylose (acid production)	+	+	+	+	0	v
Trehalose (acid production)	+	+	+	+	+	0
Indole production	+	+	v	0	0	0
Ornithine decarboxylase	+	+	+	+	+	+(+)
Voges-Proskauer test	+	+	+	+	+	+(+)
Pyrazinamidase activity	+	0	0	0	0	0
Sorbose (acid production)	+	+	+	+	+	0
Inositol (acid production)	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	0

[a] +, positive; 0, negative; (+), delayed positive; v, variable

[b] Biogroup 1B is comprised mainly of strains isolated in the United States

1.2.2 Pathogenicity of Y. enterocolitica

Virulence determinants are factors that promote bacterial survival, growth, and/or transmission by directly affecting the host through adherence, thwarting host defense responses, disrupting cellular metabolism, or acquiring essential nutrients from the host (Perry & Fetherston, 1997). A common virulence determinant amongst the three human pathogenic *Yersinia* species is iron acquisition systems. The ability to use exogenous siderophores probably allows the enteropathogens to more effectively compete with other microbes in the intestinal lumen and in the environment outside the host (Perry & Fetherston, 2004). The connection between iron metabolism and disease outcome was established by Jackson and Burrows (1956), who observed that 'nonpigmented' (Pgm-) mutants of *Y. pestis* were avirulent in mice unless injected along with iron or haemin (Burrows & Jackson, 1956). These Pgm- isolates, which failed to bind sufficient haemin to form greenish-brown colonies at 26°C, probably arose from deletion of a 102-kb chromosomal region now termed the *pgm* locus, which is required for the characteristic haemin adsorption. The *pgm* locus contains the haemin-storage (*hms*) locus that is required for blockage of the flea proventriculus but is not involved in iron or haeme acquisition by the bacterium (Hinnebusch *et al.*, 1996).

It was shown that while highly pathogenic *Y. enterocolitica* strains of serotype O:8 (biotype 1B) are lethal for laboratory animals at low doses, low-pathogenicity *Y. enterocolitica* strains become lethal if iron or an exogenous siderophore (Desferal) is administered to the animals (Robins-Browne & Prpic, 1985; Carniel, 2001). The suppressive effect of iron on the host immune response may partly account for the enhanced bacterial pathogenicity. Availability of iron, provided either exogenously or through an intrinsic high-affinity iron-chelating system (e.g., siderophore) endows the bacteria with the ability to multiply in the host and to cause systemic infections. In *Yersinia*, the machinery of siderophore biosynthesis, transport and regulation is located on a large chromosomal fragment termed a 'high-pathogenicity island' or HPI, which is part of the *pgm* locus (Carniel *et al.*, 1996; Carniel, 2001).

The *Yersinia* HPI varies in size from 36 kb in *Y. pseudotuberculosis* and *Y. pestis* to 43 kb in *Y. enterocolitica*. It encodes the yersiniabactin (Yst) system involved in siderophore-mediated iron uptake and it incorporates several repeated sequences (IS1328, IS1329, IS1222 and IS1400 or IS100) and a mobility gene (bacteriophage P4-like integrase gene). This DNA region is G+C rich (56.4%) (Carniel, 2001) compared

to the rest of the chromosome (47.27%) (Thomson *et al.*, 2006). Since most of the genes located on the HPI are involved in siderophore-mediated iron acquisition, the HPI may be regarded as an iron-uptake island. The HPI is present in the three pathogenic species of *Yersinia* but is restricted to certain subgroups of each species. In *Y. pseudotuberculosis*, a complete island is only found in strains of serotype I, while an HPI with a 9-kb truncation in its left-hand part is characteristic of serotype III strains. No HPI is detected in other serotypes of *Y. pseudotuberculosis* (Carniel, 2001). In *Y. enterocolitica*, the island is specific for strains of biotype 1B (de Almeida *et al.*, 1993) and this was recently reconfirmed in a study on the comparative phylogenomics of a diverse collection of *Y. enterocolitica* strains (Howard *et al.*, 2006). The HPI has wide distribution amongst other members of the Enterobacteriaceae family including *Escherichia coli*, *Klebsiella spp.*, *Citrobacter spp.*, *Enterobacter spp.*, *Serratia liquefaciens*, *Photorhabdas luminescens* and *Salmonella enterica*, where the core region of the island is well conserved and functional (Lesic & Carniel, 2004).

The 482-Da yersiniabactin molecule (alternatively termed "yersiniophore") is identical in *Y. enterocolitica* and *Y. pestis* and its structure is closely related to those of pyochelin and anguibactin produced by *Pseudomonas aeruginosa* and *Vibrio anguillarum*, respectively. The Yst locus is comprised of 11 genes organised into four operons and can be roughly divided into three functional groups: yersiniabactin biosynthesis, transport into the bacterial cell (outer membrane receptor and transporters) and regulation (Carniel, 2001). In one study, the importance of this gene cluster was demonstrated by transferring the HPI core genes of a highly pathogenic biotype 1B strain to a strain with low pathogenicity (biotype 2) and therefore conferring virulence to the latter strain (Pelludat *et al.*, 2002). Although the role of HPI in promoting virulence in *Yersinia* is now clearly documented, the mechanisms underlaying the enhanced capacity to cause bacterial dissemination in the host remain to be elucidated (Lesic & Carniel, 2004).

1.2.3 The pYVe plasmid

All pathogenic strains of *Yersinia* carry a conserved 70-kb virulence plasmid designated pYV (for plasmid associated with *Yersinia* virulence). Both the low- and high-pathogenicity groups of *Y. enterocolitica* carry the pYVe plasmid. This plasmid is usually absent from the nonpathogenic 1A biogroup (Marenne *et al.*, 2004). The pYVe plasmid encodes the Yop virulon that consists of a complete type III secretion (TTS) system, called Ysc-Yop (Cornelis, 2002a; Cornelis, 2002b). T3SSs are highly sophisticated virulence mechanisms used by many pathogenic Gram-negative bacteria to interact with eukaryotic cells by injecting bacterial proteins into the cytosol. The proteins are not only secreted across the bacterial membranes but are also translocated across the eukaryotic cell membrane. Inside the eukaryotic cell, these bacterial proteins will subvert and disrupt host cell signalling pathways (Marenne *et al.*, 2004). Figure 1.2 shows the map of pYVe plasmid of the *Y. enterocolitica* 8081 strain.

The T3SS in *Yersinia* can be triggered *in vitro* to release large amount of Yops (for Yersinia Outer Proteins) by incubating the bacteria at 37°C in the absence of calcium ions (Michiels *et al.*, 1990). Yop release occurs *via* a specialised structure known as the Ysc (for Yops secretion) injectisome. Secretion of some Yops requires the assistance of small individual chaperones called the Syc (specific Yop chaperone) proteins that bind specifically to their cognate Yop. The Yop proteins include intracellular 'effectors' (YopE, YopH, YopM, YpkA/YopO, YopJ/P, YopT) and 'translocaters' (YopB, YopD, LcrV) which are needed to deliver the effectors across the plasma membrane into the cytosol of eukaryotic target cells. The system also secretes proteins that seem to have an exclusive regulatory role (YopN, YopQ, YscM/LcrQ), components of the Ysc injectisome (YscP, YscF) and one protein with unknown function (YopR). Physiological secretion of Yops is triggered by intimate contact between an invading bacterium and a target cell. The whole system is tightly regulated at several levels to ensure that the cocktail of six Yop effectors are delivered into the eukaryotic cell cytoplasm (Marenne *et al.*, 2004).

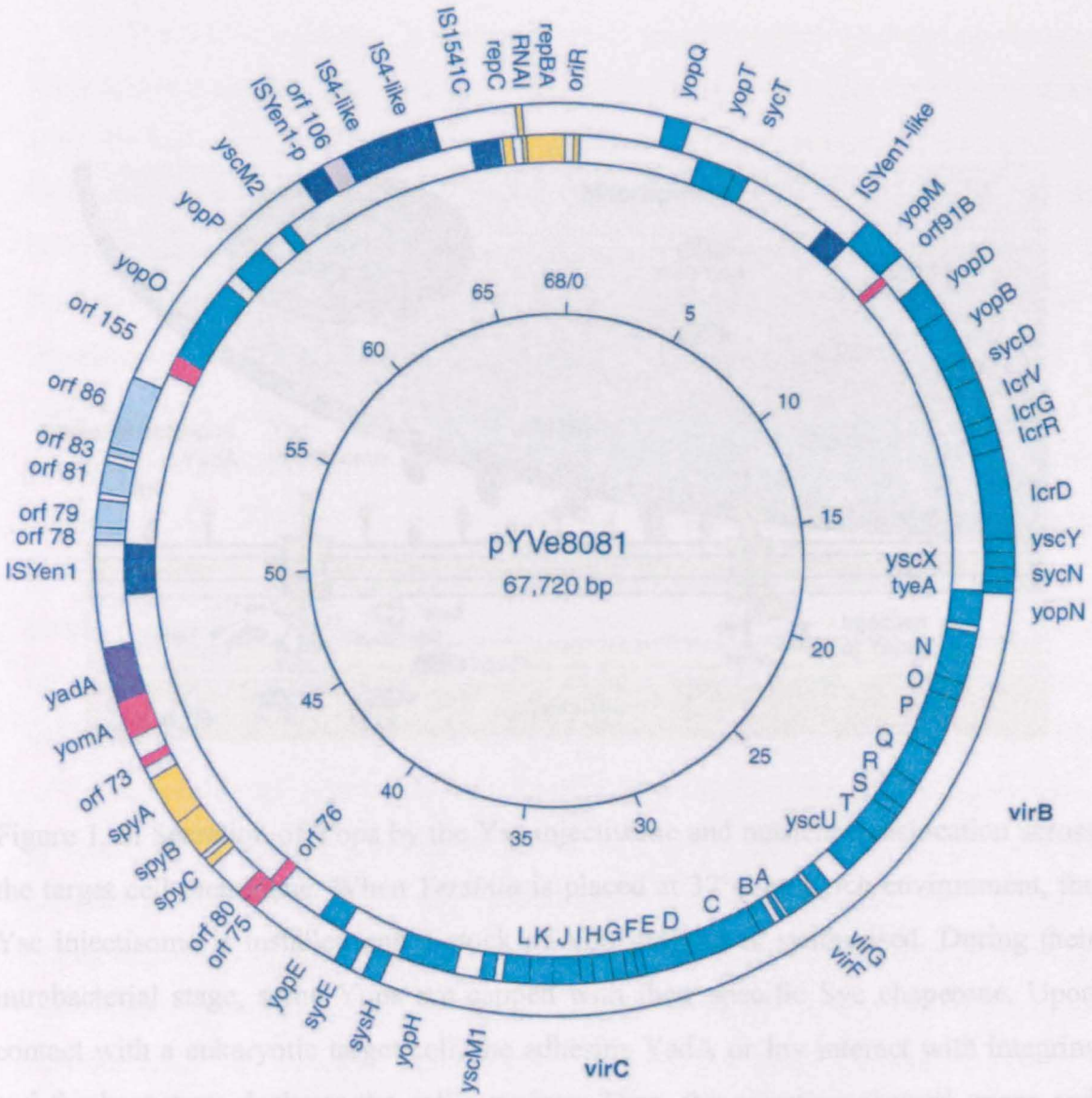


Figure 1.2. Map of pYVe plasmid of the *Y. enterocolitica* 8081 strain, showing significant genes, IS (insertion sequence) elements, and replication and partition regions. The direction of transcription is clockwise for genes shown inside the circle and counter-clockwise for genes shown outside the circle. Green boxes indicate genes comprising the LCR (for Low Calcium Response) stimulon, and a purple box indicates *yadA*. Yellow boxes indicate genes with replication and partition functions. Pink boxes indicate previously identified genes of unknown function, and potential new genes are indicated by light blue boxes. IS elements are indicated by dark blue boxes. The positions of the *virB* operon (*yscN* to *yscU*) and the *virC* operon (*yscA* to *yscM1*) are noted in boldface on the outside of the circle. The inner circle shows the scale in kilobase pairs (reproduced from Snellings *et al.*, 2001).

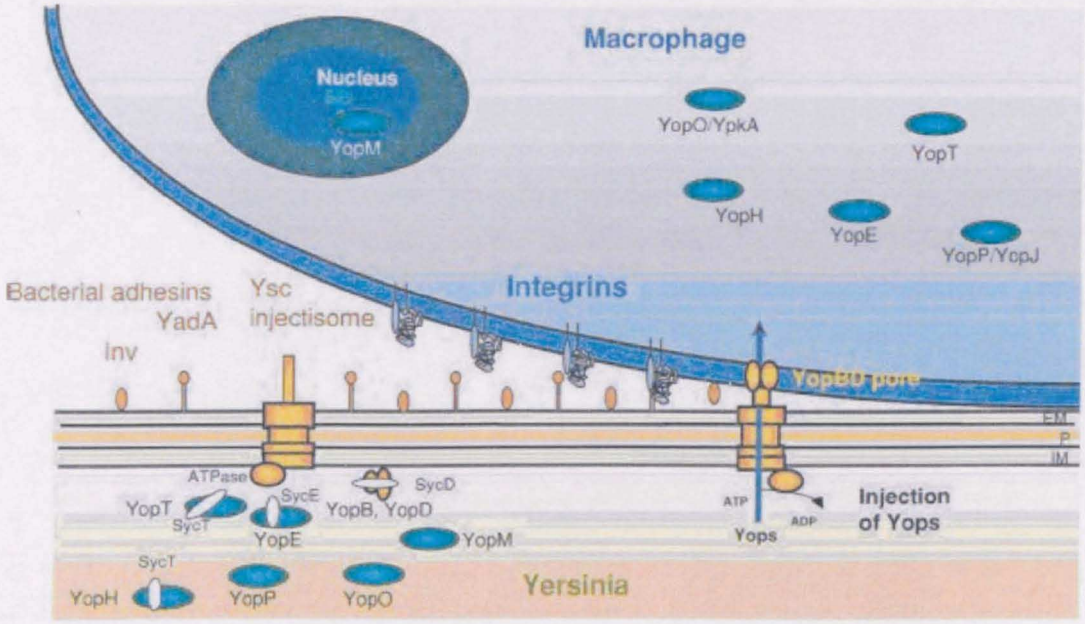


Figure 1. 3. Secretion of Yops by the Ysc injectisome and nutrient translocation across the target cell membrane. When *Yersinia* is placed at 37°C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesised. During their intrabacterial stage, some Yops are capped with their specific Syc chaperone. Upon contact with a eukaryotic target cell, the adhesins YadA or Inv interact with integrins and the bacterium docks at the cell's surface. Then, the secretion channel opens and Yops are exported. YopB and YopD form a pore in the target cell plasma membrane, and the effector Yops are translocated across this membrane into the eukaryotic cell cytosol. YopM migrates to the nucleus. EM, outer membrane; P, peptidoglycan; IM, plasma membrane (Reproduced from Cornelis, 2002a).

1.2.3.1 The Ysc injectisome

The Ysc injectisome is composed of 29 proteins which span the peptidoglycan layer and two bacterial membranes, and is topped by a needle-like structure protruding from the bacterium (Hoiczyk & Blobel, 2001). Several components of the injectisome, which includes an ATPase, share homology with structural proteins of the flagellum, indicating that the two structures have a common evolutionary origin. It is likely that the flagellum is the ancestor, given that the injectisome would be useful only after the appearance of unicellular eukaryotes (Cornelis, 2002b). The external part of the Ysc injectisome, which spans the bacterial outer membrane, is a homomultimeric ring-shaped structure with an external diameter of about 200 Å and a central pore of 50 Å (Koster *et al.*, 1997). YscC, the monomer of this complex, belongs to the family of secretins. The Ysc injectisome ends with a 6-7 nm-wide needle formed by the polymerisation of monomers of the 6-kDa YscF protein that are secreted by the Ysc apparatus itself (Hoiczyk & Blobel, 2001). The needle has a hollow centre of about 2 nm. The length of the needle varies between different *Yersinia* species. It is 58 ± 10 nm in *Y. enterocolitica* O:9 but only 41 ± 8 nm in *Y. pestis*. This length is controlled by the protein YscP acting as a molecular ruler, with an increment of 1.9 Å per residue (Journet *et al.*, 2003). When the needle reaches its normal length, YscP signals the export apparatus to stop secreting YscF subunits and to be ready to secrete Yops upon cell contact (Edqvist *et al.*, 2003; Journet *et al.*, 2003). Figure 1. 4 shows a schematic representation of the Ysc injectisome spanning the outer membrane (OM), the peptidoglycan layer (PG) and the cytoplasmic membrane (CM) of the bacterium.

The complete Ysc injectisome of *Yersinia* has not been isolated but the entire TTS complex of *Salmonella enterica* serovar Typhimurium (Kubori *et al.*, 1998; Kubori *et al.*, 2000; Kimbrough & Miller, 2000) and *Shigella flexneri* (Blocker *et al.*, 2001) has been purified and visualised by electron microscopy. These structures are composed of three domains: the export apparatus, the basal body and the needle. The sequence similarity between proteins composing the *Salmonella/Shigella* injectisome and the *Yersinia* injectisome suggests that the *Yersinia* injectisome is similar to those so far visualised. The export apparatus, installed in the basal body, exports the components of the needle as well as the Yop proteins. The basal body consists of two pairs of rings that are anchored to the inner and outer membranes of the bacterial envelope and joined by a central rod.

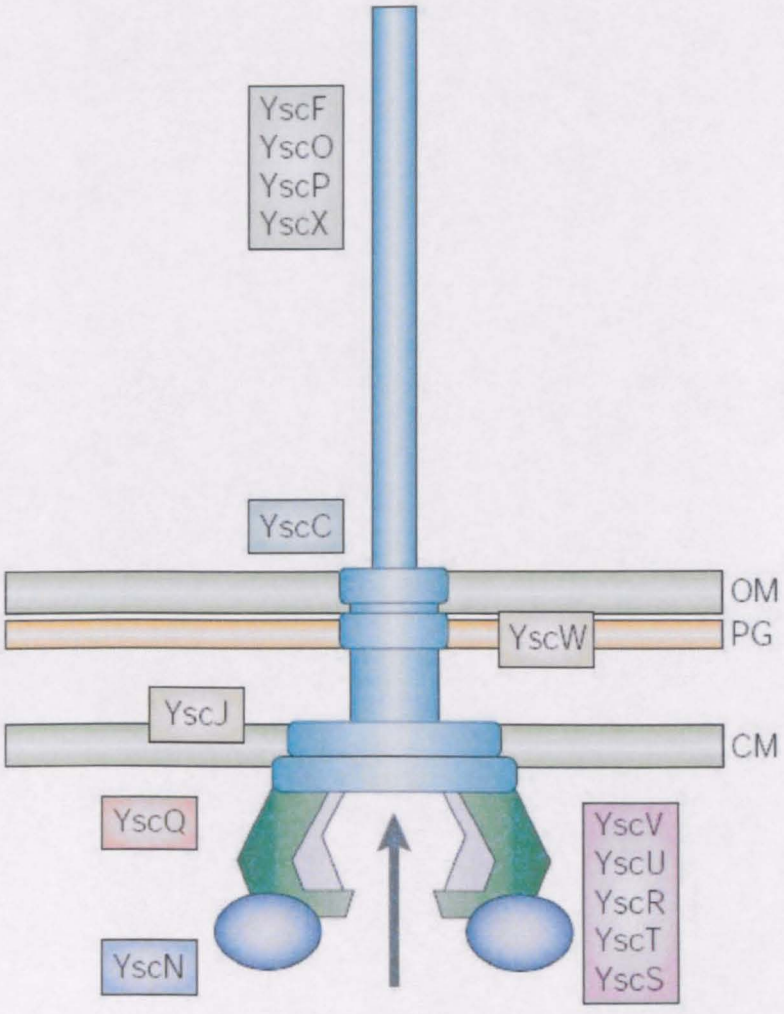


Figure 1.4. Schematic representation of the Ysc injectisome spanning the outer membrane (OM), the peptidoglycan layer (PG) and the cytoplasmic membrane (CM) of the bacterium. The ring spanning the OM is made of the secretin YscC, assisted by the lipoprotein YscW. YscJ is another lipoprotein. YscF, YscO, YscP and YscX are external parts of the injectisome. YscF is the main constituent of the needle. YscV, YscU, YscR, YscT and YscS are proteins of the basal body that are in contact with the CM. YscN is the ATPase of the pump. YscQ is probably localised to the large inner cylinder (Reproduced from Cornelis, 2002b).

1.2.3.2 Yersinia *outer proteins (Yops)*

Yop effectors can be thought of as the bacterial equivalent of viral oncogenes because they mimic or capture eukaryotic cell activity in order to modulate the host's signalling pathways. Like viral oncoproteins, bacterial effectors tend to be more potent than their eukaryotic counterparts, causing the deregulation of normal cellular functions by either over-activating or inhibiting a pathway. Unlike viral oncoproteins, effectors remain quiescent in the bacteria, only to become active in the eukaryotic target cell (Trosky *et al.*, 2008). There are six Yops found in *Y. enterocolitica*: YopH, YopM, YopE, YpkA/YopO, YopJ/YopP and YopT. These effectors work together to establish an extracellular infection that disrupts the actin cytoskeleton and deregulates signalling pathways (Navarro *et al.*, 2005; Viboud & Bliska, 2005).

YopH inactivates proteins involved in adhesion and phagocytosis *via* dephosphorylation (Viboud & Bliska, 2005) which leads to disruption of actin structures in macrophages and polymorphonuclear leukocytes (Andersson *et al.*, 1996). YopH was proposed to be held quiescent in the bacterial cytoplasm by the association with the chaperone, SycH, which binds to the N-terminus of YopH (Trosky *et al.*, 2008).

YopE is cytotoxic and disrupts actin cytoskeleton assembly in infected cells (Holmstrom *et al.*, 1997). In a mouse model of *Yersinia* infection, YopE is essential for virulence indicating the importance of its antiphagocytic activity in the pathogenesis of *Yersinia* spp. (Black & Bliska, 2000). Cells infected by wild type *Yersinia* undergo apoptosis, which does not result in the release of cellular contents and induction of inflammatory signals by the host immune system. However, in cells infected with a YopE-deficient *Yersinia* strain, there is release of cytosolic proteins upon apoptosis. These results suggest that YopE is involved in resealing pores made by the T3SS *via* actin polymerization, (Viboud & Bliska, 2005). YopE may also play a role in regulating how much effector is delivered into a target cell. Infection with YopE mutant strains results in a 'hyper translocation phenotype' (Aili *et al.*, 2008).

YopM effectors are composed of ~20 amino acid leucine-rich repeats (LRRs) which are known to mediate protein-protein interactions. The crystal structure of YopM revealed that the monomers form a horseshoe shape and stack together to form a hollow cylinder comprised of 4 YopM monomers (Evdokimov *et al.*, 2001). Mice infected with a YopM mutant strain experienced decreased virulence suggesting that YopM is involved in pathogenesis but the exact mechanism of this is unclear (Mulder *et al.*,

1989; Gallant *et al.*, 2005). Because of its nuclear localization, it has been suggested that YopM affects gene transcription but this is still to be confirmed (Sauvonnet *et al.*, 2002; Hoffmann *et al.*, 2004).

Studies on YopT mutants demonstrated that YopT induces cytotoxicity by disrupting actin microfilaments during infection (Zumbihl *et al.*, 1999). YopT is a protease which cleaves Rho family GTPases at the site of anchorage to the cell membrane (Shao *et al.*, 2005). Mislocalization of Rho GTPases from the cell membrane disrupts their function, resulting in interference with actin assembly (Shao *et al.*, 2005).

YpkA (for Yersinia protein kinase A) is an autophosphorylating serine-threonine kinase (Galyov *et al.*, 1993; Dukuzumuremyi *et al.*, 2000) which localizes to the cytoplasmic face of the plasma membrane in eukaryotic cells (Holmstrom *et al.*, 1997; Dukuzumuremyi *et al.*, 2000; Viboud & Bliska, 2005; Navarro *et al.*, 2005). It disrupts the actin cytoskeleton and contributes to the ability of *Yersinia* to resist phagocytosis by macrophages (Grosdent *et al.*, 2002; Navarro *et al.*, 2005; Rogers *et al.*, 2008; Trasak *et al.*, 2007). YpkA is hypothesised to contribute to the abnormal 'bleeding' observed in patients afflicted with the plague, which causes increased bleeding times and defective platelet activation (Offermanns *et al.*, 1997; Navarro *et al.*, 2007).

YopP inhibits the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor known to be of central importance in the onset of inflammation (Boland & Cornelis, 1998; Schesser *et al.*, 1998). This causes the reduced release of TNF-α (tumor necrosis factor-alpha) (Boland & Cornelis, 1998) by macrophages, IL-8 (Interleukin 8) by epithelial (Schesser *et al.*, 1998) and endothelial cells (Denecker *et al.*, 2002). It also reduces the presentation of adhesion molecules at the surface of endothelial cells (Denecker *et al.*, 2002) and presumably reduces neutrophil recruitment to the site of infection. YopP also induces apoptosis of macrophages but it is not clear yet whether apoptosis results from a YopP-induced early cell death signal or from the YopP-induced loss of NF-κB activity known to protect cells from apoptosis (Ruckdeschel *et al.*, 1997; Denecker *et al.*, 2001).

1.2.3.3 The Yop chaperones

The chaperones in the Yersinia Ysc-Yop type III secretion system are called Syc proteins (for specific Yop chaperone). Chaperones have been described for three of the effector Yops; SycE for YopE, SycH for YopH and SycT for YopT. The translocator Yops, YopB, and YopD share the same chaperone, SycD. The regulatory YopN has two chaperones, SycN and YscB, and YscY may be a chaperone for YscX, an element of the Ysc injectisome. Generally, each Syc chaperone gene is located next to the gene encoding their cognate protein. There are no obvious sequence similarities between the different chaperones but they are characteristically small (less than 20 kDa), acidic (pH ~4-5), bear a C-terminal amphiphilic α-helix, and specifically bind with high affinity to their cognate Yop (Michiels *et al.*, 1990).

The Syc chaperones were hypothesised to help the secretion of their cognate Yop, by acting as a kind of secretion pilot to drive nascent Yops through the secretion machinery (Michiels *et al.*, 1990; Wattiau & Cornelis, 1993). However, using adenylate cyclase (Cya) reporter enzyme strategies, a YopE-Cya hybrid protein devoid of the SycE chaperone-binding site was shown to be secreted normally in the presence or absence of SycE and a YopE-Cya hybrid protein that possesses the SycE chaperone-binding site is secreted only when SycE is present. These results suggested that the chaperone is not indispensable for the secretion of its cognate Yop, but becomes necessary when its binding site is present (Woestyn *et al.*, 1996).

Another hypothesis suggests that the Syc chaperones could also play an anti-folding role (Edqvist *et al.*, 2003). YopE is rapidly secreted when conditions are favourable and the secretion of stored YopE does not require *de novo* protein synthesis but is completely dependent on SycE. This observation suggests that SycE may maintain preformed, stored YopE in a secretion-competent (presumably unfolded or partially folded) state. This is supported by the fact that the diameter of the needle end of the injectisome is 2 nm (Hoiczyk & Blobel, 2001), whereas the diameter of YopE is nearly 2.5 nm, a size that should not allow folded YopE to travel through the needle (Evdokimov *et al.*, 2002). If the Yops travel through the needle, then they have to be at least partially unfolded. Therefore, SycE might prevent YopE from folding prematurely until it has passed through the needle.

This anti-folding role is supported by observations in a study using YopE-DHFR hybrid protein (Feldman *et al.*, 2002). DHFR is mouse dihydrofolate reductase, a cytosolic globular protein secreted by the *Yersinia* Ysc injectisome. A fusion between

the first 52 residues of YopE and the wild type DHFR was secreted, and this secretion was dependent on SycE. However, in another study, it was shown that the folding state of YopE is not affected by the binding of SycE (Birtalan *et al.*, 2002). It was also observed that the purified SycE-YopE complex produced in *E. coli* displays GTPase activating protein activity, which indicates that the folding of the C-terminal domain of YopE was correct.

It was proposed that chaperones could orchestrate a defined order of secretion (Boyd *et al.*, 2000). In wild type *Yersinia* strain, the YopE-Cya hybrid protein devoid of the SycE chaperone-binding site is not translocated. However, in a polymutant *Yersinia* strain deleted of all the Yop effectors, the same YopE-Cya hybrid protein is translocated at the same level as the YopE-Cya hybrid protein which carries the chaperone-binding site. These results suggest that SycE is required for the secretion of YopE when the other Yop proteins are present. The SycE chaperone may be the dominant factor in a hierarchy which controls the secretion of the Yop proteins in a defined order. In support of Birtalan *et al.* (2002), it was indicated that the SycE-YopE complex could provide a 3-dimensional secretion signal, helping secretion initiated by the amino-terminal signal. These authors speculate that the SycE-YopE complex could interact with the Ysc machinery, probably with the ATPase YscN.

The majority of the TTS chaperones contribute to the cytosolic stability of their cognate Yops. For example, SycE plays an antidegradation role since the half-life of YopE is longer in the wildtype *Yersinia* than in *sycE* mutant bacteria (Frithz-Lindsen *et al.*, 1995). There are some exceptions, however. SycH is the chaperone required for the secretion not only for YopH, but also of YscM1-2, two negative regulators of the system (Michiels *et al.*, 1990; Cambronne *et al.*, 2000). Instead of stabilising its partners, SycH induces the inactivation of the negative regulators, YscM1-2, and allows an increase in the transcription of *yop* genes (Michiels *et al.*, 1990; Persson *et al.*, 1995; Cambronne *et al.*, 2000).

The situation of the SycN and YscB chaperones is special because these two chaperones possess the same substrate (YopN). Cross-linking experiments followed by co-immunoprecipitation showed that SycN and YscB bind together to YopN (Day & Plano, 1998). SycN and YscB can interact together in the absence of YopN, but either chaperone individually does not bind to YopN. SycN and YscB facilitate the secretion and the subsequent translocation of YopN (Day *et al.*, 2003).

YscY is a protein that shows the same properties as the chaperones. This protein interacts with YscX, a secreted element of the type III secretion machinery (Iriarte *et al.*, 1995; Day & Plano, 2000).

1.2.3.4 The regulation of type III secretion system

Yersinia does not secrete Yops when they are incubated in a cell-free eukaryotic cell culture medium. However, in the presence of target cells, they inject their Yop effectors, indicating that physical contact with cells triggers this process (Holmstrom *et al.*, 1997). This hypothesis was confirmed by a gene fusion experiment. The *yopE* promoter is fused to a luciferase gene resulting in a recombinant *Y. pseudotuberculosis* which is then used to infect HeLa cells. Only adhering bacteria became luminescent, demonstrating clearly the necessity of contact in triggering the system (Pettersson *et al.*, 1996).

In vitro, *Yersinia* secrete Yop proteins when placed at 37°C in a rich medium devoid of calcium ions. The isolation of calcium-blind mutants allowed the identification of three genes whose products are involved in the control of Yop release: *yopN*, *tyeA* and *lcrG* (Yother & Goguen, 1985; Forsberg *et al.*, 1991; Skryzpek & Straley, 1993; Iriarte *et al.*, 1995; Boland *et al.*, 1996; Cheng & Schneewind, 2000). Figure 1.5 shows a schematic representation of the transcriptional regulation of type III genes.

YopN is a 32.6-kDa protein with two coiled-coil domains. It is secreted at 37°C in the absence of calcium ions. In the presence of calcium ions, the protein is not released but it is exposed at the bacterial surface (Iriarte *et al.*, 1995). A *yopN* mutant secretes Yops at 37°C, even in presence of calcium ions. Upon contact with eukaryotic cells, the *yopN* mutant can still deliver Yops into the cytosol of the target cell, but it secretes more Yops into the eukaryotic cell medium than wild type *Yersinia* strains (Boland *et al.*, 1996). YopN is translocated into the cytosol of eukaryotic cells (Day *et al.*, 2003) and it has been suggested that YopN could function as a sensor and a plug controlling Yop secretion. After contact with the eukaryotic cell, the YopN sensor could interact with a ligand on the target cell surface, be removed, and allow Yop secretion and delivery into the target cell (Holmstrom *et al.*, 1997). However, YopN has never been shown to interact with a cell receptor.

TyeA is a 10.8-kDa protein which was initially thought to play a role only in the translocation of YopE and YopH (Iriarte *et al.*, 1995). However, Cheng and

Schneewind (2000) have observed by fractionation experiments that TyeA is involved in the translocation of all Yop effectors. TyeA is detected in the bacterial cytosolic fraction but not in the culture supernatant, irrespective of the presence of calcium ions in the culture medium. TyeA is located in the bacterial cytosol and has the capacity to bind to the second coiled coil of YopN and to interact with YopD (Iriarte *et al.*, 1995; Cheng & Schneewind, 2000).

LcrG is a 11 kDa protein that controls the release of Yops *in vitro* (Skryzpek & Straley, 1993; Sarker *et al.*, 1998), and it is also required for efficient translocation of Yop effectors (Sarker *et al.*, 1998). This protein has been shown to be primarily cytosolic, but it has also been detected in the membrane and in the extracellular medium (Skryzpek & Straley, 1993; Nilles *et al.*, 1997). Impaired Yop secretion in *yopN*, *tyeA* and *lcrG* mutants (Skryzpek & Straley, 1993; Iriarte *et al.*, 1995; Sarker *et al.*, 1998) suggests that the control of the delivery of the effectors requires a complex system comprised of at least YopN, TyeA and LcrG. This control also requires that the complex of these three proteins be located in the bacterial cytosol.

Another key component of the *Yersinia* TTSS is the LcrV, a 37-kDa protein. Level of intracellular LcrV increases in secretion-permissive conditions and LcrV titrates LcrG by formation of a stable LcrG-LcrV complex (Nilles *et al.*, 1998; Matson & Nilles, 2002) through coiled-coil interaction (Lawton *et al.*, 2002). This complex removes the plug of the Ysc apparatus, formed by YopN, TyeA and LcrG, consequently enabling Yop secretion to occur (Lawton *et al.*, 2002). In other words, the role of LcrV is indirect; counteracting the negative regulatory role of LcrG. However, it has been hypothesised that LcrV could insert into the eukaryotic cell membrane since purified LcrV can form channels in planar lipid bilayers. Initiation of pore formation by LcrV is then followed by the subsequent insertion of YopB and YopD (Holmström *et al.*, 2001) A *lcrV*, *yopQ* double mutant is deficient in pore formation, which could be restored by the introduction of an *in trans* copy of *lcrV* but not *yopQ*, confirming that LcrV itself is directly required for pore formation (Marenne *et al.*, 2003). In agreement with its role as a translocator, LcrV is also a translocator and is shown to be exposed on the bacterial surface before host contact and is involved in the targeting of the effector Yops to the cytosol of host cells (Nilles *et al.*, 1998; Fields *et al.*, 1999; Pettersson *et al.*, 1999).

In vitro, Yop secretion occurs only at 37°C in the absence of calcium ions. This 'low-calcium response' (LCR) correlates with growth arrest of the bacterium. At 26°C, Yop expression is repressed. The activation of Yop expression by temperature and repression by calcium ions are two distinct phenomena. Temperature control acts directly at the level of the transcription of the *yop* genes (Cornelis *et al.*, 1989; de Rouvroit *et al.*, 1992), while calcium ions inhibit Yop secretion by the type III machinery (Forsberg *et al.*, 1987). However, when secretion is inhibited by the presence of calcium ions, a feedback inhibition mechanism blocks transcription of *yop* genes (Cornelis *et al.*, 1987; Forsberg & Wolf-Watz, 1988; Straley *et al.*, 1993). This feedback mechanism allows a strict control of the amount of intrabacterial Yops.

VirF is a 30.9 kDa protein that belongs to the AraC family of regulators (Cornelis *et al.*, 1989) which controls the transcription of all the *yop* genes, *sycE*, *ylpA*, *yadA*, and the *virC* operon (Cornelis *et al.*, 1989; China *et al.*, 1990; Michiels & Cornelis, 1991; Skurnik & Toivanen, 1992). VirF does not appear to control the transcription of the *virA* and *virB* operons (encoding the Ysc injectisome), and *sycH* (Michiels *et al.*, 1990; de Rouvroit *et al.*, 1992). Regardless of VirF dependency, all Yop virulon gene expression is suppressed at low temperature (22°C to 26°C) but strongly expressed at 37°C. It was shown that the promoter regions of *yopE*, *yopH*, *virC*, and *IcrGVsycDyopBD* bind to VirF on a 40-bp region immediately upstream from the RNA polymerase binding site (Michiels *et al.*, 1990). VirF self-activates through a positive feedback mechanism. It was found that *virF* itself is strongly thermoregulated *via* the chromosomally encoded YmoA (Cornelis *et al.*, 1987).

Dorman *et al.* (1999) showed that YmoA (for Yersinia modulator) is the regulator of *virF*. YmoA is an 8.1 kDa protein which is rich in positively and negatively charged residues, a feature also found in the *E. coli* histone-like protein H-NS. H-NS is involved in the temperature regulation of virulence gene expression through modifications in DNA structure (Dorman *et al.*, 1999). Although there is no sequence similarity between YmoA and H-NS, it is very likely that YmoA is involved in DNA supercoiling as the level of supercoiling is higher in *ymoA* mutant bacteria than in wild type (Cornelis *et al.*, 1991). Further studies showed that chromatin structure influences expression of *yop* genes (de Rouvroit *et al.*, 1992) and that temperature could modify the structure of chromatin, making the promoters more accessible to VirF. Raising the temperature dislodges the repressor YmoA, bound on promoter regions of VirF-dependant genes (Rohde *et al.*, 1999).

Calcium ions not only inhibit secretion of the Yop proteins at 37°C but also strongly reduce the expression of the *yop* genes. Transcription of the *ysc* genes is only weakly affected by the presence of calcium ions (Goguen *et al.*, 1984; Cornelis *et al.*, 1987; Forsberg & Wolf-Watz, 1988; Mulder *et al.*, 1989). It was hypothesised that when calcium ions on the bacterial surface inhibit Yop secretion, a negative feedback mechanism reduces transcription of the *yop* genes to prevent a cytosolic accumulation of the Yop proteins that could be toxic for the bacterium (Cornelis *et al.*, 1987).

LcrQ in *Y. pseudotuberculosis* is a negative regulator of Yop secretion (Rimpilainen *et al.*, 1992; Pettersson *et al.*, 1996). The overproduction of this 12.4kDa secreted protein abolishes Yop production (Rimpilainen *et al.*, 1992). In contrast, a *lcrQ* mutant synthesizes more Yops than the wildtype strain. In the presence of calcium ions, this *lcrQ* mutant secretes YopD and LcrV. LcrQ is rapidly secreted when bacteria are shifted from a medium containing calcium ions (nonpermissive conditions for Yop secretion) to a medium containing a calcium chelator (permissive conditions for Yop secretion). LcrQ accumulates in the bacterial cytosol in conditions that are nonpermissive for Yop secretion and suppress *yop* transcription. The LcrQ level in the bacterium is inversely proportional to the expression level of Yops genes.

In *Y. enterocolitica*, two homologues of LcrQ were found and named YscM1 and YscM2 (Allaoui *et al.*, 1995; Iriarte *et al.*, 1995). The *yscM1/yscM2* double mutant in *Y. enterocolitica* shows the same phenotype as the *lcrQ* mutant of *Y. pseudotuberculosis*. Therefore, YscM1 and YscM2 in *Y. enterocolitica* are negative regulators of *yop* transcription. However, *lcrQ* and *yscM1-2* mutants still secrete YopD and LcrV in the presence of calcium ions (Rimpilainen *et al.*, 1992; Iriarte *et al.*, 1995). It was suggested that YopD is also involved in this feedback inhibition mechanism (Williams & Straley, 1998).

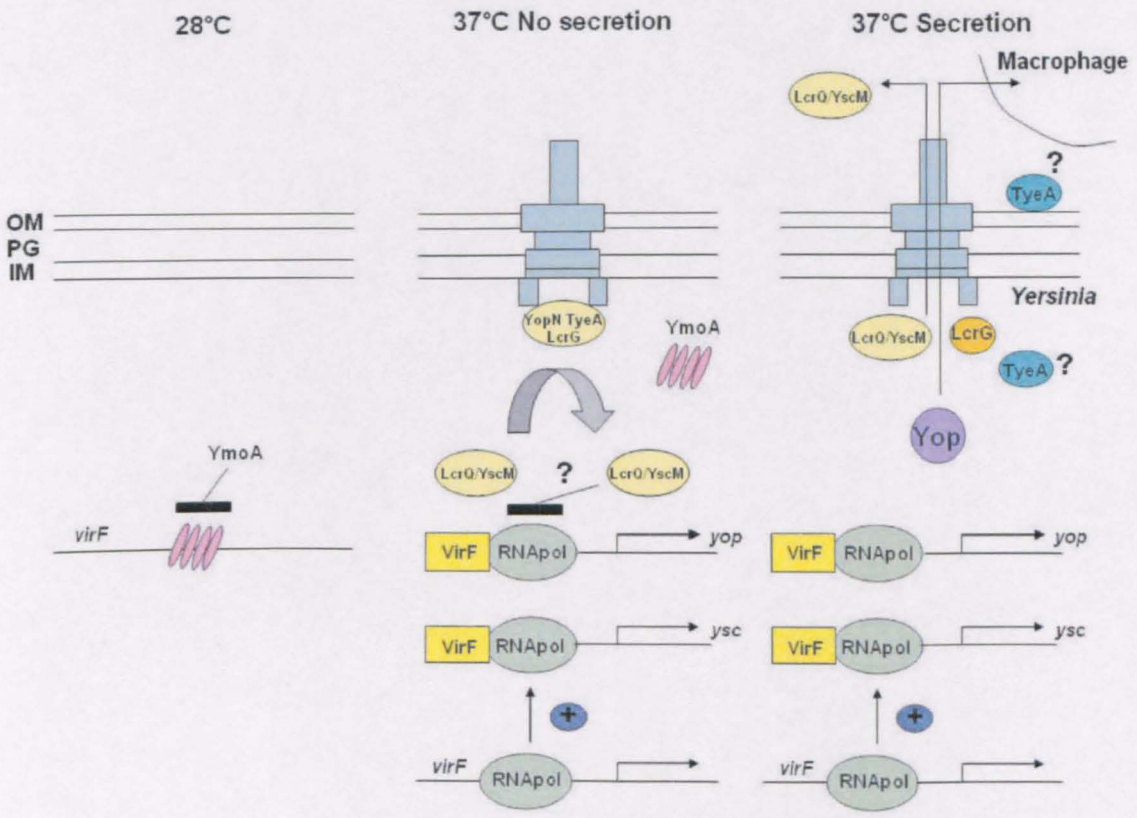


Figure 1.5. Schematic representation of transcriptional regulation of the type III genes in *Yersinia*. At 28°C, YmoA repress the transcription of *virF*. At 37°C, and in absence of contact between *Yersinia* and a eukaryotic cell, *virF* is transcribed. Transcription of all *yop* genes and some *ysc* genes requires the activator VirF. The plug, composed of the YopN, TyeA and LcrG proteins, prevents the release of LcrQ/YscM and of Yops. LcrQ/YscM accumulates inside the bacterial cytosol and indirectly represses *yop* transcription. At 37°C, upon contact between *Yersinia* and the eukaryotic cell, the *yop* and *ysc* genes are transcribed allowing for the injection of Yops into the cytosol of the target cell. The release of LcrQ/YscM amplifies *yop* transcription. The subcellular localization of TyeA is not clear (reproduced from Marenne *et al.*, 2004).

1.2.3.5 Replication and partition of the pYVe plasmid

The current classification scheme of plasmids is based on RepA, which is the product of the replication initiator gene, *repA* (del Solar *et al.*, 1998; del Solar & Espinosa, 2000; Sesma *et al.*, 2000). Plasmids which share one or more elements in their replication or partition systems are classed in the same incompatibility (Inc) group. Incompatibility is defined as the inability of two plasmids to coexist in the same cell in the absence of external selection (Lyon *et al.*, 2000) and is an indicator of relatedness (Couturier *et al.*, 1988). Replicons from a number of different incompatibility groups have been sequenced and characterised based on DNA and protein homologies as well as the shared use of counter transcript RNA (ctRNA) to control replication (Praszkier *et al.*, 1991).

In the pYVe plasmid of *Y. enterocolitica* 8081, *repA* is encoded on bp 66548 to 67570. The predicted 340 amino acid long protein was 54% identical over 96% of its length to RepA of pMU407.1, a naturally occurring conjugative plasmid belonging to the IncL/M group (Davey *et al.*, 1984). However, no homologues of *repA* were found in pCD1 (virulence plasmid of *Y. pestis*), pIB1 (virulence plasmid of *Y. pseudotuberculosis*, or pYVe227 (pYVe plasmid of a *Y. enterocolitica* serotype O:9) in the GenBank database. The sequence of *Y. enterocolitica repA* appears to be representative of *Y. enterocolitica* serotype O:8 plasmids (Snellings *et al.*, 2001). *repB* homologues upstream of *repA* were also identified which encode small leader peptides involved in the regulation of *repA* expression which were 55% identical over 94% of its length to *repB* from IncL/M plasmid pMU407.1. The *repB* nonsense codon (TAA) overlapped the *repA* start codon, suggesting translational coupling of these two genes, as has been observed previously for IncL/M plasmids (Athanasopoulos *et al.*, 1995). A homologue of *repC* gene is located in the corresponding position in the pMU407.1 replicon. However, RepC was only 36% identical over 78% of its length to RepC encoded on pMU407.1. Both RepB and RepC appear to be involved in the control of *repA* expression in their respective replication systems (Cornelis *et al.*, 1991; Athanasopoulos *et al.*, 1995; Perry *et al.*, 1998).

In contrast to the replication region, the partitioning loci encoded in pYVe plasmid have homologous counterparts on pCD1 (Perry *et al.*, 1998) and pYVe227 (Iriarte *et al.*, 1995). The partitioning (*par*) locus is located at bp 42557 to 45058 on the pYVe and showed 98% nucleotide identify to the *sopABC* locus in pCD1 and approximately 97% nucleotide identity to the *spyABC* locus in pYVe227. The high

DNA similarity of the virulence plasmid partition systems is further evidence that they have a common evolutionary origin and suggests that these systems have not diverged significantly since they were acquired (Snellings *et al.*, 2001).

Another putative partition gene was identified in the pYVe sequence. *orf79*, located on bp 51244 to 51873, encode a 211 amino acid long partition protein which is 26% identical to an *Actinobacillus actinomycetemcomitans* partition protein. The *Actinobacillus* protein is a Walker-type ATPase from a type 1B partition locus. Type 1B partition loci are found in Gram-positive and Gram-negative bacteria (Gerdes *et al.*, 2000). The finding of a remnant of a broad-host-range plasmid, specifically a protein associated with partitioning, suggests that the pYVe plasmid may have been generated by cointegration of distinct plasmids during its evolution (Snellings *et al.*, 2001).

1.3 Quorum sensing

1.3.1 Introduction

Bacteria were for a long time thought to be primitive cells that sought primarily to find nutrients and to multiply independently of other members of their species (de Kievit & Iglewski, 2000, Donabedian, 2003). This simplistic view was altered with the growing appreciation of the phenomenon of quorum sensing (QS). The discovery of QS led to the realisation that bacteria are capable of coordinated activity to facilitate their adaptation to changing environmental conditions, which was once believed to be restricted to multicellular organisms. In essence, QS allows bacteria to behave as multicellular organisms and to reap the benefits that would be unattainable to them as individuals (Schauder & Bassler, 2001).

QS is a bacterial cell-to-cell communication process which relies upon the interaction of a small diffusible signal molecule (autoinducers) with a sensor or transcriptional activator to couple gene expression with cell population density (Withers, 2001). At a threshold level when the bacterial population is considered quorate, the QS signal molecules will alter the expression of target genes to control a diverse array of physiological processes including bioluminescence, pigment production, symbiosis, swarming, swimming and sliding motility, cell division, polysaccharide or antibiotic biosynthesis, plasmid conjugation, sporulation, genetic competence, biofilm differentiation, plant-microbe interaction, and the production of virulence determinants (For reviews, see Fuqua *et al.*, 1996; Swift *et al.*, 1996b; Cámara *et al.*, 2002; Smith *et al.*, 2006; Parker & Sperandio, 2009).

The best-studied mechanisms of QS are found in the gram-negative proteobacteria, which use small extracellular signalling molecules known as AHLs (*N*-acyl-homoserine lactones) (Fuqua *et al.*, 1996; Greenberg, 1997; Withers *et al.*, 2001). Some gram-positive bacteria communicate using oligopeptide-based molecules, named autoinducer proteins (AIPs), as signalling molecules (Dunny & Winans, 1999).

1.3.2 Diversity of QS signal molecules

QS signal molecules are chemically diverse and the detection of these chemically distinct autoinducers, and the resulting alteration in gene expression, is specific to each system (Parker & Sperandio, 2009). Besides AHLs, 2-alkyl-3-hydroxy-4-quinolones (AHQs), long-chain fatty acid derivatives such as diffusible signal factor (DSF) and fatty acid methyl esters have been documented as signal molecules in Gram-negative bacteria. In Gram-positive bacteria such as the staphylococci, streptococci and *Streptomycetes*, the autoinducing polypeptides (AIPs) which include linear, modified or cyclic peptides and γ-butyrolactones such as A-factor are used as signalling molecules (reviewed by Williams, 2007).

There may also be a generalised signalling system found in both Gram positive and Gram negative bacteria, which utilises dihydroxypentandione (DPD) derivatives collectively termed autoinducer 2 (AI-2) (Bassler, 2002). There is also recent evidence that QS signalling is not restricted to bacterial cell-to-cell communication, but also allows communication between microorganisms and their hosts *via* a class of molecules which may act like hormones (eukaryotic cell-to-cell signalling molecule), termed autoinducer-3 (AI-3), which is distinct from AI-2. However, the structure and synthesis of this putative signal is unclear (Sperandio *et al.*, 2003; Hughes & Sperandio, 2008).

Here, the four main categories of cell-to-cell signalling systems characterised so far will be discussed: the AHLs and AI-3 systems found in Gram negative bacteria; the AIP system in Gram-positive bacteria and the fourth system, the AI-2 system, which is found in both Gram-positive and Gram-negative bacteria. Figure 1.6 shows three autoinducing strategies employed by Gram-negative cells, AHLs, AI-2 and AI-3. Figure 1.8 shows the AIP-based QS system in Gram-positive bacteria.

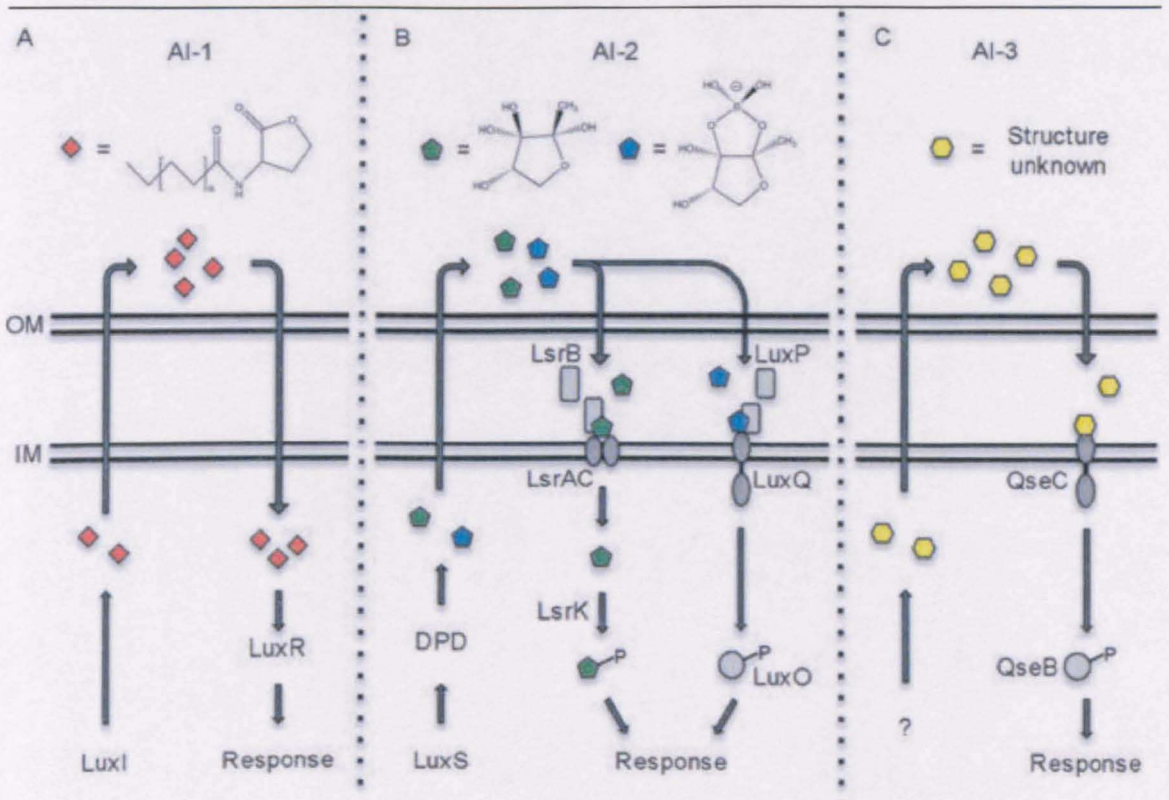


Figure 1. 6. Three autoinducing strategies in Gram-negative cells. A. AHL-based strategy. AHLs (red diamonds) are synthesised *via* LuxI and released into the environment. As the AHLs diffuse back into the cell, they interacts with LuxR-type protein resulting in a cellular response. B. AI-2-based strategy using either R-THMF (green pentagons) or furanosyl borate diester (blue pentagons), both derived from LuxS-derived DPD. In *Vibrio* spp., furanosyl borate diesters interact with LuxP in the periplasm and initiate the LuxQ/LuxO phosphoryl cascade, but in other organisms R-THMF is imported into the cell and phosphorylated by the Lsr system. C. AI-3-based strategy using an autoinducer of unknown structure (yellow hexagons). AI-3 is released into the environment and sensed by QseC as it diffuses back into the periplasm. QseC then phosphorylates the response regulator QseB (reproduced from Parker & Sperandio, 2009).

1.3.2.1 AI-1/LuxIR-based systems

The earliest reported bacterial AHL-dependent cell-to-cell communication system was identified in the bioluminescent symbiotic marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970). When free-living in seawater (i.e., at low population densities), *V. fischeri* is non-luminescent. However, when grown to high cell densities in the laboratory, a *V. fischeri* culture bioluminesces with a blue-green light. This *in vitro* condition is analogous to the autoinduction of bacterial luciferase gene when *V. fischeri* occurs at very high densities (10^{10} to 10^{11} cells per ml) in light organs of certain fish and squid in a symbiotic relationship (Whitehead *et al.*, 2001). QS allows *V. fischeri* to sense its elevated density in the light organ and express the luminescence system where it is required for symbiosis (Greenberg, 1997; Bourgois *et al.*, 2001).

The bioluminescence gene cluster of *V. fischeri* consists of eight *lux* genes (*luxA-E*, *luxG*, and *luxR*) arranged in bi-directionally transcribed operons (Engebrecht & Silverman, 1984). The *luxCDABE* operon encodes the proteins responsible for catalyzing the bioluminescence reaction. LuxI is the *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) molecule generator and LuxR is the transcriptional regulator (Eberhard *et al.*, 1981). As a population of *V. fischeri* cells grow, the concentration of the external AHL increases as a function of cell population density. When the AHL concentration reaches a threshold level, the AHL interacts with LuxR, and the LuxR-AHL complex binds the luciferase promoter and activates transcription of the *luxCDABE* operon. This QS regulatory circuit allows light production to be tightly correlated with cell population density (Kolibachuk & Greenberg, 1993).

Over 70 species of proteobacteria communicate by using homologous LuxR-LuxI signalling circuits as QS systems to control gene expression (Taga & Bassler, 2003). LuxI-type proteins generate AHLs. The homoserine lactone moiety in AHLs is derived from *S*-adenosyl methionine (SAM) (Moré *et al.*, 1996; Jiang *et al.*, 1998) and the *N*-acyl chains, which range in length from 4 to 14 carbons (mostly even-numbered), may be saturated or unsaturated, may or may not contain a C_3 hydroxy or oxo group and are provided *via* the appropriately charged acyl–acyl carrier protein (acyl-ACP) or acyl–coenzyme A (acyl-CoA) (Withers *et al.*, 2001). Figure 1.7 shows the structure of a generic AHL structure. AHLs with odd-numbered *N*-linked acyl chains have been identified only recently as *N*-heptanoyl-L-homoserine lactone (C7-HSL) in *Rhizobium leguminosarum* (Lithgow *et al.*, 2000) and in *Y. pseudotuberculosis* (Atkinson, 1999; Atkinson *et al.*, 2004). LuxR-type proteins can be subdivided into two functional

domains, an AHL-binding domain (N terminal) and a DNA binding domain (C terminal) and they are able to discriminate between cognate and non-cognate AHLs, suggesting that they are molecule-specific (Fuqua *et al.*, 1996).

A DNA element known as the *lux* box, situated within the intergenic region between *luxR* and *luxI*, is required for LuxR-dependent induction of the luminescence genes (Egland & Greenberg, 1999). This DNA element is a 20 nucleotide inverted repeat and has features that are conserved within binding sites of other LuxR-type proteins (Stevens & Greenberg, 1997; Stevens & Greenberg, 1999). In *P. aeruginosa*, *lux* box-like elements have been identified in the promoter regions of many different target structural genes (Whiteley & Greenberg, 2001). Purified LuxR proteins such as TraR (from *A. tumefaciens*) and ExpR (from *Erwinia chrysanthemi*) have been shown to bind *in vitro* to *lux* box-type sequences (Nasser *et al.*, 1998; Reverchon *et al.*, 1998; Zhu & Winans, 1999).

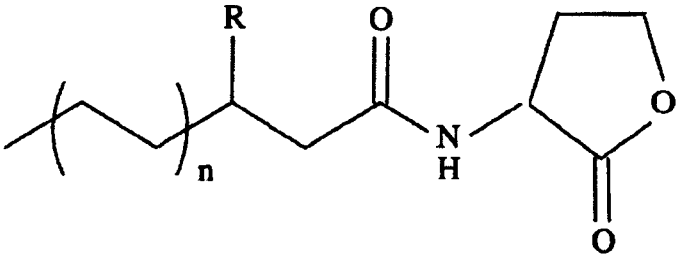


Figure 1.7. Representation of a generic *N*-Acyl homoserine lactone with n representing the *N*-acyl chain (range in length from 4 to 14 carbons) and R may be unsubstituted or represent a hydroxy or oxo group (adapted from Dong & Zhang, 2005).

1.3.2.2 AI-2/LuxS-based system

AI-2 signalling was first described in *Vibrio harveyi*. This signal is different from the AHLs described previously and is synthesised from a by-product of SAM metabolism. LuxS (AI-2 signal molecule generator) converts *S*-ribosehomocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), a compound that, in the presence of water, cyclises into several related furanones including (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF-borate) (Surette *et al.*, 1999; Schauder *et al.*, 2001). *S*-THMF-borate is the AI-2 used by *V. harveyi* to control luminescence.

AI-2 is released to the environment where it accumulates and is detected *via* its association with the autoinducer-specific binding protein, LuxP. This AI-2/LuxP complex is detected in the periplasm where it interacts with a sensor kinase, LuxQ, initiating a phospho-transfer cascade that results in luciferase production and luminescence *via* deactivation of LuxO, a response regulator (Figure 1.6B). AI-2 signalling has also been documented in *V. cholerae* (Schauder *et al.*, 2001), *Salmonella typhimurium* (Taga *et al.*, 2001) and enterohemorrhagic *E. coli* (EHEC) (Sperandio *et al.*, 1999; Sperandio *et al.*, 2001). Figure 1.8 shows the structures of the AI-2 molecules of *V. harveyi* and *S. typhimurium* (Bassler, 2002; Miller *et al.*, 2004).

The existence of an interspecies signalling system which is molecularly conserved throughout the bacterial kingdom is still debated. AI-2 based signalling has been suggested to play such a role (Bassler *et al.*, 1997; Bassler, 2002). It was speculated that because of the mixed nature of most natural bacterial communities, such as that found in the gastrointestinal tract of mammals, A1-2 might be used to regulate genes needed to survive in the presence of other bacteria and be used by pathogenic bacteria to activate genes that would allow it to gain an advantage in this same environment. However, to date, the function of LuxS as a QS signal molecule synthase has only been experimentally documented *V. harveyi*. Moreover, many bacteria (e.g. the pseudomonads) do not possess *luxS* and hence do not produce AI-2 (Winzer *et al.*, 2002a). In addition, LuxS is a key metabolic enzyme in the activated methyl cycle responsible for recycling SAM, which is also substrate for AHLs synthesis (Winzer *et al.*, 2002a; Winzer *et al.*, 2002b). Phenotypes associated with mutation of *luxS* are often not a consequence of a defect in cell-to-cell communication but the result of the failure to recycle SAM metabolites (Winzer *et al.*, 2002a; Winzer *et al.*, 2002b).

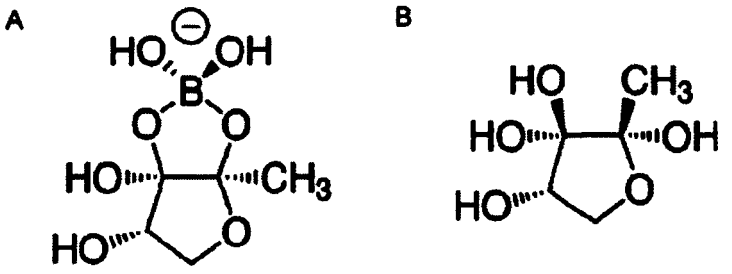


Figure 1.8. Structures of AI-2 molecules. A. (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxy tetrahydrofuran-borate (*S*-THMF-borate) produced by *Vibrio harveyi*. B. (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) produced by *Salmonella typhimurium* (reproduced from Bassler, 2002; Miller *et al.*, 2004).

1.3.2.3 AI-3/QseC system

AI-3 was first described as a compound found in spent media, distinct from AI-2, which activated the expression of genes involved in attaching and effacing by enterohaemorrhagic *E. coli* (EHEC) to host eukaryotic cells which subsequently causes actin rearrangement in the eukaryotic host cell (Sperandio *et al.*, 2003). The structure and synthesis of this signal is unclear and might represent a family of molecules that may act like hormones in eukaryotic cell-to-cell signalling. It was speculated that A1-3 might be a 'language' that allows communication between microorganisms and their hosts (Sperandio *et al.*, 2003; Hughes & Sperandio, 2008).

In the periplasm, the sensor kinase QseC undergoes autophosphorylation in the presence of AI-3 (Figure 1.6C) and then transfers this phosphate to QseB, a response regulator. QseB activates genes in flagella biosynthesis and motility by upregulating the master flagellar regulator, *flhDC* (Clarke & Sperandio, 2005). AI-3 is also linked to the formation of attaching and effacing (AE) lesions by EHEC, which is achieved through upregulation of five separate loci of enterocyte effacement (LEE) operons located within the EHEC chromosome (Sperandio *et al.*, 2003). The regulation of these genes is likely to involve QseA, a LysR-family regulator that is influenced by cell-to-cell signalling and directly upregulates LEE genes (Sperandio *et al.*, 2002). It has been proposed that enteropathogens might use AI-3 produced by the host's flora to gauge the suitability of the habitat and that upregulation of flagella and motility are needed to penetrate the mucosal lining of the colon in order to reach underlying epithelial cells. Once in contact with epithelial cells, proteins encoded by the LEE genes enable the pathogen to attach to the eukaryotic cells for the purpose of colonization (Parker & Sperandio, 2009).

Intriguingly, the QseBC cascade also responds to the hormone signals, norepinephrine and epinephrine, both found in the gastrointestinal tract (Clarke *et al.*, 2006). Norepinephrine and epinephrine are stress hormones underlying the 'fight-or-flight' response in animals, The hormones boost the supply of oxygen and glucose to the brain and muscles, while suppressing other non-emergency bodily processes, particularly digestion in the gut (Lovallo & Thomas, 2007). This suggests that QseC responds to bacterial and host signals simultaneously. Conversely, this presents the possibility that adrenergic receptors on eukaryotic cells might respond to AI-3 as well, and thus resulting in 'conversations' between enteropathogens and host cells (Parker & Sperandio, 2009).

1.3.2.4 AIP/Agr system

The AIP or Agr system is found exclusively in Gram-positive bacteria. It was first identified in *Staphylococcus aureus,* which uses cyclic peptide signals as autoinducers (Peng *et al.*, 1988; Ji *et al.*, 1995). Figure 1.9 shows a schematic representation of the AIP-based autoinduction and inhibition. AIP is derived from the AgrD propeptide which once translated, is targeted to the membrane by an N-terminal signal sequence, where AgrB (an endopeptidase) cleaves the AgrD C-terminus (Ji *et al.*, 1995). The N-terminus of AgrD is then removed by another peptidase, possibly SpsB (Jeffrey *et al.*, 2007). Finally, the C-terminus of the processed polypeptide is covalently linked to a conserved, centrally located cysteine to form a thiolactone ring with a free N-terminal tail (Ji *et al.*, 1997; Novick, 2003) (See Figure 1.10). After release into the environment, the AIP is recognised by a signal receptor, AgrC, which phosphorylates AgrA (a response regulator) (Novick *et al.*, 1995). Phosphorylated AgrA activates transcription of selected genes by binding to promoter regions (Rescei *et al.*, 1985; Novick *et al.*, 1995; Ji *et al.*, 1997).

AIP-directed regulation of genes by AgrA results in production and release of numerous toxins by *S. aureus* such as alpha-, beta- and delta-haemolysins, serine proteases and toxic shock syndrome toxin 1 (TSST-1) (Rescei *et al.*, 1985; Lindberg *et al.*, 1990). In addition, AIP is also responsible for downregulation of surface-exposed proteins such as fibronectin-binding protein and protein A, two proteins involved in Staphylococcal adhesion, causing a negative effect on the architecture of biofilm-associated communities (Lindberg *et al.*, 1990). As a result, the bacterial cells are released from their communities and are free to migrate to other locations within the host. Once a new, suitable location is found, the cell can reattach and begin multiplying again, possibly resulting in a secondary infections.

Agr polymorphism occurs in the staphylococci with *S. aureus* having 4 different specificity groups. Such polymorphism enables the AIP produced by one strain of *S. aureus* to activate its own system while simultaneously repressing that of other strains which can also play a role as inhibitors in other *S. aureus* strains (Ji *et al.*, 1997; Dufour *et al.*, 2002). Figure 1.10 shows the slightly different AIPs produced by different strains of *S. aureus*.

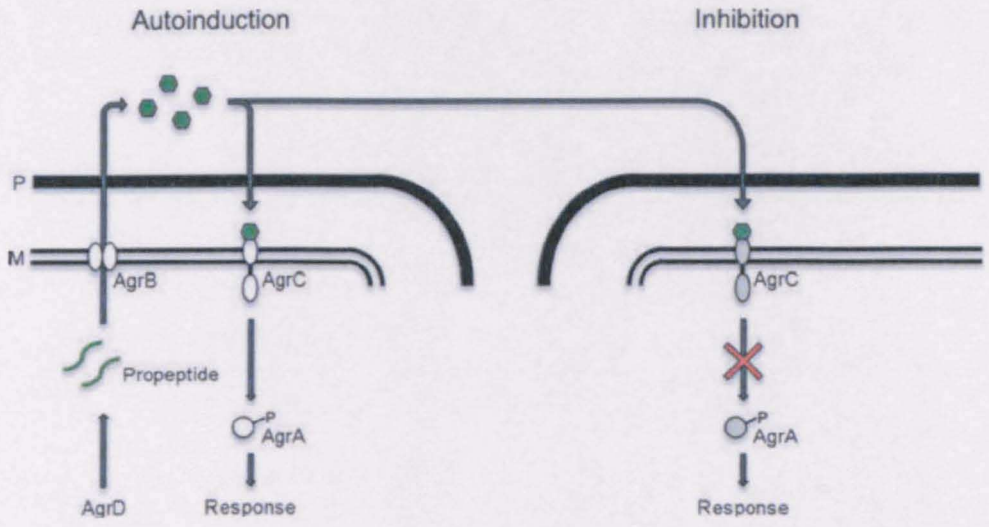


Figure 1.9. AIP-based autoinduction and inhibition in Gram-positive cells using polypeptides (green hexagons). Propeptide, encoded by AgrD, is processed by AgrB and released into the environment where it can either activate the AgrA/AgrC cascade or inhibit the same cascade of a different species. M, membrane; P, peptidoglycan. (reproduced from Parker & Sperandio, 2009).

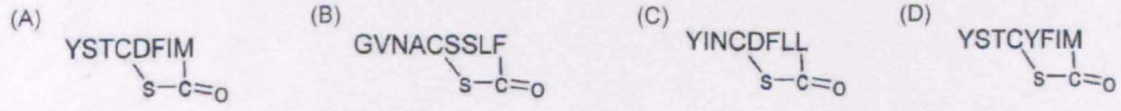


Figure 1.10. Polypeptide structures of AIPs produced by *Staphylococcus aureus*. A, B, C and D are produced by different subgroups of *S. aureus* (adapted from Bassler, 2002).

1.3.3 QS in Yersinia

AHL-dependent QS is highly conserved in both pathogenic and nonpathogenic members of the genus *Yersinia.* In all *Yersinia* species so far examined, the genes coding for LuxR and LuxI homologues are characteristically arranged convergently and overlapping by either 8 or 20 bp (Atkinson, 1999). *Y. pseudotuberculosis* has two *luxIR* homologues, *ypsIR* and *ytbIR*, and the *ypsIR* system is involved in the hierarchical regulation of the *ytbIR* locus (Atkinson *et al.*, 1999; 2008). *Y. pestis* also has two pairs of *luxRI* homologues, termed *ypeRI* and *yepRI* (Swift *et al.*, 1999a; Isherwood, 2001). Only one pair of *luxIR* homologues were previously described for *Y. enterocolitica*, which was named *yenIR* (Throup *et al.*, 1995) although it is now known that it possesses an additional *luxR* homologue named *ycoR* (this study).

1.3.3.1 QS in Y. enterocolitica

Throup *et al.* (1995) constructed a recombinant AHL reporter plasmid which coupled *luxR* and the *luxI* promoter region of *V. fischeri* to the *luxCDABE* structural operon of *Photorhabdus luminescens*. When introduced into *E. coli*, this *lux*-based AHL reporter (termed pSB401) is dark but responds to the presence of exogenous AHLs by emitting light. With this AHL reporter, cell free supernatants of several species of *Yersinia* including *Y. enterocolitica* serotypes O:3, O:8, O:9, O:10K and O:1(2a, 3) were screened (Throup *et al.*, 1995). For *Y. enterocolitica* 90/54 (serotype O:9), the types of AHLs present were identified by subjecting the isolated AHLs mixture to high-resolution tandem mass spectrometry (MS-MS) which revealed them to be two AHLs (~50:50 ratio) known as 3-oxo-C6-HSL and C6-HSL.

A plasmid-based *in trans* complementation strategy was used to detect the presence of an AHL synthase in *Y. enterocolitica* 90/54 (Throup *et al.*, 1995). This approach yielded two convergently transcribed genes with overlapping open reading frames (ORFs) which were subsequently cloned and shown to produce both 3-oxo-C6-HSL and C6-HSL. The first, termed *yenI*, when translated exhibited homology to the LuxI protein family; while the second, termed *yenR* belongs to the LuxR protein family. TLC using the CV026 biosensor showed that inactivation of the *yenI* by deletion mutagenesis abolished AHL production in *Y. enterocolitica*, thus confirming the function of *yenI* as an AHL synthase (Throup *et al.*, 1995).

AHLs are often referred to as 'autoinducers', implying a positive feedback or autoregulatory mechanism of action. However, the generic use of this term can be misleading because this is not the case for *Y. enterocolitica* (Cámara *et al.*, 2002, Atkinson *et al.*, 2004). In *Y. enterocolitica* 90/54, *yenI* is not subject to autoinduction but is expressed constitutively since inactivation of *yenI* did not abolish production of *yenI* mRNA (Throup *et al.*, 1995). This observation was confirmed by examining the expression of *yenI::luxAB* transcriptional fusions as chromosomal insertions in both the wildtype and *yenI* mutant respectively (Throup *et al.*, 1995).

The swimming motility of a *yenI* mutant in *Y. enterocolitica* 90/54 is temporally delayed while swarming motility is abolished (Atkinson *et al.*, 2006). Since both swimming and swarming are flagellar-dependent, Atkinson *et al.* (2006) purified the flagellin protein from the wildtype and *yenI* mutant. SDS-PAGE revealed that in contrast to the wildtype, the *yenI* mutant grown for 17 h at 26°C lacked the 45 kDa flagellin protein, FleB. RT-PCR indicated that while mutation of *yenI* had no effect on *flhDC* or *fliA* expression, *fleB* was down regulated suggesting that motility in *Y. enterocolitica* is, at least in part, QS-dependent at the level of *fleB* transcription. The exogenous provision of AHLs either individually or in combination to the *yenI* mutant did not restore swimming or swarming motility (Atkinson *et al.*, 2006).

On re-examination of the *Y. enterocolitica* 90/54 AHL profile using AHL biosensors capable of detecting both short and long chain AHLs, Atkinson *et al.* (2006) discovered that in addition to short chain AHLs, *Y. enterocolitica* 90/54 also synthesises three long chain AHLs which were identified using HPLC as 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL. It was determined that the inability of exogenous AHLs to restore motility to the *yenI* mutant was not due to a lack of AHL-uptake, as demonstrated using radiolabelled AHLs. However, both AHL synthesis and motility could be restored by complementation of the *yenI* mutant with a plasmid borne copy of *yenI*. However, the contribution of YenR to swimming and swarming in *Y. enterocolitica* 90/54 could not be studied because a *yenR* mutant strain could not be constructed, despite repeated attempts (Atkinson *et al.*, 2006).

1.3.3.2 QS in Y. pseudotuberculosis

Recently, a method for the comprehensive profiling of the AHLs using liquid chromatography (LC) coupled to hybrid quadrupole–linear ion trap (QqQLIT) mass spectrometry revealed that *Y. pseudotuberculosis* YPIII produces 24 different AHLs with acyl chains ranging from C4 to C15 with or without 3-oxo or 3-hydroxy substituents (Ortori *et al.*, 2007). Only six of these AHLS had been previously documented (Buckley, 2002). The LC-QqQLIT technique was used to determine the AHLs produced by *Y. pseudotuberculosis* lacking either or both of the AHL synthase genes (*ypsI* and *ytbI*). In the *ypsI/ytbI* mutant, AHL synthesis was abolished. YtbI was found to be able to direct the synthesis of the full range of AHLs whereas YpsI was only responsible for producing primarily 3- oxo-C6-HSL, and at lower levels, C6-HSL and 3-oxo-C7-HSL. AHL production was unaffected in a *ypsR/ytbR* double mutant but substantially reduced in a *ypsI/ytbR* mutant, indicating that neither YpsR nor YtbR is essential for AHL synthesis (Ortori *et al.*, 2007). When compared with the parent strain, the *QS* mutants of YPIII exhibit a number of phenotypes, including clumping (*ypsR* mutant), overexpression of a major flagellin subunit (*ypsR* mutant) and increased motility (both *ypsR* and *ypsI* mutants) (Atkinson *et al.*, 1999).

Using chromosomal *lux*-promoter fusions to *ypsR*, *ypsI*, *ytbR* and *ytbI*, the interrelationship between YpsRI and YtbRI were determined. The YpsRI system negatively autoregulates itself but positively regulates the expression of the *ytbRI* system whereas the *ytbRI* system is positively autoregulated but only at the level of *ytbI* expression. YtbRI does not control expression of *ypsR* or *ypsI* (Atkinson *et al.*, 2008). However, a subsequent re-examination revealed that the YpsRI system actually positively autoregulates itself (Patrick, H. L.; personal communication)

Analysis of the regions upstream of the translational start sites of *ypsI*, *ypsR*, *ytbI* and *ytbR* revealed the presence of *lux* box-like consensus sequences in the promoters. Potential *lux* boxes in the promoter regions of the *flhDC* and *fliA* genes were also found. Again using chromosomal *lux*–promoter fusions strategy, the impact of QS on *flhDC* and *fliA* expression was studied. It was found that the hierarchical QS system of *Y. pseudotuberculosis* controls swimming motility *via* regulation of *flhDC* and *fliA*. The AHLs synthesised *via* YtbI play a dual role, activating *flhDC* in conjunction with YpsR but repressing *fliA* in conjunction with YtbR and YpsR. In liquid and plate assays, the early onset of motility observed in *ypsR* and *ypsI* mutants was abolished in *ytbI*, *ytbR ypsI/ytbI* and *ypsR/ytbR* mutants, indicating that QS regulates motility both

positively (*via* YtbRI) and negatively (*via* YpsRI). Figure 1.11 shows a simplified model for the regulatory cascade in *Y. pseudotuberculosis* (Atkinson *et al.*, 2008).

Darby *et al.* (2002) showed that *Y. pseudotuberculosis* could form biofilms on *Caenorhabditis elegans*, a free-living nematode found in the soil (Darby *et al.*, 2002). When *C. elegans* was exposed to *Y. pseudotuberculosis* or *Y. pestis*, biofilms developed on the head of the nematodes within 1 h. The biofilms increase in size with continuing exposure, eventually covering the mouth completely and blocking food intake (Darby *et al.*, 2002). Atkinson *et al.* (unpublished data) recently showed that QS is involved in biofilm formation by *Y. pseudotuberculosis* on *C. elegans* by introducing a green fluorescence protein-based (GFP) AHL biosensor pJBA89 (Andersen *et al.*, 2001) into YPIII. Biofilms of *Y. pseudotuberculosis* on *C. elegans* fluoresce green indicating that the bacteria within the biofilm matrix are actively synthesising AHLs (Atkinson *et al.*, unpublished data). When the gene coding for the AHL-inactivating lactonase, *aiiA*, was introduced into *Y. pseudotuberculosis*, the severity of biofilm infection on *C. elegans* was markedly reduced (Atkinson *et al.*, unpublished data).

An analysis of QS mutants revealed that *ypsI* and *ypsR* mutants produced slightly reduced biofilms at the anterior end and worms exhibited exaggerated body bends but were unable to translocate within 1.5 h and had become moribund by 5 h. The worms also exhibited aberrant movement on *Y. pseudotuberculosis* lawns as revealed by the characteristic tracks on the agar surface which contrast those made by worms grown on *E. coli* OP50. The *ypsI/ytbI* and *ypsR/ytbR* double mutants formed very little biofilm when compared with the wildtype. Single *ytbI* and *ytbR* mutants also produced small but visible biofilms on the worm surface. However, a *fliC* mutant did not show any difference in disease severity of *C. elegans* compared with the wildtype *Y. pseudotuberculosis* strain. This indicates that neither motility nor flagella filaments are required for forming biofilms on *C. elegans* by *Y. pseudotuberculosis*. However, mutation of the *Y. pseudotuberculosis* motility master regulator, *flhDC* resulted in a non-motile strain severely impaired in its ability to form biofilms on *C. elegans* which could be restored by a genetic complementation with a plasmid-borne copy of *flhDC* (Atkinson *et al.*, unpublished data). These data indicate that FlhDC but not flagellar-mediated motility is essential for biofilm formation on *C. elegans* and that the biofilm phenotype is mediated *via* FlhDC functioning as a global regulator rather than specifically as an activator of motility genes although the identity of the QS/FlhDC target genes involved remain to be elucidated.

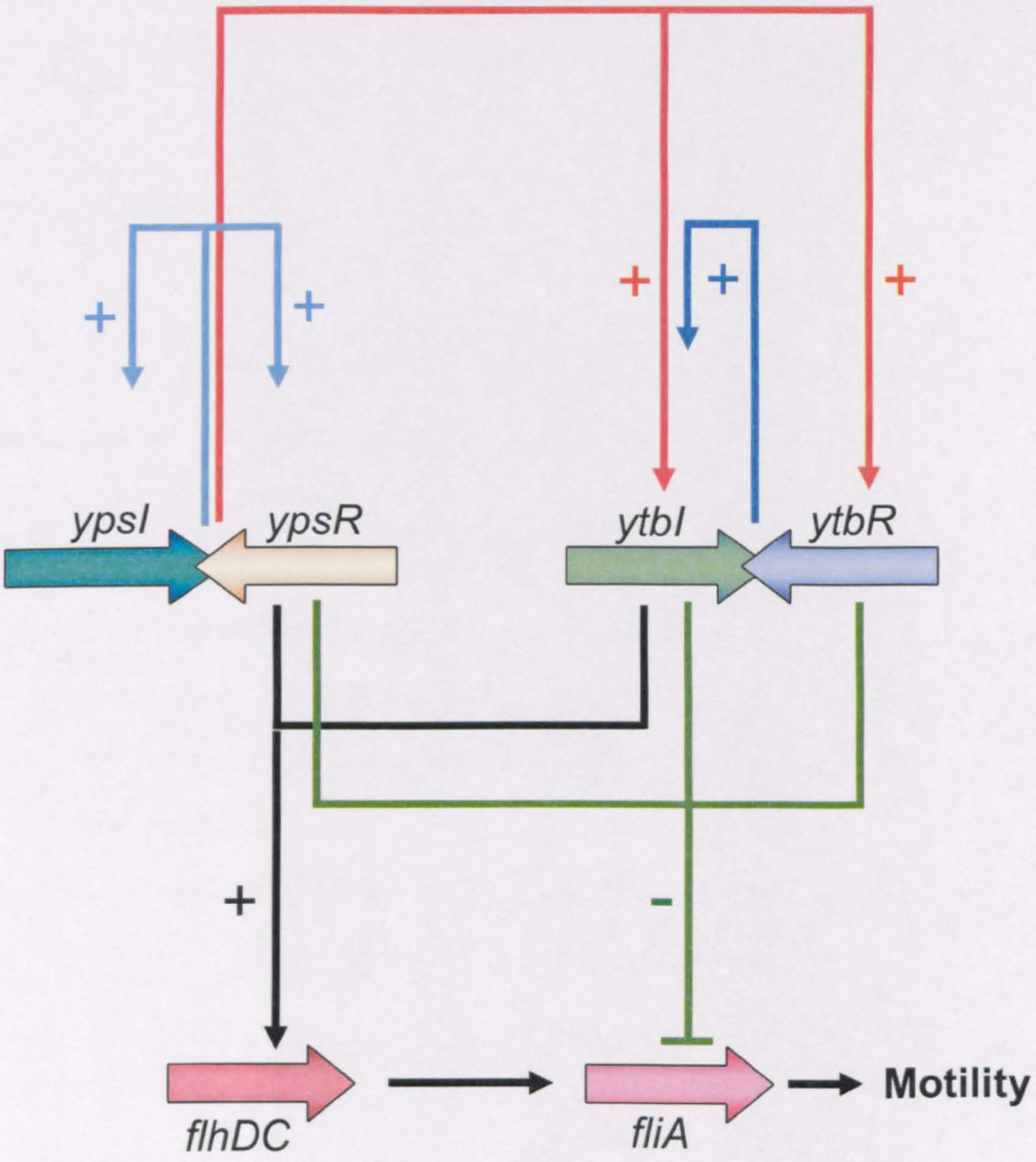


Figure 1.11. A simplified model for the regulatory cascade linking the *ypsRI* and *ytbRI* QS systems to *flhDC*, *fliA* and swimming motility in *Y. pseudotuberculosis*. Lines ending with arrowheads indicate activation, those ending as a dash indicate repression. YpsR/I positively autoregulates and activates the expression of *ytbR/I* while YtbRI positively autoregulates *ytbI* expression. Both *flhDC* and *fliA* expressions are under the dual control of the YpsRI and YtbRI systems. YpsR and AHLs produced via YtbI are required for positive regulation of *flhDC* expression. Both YpsR and YtbR, in conjunction with AHLs produced via YtbI, are required for *fliA* repression (adapted from Atkinson *et al.*, 2008; Patrick, H. L., personal communication).

1.3.3.3 QS in Y. pestis

As discussed in Section 1.1, *Y. pestis* is a clone of *Y. pseudotuberculosis* and this similarity extends to the QS system where two pairs of *luxRI* homologues, termed *ypeRI* and *yepRI* were characterised (Swift *et al.*, 1999a; Isherwood, 2001). *Y. pestis* produces predominantly 3-oxo-C8-HSL and 3-oxo-C6-HSL, as well as C6-HSL, C8-HSL and 3-oxo-C10-HSL (Swift *et al.*, 1999a; Kirwan *et al.*, 2006). YpeI (which is more closely related to YpsI and YenI than YtbI), directs the synthesis of C6-HSL and 3-oxo-C6-HSL and YepI is responsible for C8-HSL synthesis.

Y. pestis has not been observed to be motile even though the genome contains one full set of flagella genes plus a second partial set (Parkhill *et al.*, 2001). Isherwood (2001) corroborated this immotility by incubating a *Y. pestis* strain on 3% (w/v) LB agar at room temperature, 28°C and 37°C for 72 h. Despite the observation that QS is a key regulator of motility in both *Y. pseudotuberculosis* and *Y. enterocolitica*, mutation of both *ypeI* and *ypeR* in *Y. pestis* did not affect its non-motile phenotype (Isherwood, 2001). Analysis of *Y. pestis* virulence gene expression in *ypeI* and *ypeR* mutants grown at 28°C and 37°C did not reveal any differences to V-antigen, pH 6 (an adhesin), the coagulase/fibrinolytic protein Pla or lipopolysaccharide profile compared to wildtype (Swift *et al.*, 1999a). However, in a mouse infection model, a Δ*ypeR* strain showed an slower death rate for mice challenged with the mutant when compared to wildtype, therefore implying a possible role for QS in the pathogenicity of *Y. pestis* infections (Swift *et al.*, 1999a).

Recently, a protein microarray that consists of virulence-associated proteins of *Y. pestis* was used to compare antibody profiles elicited by the wildtype and a QS mutant strain (Δ*ypeRIyepRI*) (Chen *et al.*, 2006). The antibody profile that was induced in the sera of rabbits immunised with the QS mutant differed from that of the wildtype. It was shown that QS affects the expression of many virulence associated proteins of *Y. pestis*, which is in disagreement with an earlier study by Swift *et al.* (1999a). These virulence proteins, including F1 (a protective antigen), LcrV (putative V antigen and antihost protein/regulator), KatY (a catalase-peroxidase) and pH6 (antigen precursor and adhesin) were not expressed or expressed at relatively lower levels in the QS mutant (Chen *et al.*, 2006).

1.4 Objective and aims

Although an initial study has been published documenting the existence of a QS system in *Y. enterocolitica*, the exact conservation of this system in the species remains unknown. Much is still not clear on how this QS system is regulated and what biological functions and virulence factors it controls.

The objective of this project was to increase our understanding of the QS system of *Y. enterocolitica*. The work described in this study was primarily done using the fully sequenced 8081 strain. The advantage to this was that it allowed detailed *in silico* genomic/genetic studies of the bacterium and therefore aid the advancement of this study. In order to achieve the objective, the aims of following work were:

- to elucidate any additional component gene(s) of the QS system in *Y. enterocolitica* by *in silico* studies
- to construct mutants in the QS genes; *yenI*, *yenR* and any additional QS component(s)
- to conduct a species-wide study of the QS systems in a diverse collection of *Y. enterocolitica* strains
- to study the AHL profiles of the *Y. enterocolitica* wildtype strain and QS mutants *via* thin-layer-chromatography and mass spectrometry
- to study the transcriptome profiles of *Y. enterocolitica* wildtype and QS mutants and identify the QS regulon and the role of each QS gene
- to investigate phenotype and virulence regulation by the QS system using the constructed QS mutants

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

All bacterial strains used in the study are listed in Table 2.1.

Table 2.1. Bacterial strains used in this study

Strain	Genotype/Characteristics	Reference/Source
Escherichia coli:		
DH5α	F'/*endA1*, *hsdR17*(R- M+), *supE44*, *thi-1*, *recA1*, *gyrA*, *relA1*, Δ(*lacZYA-argF*)*U169*, *deoR*[Φ80d *lac*Δ(*lacZ*)M15]	(Hanahan, 1983)
JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ(*lac-proAB*), *mcrA*, [*F' traD36 proAB laclq lacZ ΔM15*]	(Yanisch-Perron *et al.*, 1985)
S17-1	λ*pir*, *recA*, *thi*, *pro*, *hsdR-M+*, RP4-2-Tc::Mu-Km::Tn*7*	(Miller, 1988)
CC118	λ*pir*, Δ(*ara-leu*), *araD*, Δ*lac*X74, *galE*, *galK*, *pho*A20, *thi*-1, *rps*-1, *rpoB*, *argE*(Amp), *recA*, *thi*, *pro*, *hsdR-M+*, RP4-2-Tc::Mu-Km::Tn*7*	(Miller & Mekalanos, 1988)
E. coli pSB1075	bioluminescent reporter strain sensitive to long chain AHLs (C10 to C14)	(Winson *et al.*, 1998a)
Yersinia enterocolitica:		
90/54	Wild-type, serotype O:9, isolated in the UK	(Throup *et al.*, 1995)
90/54 Δ*yenI*	*yenI* deletion/insertion mutant, Km^R	(Throup *et al.*, 1995)
8081	Wild-type, serotype O:8, biotype 1B, pYV^+, sequenced strain	(Portnoy *et al.*, 1981)

8081 Δ*yenI*	*yenI* chromosomal deletion mutant derived from 8081, Cm^R	This study
8081 Δ*yenR*	*yenR* chromosomal deletion mutant derived from 8081, Km^R	This study
8081 Δ*ycoR*	*ycoR* chromosomal deletion mutant derived from 8081, Sm^R	This study
8081 Δ*yenR*Δ*ycoR*	*yenR* and *ycoR* chromosomal deletion mutant derived from 8081, Km^R, Sm^R	This study
8081 Δ*yenI*Δ*yenR*Δ*ycoR*	*yenI, yenR and ycoR* chromosomal deletion mutant derived from 8081, Cm^R, Km^R, Sm^R	This study
Chromobacterium violaceum:		
C. violaceum	Wild type, ATCC 31532	American Type Culture Collection (ATCC)
CV026	Tn*5* insertions in *civI* and a putative repressor locus derived from *C. violaceum* wild type, addition of external AHL activates the QS-controlled pigment production	(McClean *et al.*, 1997)

2.2 Cell lines

Table 2.2. Cell line used in this study

Strain	Genotype/Characteristics	Reference/Source
Caco-2	Human colonic adenocarcinoma cell line	(Fogh *et al.*, 1977)

2.3 Plasmids

All plasmids used in the study are listed in Table 2.3.

Table 2.3. Plasmids used in this study

Name	Description	Reference/Source
pBluescript KS^+	ColE1 based replicon for cloning (Amp^R)	Stratagene
pGEM-t easy	f1 based replicon for cloning (Amp^R)	Promega Corp.
pSU18	General vector for cloning (Amp^R)	(Martinez *et al.*, 1988)
pSU18::*yenI*	pSU18 vector carrying *yenI* (Amp^R)	(Atkinson *et al.*, 2006)
pAJD434	Red recombinase expression plasmid used in gene mutagenesis (Tp^R)	(Maxson & Darwin, 2004)
pDM4	Suicide vector carrying the *sacBR* genes for sucrose sensitivity, (Cm^R)	(Milton *et al.*, 1996)
pUC4K	Source of Km^R cassette amplification	(Yanisch-Perron *et al.*, 1985)
pACYC184	Source of Cm^R cassette amplification	(Chang & Cohen, 1978)
pHP45Ω	Source of Sm^R cassette amplification	(Prentki & Krisch, 1984)
pBluelux	A promoter-less *luxCDABE* cassette cloned into a SmaI site at the multiple cloning site of pBluescript KS^+	(Atkinson *et al.*, 2008)
pYK800	P_{virF}::*luxCDABE* cassette cloned into ApaI and NotI sites of pDM4	This study
pYK801	P_{spyA}::*luxCDABE* cassette cloned into ApaI and NotI sites of pDM4	This study

2.4 Oligonucleotide primers

All Oligonucleotide primers were synthesised by Sigma-Genosys Biotechnologies, Cambridge, UK. All the primers used in this study are listed in Table 2.4.

Table 2.4. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')	Function
yenRf	GTGAGGATATGTTATACC	Amplification of *yenR* gene for cloning into pGEM-T easy
yenRr	GAGAGTACATCAGGTTG	Amplification of *yenR* gene for cloning into pGEM-T easy
yenIf	CTGCACTCGCTAAGTCTC	Amplification of *yenI* gene for cloning into pGEM-T easy
yenIr	CCAAGCACGCAATAAGG	Amplification of *yenI* gene for cloning into pGEM-T easy
ycoRf	GGATTTTATTAAGGAGGTG	Amplification of *ycoR* gene for cloning into pGEM-T easy
ycoRr	CCAAGTAAGGGAGCATAG	Amplification of *ycoR* gene for cloning into pGEM-T easy
ycoRflankF	CACAATCTCACTCAAGGC	Amplification of the flanking region of *ycoR* gene for cloning into pGEM-T easy
ycoRflankR	GCGTATCCAGATCCATC	Amplification of the flanking region of *ycoR* gene for cloning into pGEM-T easy
yenRKan-f	AATTGTATTGTTACATTATA CACAGAGTAGAATTGGCCTA TTATGATAATTGAAAGCCAC GTTGTGTCTCAA	Amplification of Km^R cassette with flanking region of *yenR* gene for mutagenesis
yenRKan-r	AAGTTTCAACTCTATGCCAA GCCTTATTGCGTGCTTGGCA TTTAAAACACCTTAGAAAAA CTCATCGAGCAT	Amplification of Km^R cassette with flanking region of *yenR* gene for mutagenesis
yenICm-f	CGTGTACGATGTTGTTTTAA	Amplification of Cm^R cassette

	TTAAATAACTTTGGTTTTTAT TATGTTAAAAGTTGATCGGC ACGTAAGAGGT	with flanking region of *yenI* gene for mutagenesis
yenICm-r	AGCACGCAATAAGGCTTGG CATAGAGTTGAAACTTATTA AACCTATTTAATTTACGCCC CGCCCTGCCACTC	Amplification of Cm^R cassette with flanking region of *yenI* gene for mutagenesis
ycoRSm-f	CTCGAAAAATACAGAAAAA TCAGATATGCATATGCAATA ATGAATAAGAGAGTTTTCAT GGCTTGTTATGAC	Amplification of Sm^R cassette with flanking region of *ycoR* gene for mutagenesis
ycoRSm-r	GGATGGATCAAGAAAACAC TTGGCCATTATCTTTGTATA CTAGGAATAAACTTATTTGC CGACTACCTTGGT	Amplification of Sm^R cassette with flanking region of *ycoR* gene for mutagenesis
dnaE-f	CCACCGGACAGGTCAGCTT	Amplification of *dnaE* gene for RQ-PCR analysis as a control gene
dnaE-r	AACTCACGGGCGGTCATTT	Amplification of *dnaE* gene for RQ-PCR analysis as a control gene
virF-f	GGCAACCGCCCAGAAGA	Amplification of *virF* gene for RQ-PCR analysis
virF-r	CACCCTTGTAGATAATTTTC CTCCAT	Amplification of *virF* gene for RQ-PCR analysis
yscF-f	GGTGGCTCAAACGCTCAAG A	Amplification of *yscF* gene for RQ-PCR analysis
yscF-r	CTATCGAGTCATTAACCGCT TTGTT	Amplification of *yscF* gene for RQ-PCR analysis
yadA-f	CATTGCGGTTGGTGCTAGTG	Amplification of *yadA* gene for RQ-PCR analysis
yadA-r	AGCGCCCACAGCAACTG	Amplification of *yadA* gene for RQ-PCR analysis
invA-f	CGGTGACCACAGGGCTTATT	Amplification of *invA* gene for RQ-PCR analysis

invA-r	TGATCGACCCCCAGTGTAATG	Amplification of *invA* gene for RQ-PCR analysis
repA-f	TGCGACATGCCACGTTAGTT	Amplification of *repA* gene for RQ-PCR analysis
repA-r	TTCGCACGTTTTTGTTTGATG	Amplification of *repA* gene for RQ-PCR analysis
spyA-f4	GATGCGACTGATCCTCAAGCT	Amplification of *spyA* gene for RQ-PCR analysis
spyA-r4	TGCAGGTCGGGAACATAGC	Amplification of *spyA* gene for RQ-PCR analysis
tyeA-f	GCGTACGACCTTTCTGAGTTTATG	Amplification of *tyeA* gene for RQ-PCR analysis
tyeA-r	CCGCCCAGCGCTTGT	Amplification of *tyeA* gene for RQ-PCR analysis
pSpyA-ApaI-f	GGGCCCTCCCTCCTCCTCGT	Amplification of the promoter region of *spyA* for cloning into pBluescript
pSpyA-Not1-r	GCGGCCGCATGTATTATCTC	Amplification of the promoter region of *spyA* for cloning into pBluescript
pVirF-ApaI-f	GGGCCCGTTGAATACAAATA	Amplification of the promoter region of *virF* for cloning into pBluescript
pVirF-Not1-r	GCGGCCGCGGACAGTATAACAT	Amplification of the promoter region of *virF* for cloning into pBluescript
pRepA-ApaI-f	GGGCCCTCCATAAATGCAAATGTCAG	Amplification of the promoter region of *repA* for cloning into pBluescript
pRepA-Not1-r	GCGGCCGCCCACACTGCGCTTA	Amplification of the promoter region of *repA* for cloning into pBluescript

2.5 Chemical reagents and media

2.5.1 Chemical reagents

Unless otherwise stated all chemicals used in this study were obtained from Sigma-Aldrich (Poole, UK).

2.5.2 Antibiotics/X-Gal/IPTG

Stock solutions of antibiotics were prepared as following (Sambrook *et al.*, 1989) and stored at -20°C. Their abbreviation and working concentrations were: ampicillin (Amp) at 50 μg ml^{-1}, chloramphenicol (Cm) at 30 μg ml^{-1}, streptomycin (Strep) at 25 μg ml^{-1}, kanamycin (Km) at 50 μg ml^{-1}, nalidixic acid (Nal) at 15 μg ml^{-1}. 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were included in the media, when necessary, at 50 μg ml^{-1} and 40 μg ml^{-1} respectively for detection of β-galactosidase activity (Sigma Ltd).

2.5.3 Synthetic AHLs

All synthetic AHLs were made by S. R. Chhabra at the School of Pharmacy School, University of Nottingham and kept as 10 mM stocks in acetonitrile and stored at -20°C (Chhabra *et al.*, 2003).

2.5.4 Growth media for bacterial stocks

All media were obtained from Oxoid Ltd. (UK), prepared using distilled water according to the manufacturers' instructions and autoclaved at 121°C for 20 min at 15 p.s.i. unless otherwise stated (Sambrook *et al.*, 1989). Luria Bertani (LB) broth was prepared as described by Sambrook *et al.* (1989) containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl. Buffered LB was prepared by the addition of 50 mM MOPS (4-Morpholinepropanesulfonic acid) and adjusted to pH 6.8 with NaOH (LB_{MOPS}). LB agar was prepared by addition of 1.5% (w/v) Technical Agar No. 1 (Oxoid) to LB broth. Yersinia LB (YLB) broth is a modified version of LB broth used for improved growth of *Yersinia* spp. containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl. YLB agar was prepared by addition of 1.5% (w/v) Technical Agar No. 1 (Oxoid) to YLB broth. *Yersinia* selective agar (Oxoid) was prepared according to the manufacturer's instruction. Semi-solid overlay agar consisted of 10 g L^{-1} tryptone, 5 g L^{-1} NaCl and 0.5% (w/v) Technical Agar No. 1.

Semi solid swimming motility agar plates contained 0.3% w/v Bacto™ agar (Becton, Dickinson Co., USA), 1.0% w/v tryptone, and 0.5% w/v NaCl in distilled water. Minimal swarm motility plates contained 10 g L^{-1} tryptone, 5 g L^{-1} NaCl, 0.6% w/v Bacto™ agar and 10 mM glucose which was filter-sterilised and then added into the medium immediately before pouring once the swarm agar had been sterilized by autoclaving and cooled to 50°C.

Tryptone Soya Agar (TSA) (Oxoid) consists of pancreatic digest of casein (15.0 g l^{-1}), enzymatic digest of soya bean (5.0 g L^{-1}), NaCl (5.0 g L^{-1}) and agar (15.0 g L^{-1}). 40g of TSA powder was added to 1L of distilled water.

2.5.5 Growth media for cell lines

Caco-2 cells were cultured in minimal essential medium (MEM; Dibco), supplemented with $NaHCO_3$ 1.2 g L^{-1}, 2mm glutamine, penicillin 100 IU ml^{-1}, streptomycin 0.1 mg $ml^{-1}$and heat-inactivated fetal calf serum (FCS) (Sigma) 10% in a 5% CO_2 incubator.

2.6 Long term storage of stocks

Long term stocks of the bacterial strains were prepared by taking 1.5 ml of overnight bacterial culture, centrifuging the cells in a microfuge (MSE Micro Centaur, Sanyo, UK) at 21,000*g* for 2 min then resuspending them in a 30:70 solution of glycerol: LB broth before freezing at -80 °C.

For Caco-2, the cells were cultured until they reached ~80-85 % confluency. The culture medium was discarded and the cells were then trypsinised. Ten millilitres of fresh culture medium was added and the solution was centrifuged at ~500*g* for 5 min. The supernatant was gently removed and the cells were resuspended in 1 ml freezing solution (10 % DMSO, 90 % FCS), then frozen at -80°C.

2.7 DNA manipulations

2.7.1 Isolation of genomic DNA

Genomic DNA was isolated using the Wizard DNA Extraction Kit (Promega, US) following manufacturer's instructions.

2.7.2 Isolation of plasmid DNA

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, UK) following the manufacturer's instructions.

2.7.3 Digestion of DNA by restriction enzymes

Reactions contained 0.05 to 1 μg DNA, 0.5 to 1 μl restriction endonuclease (Promega, US; New England Biolabs, UK; and Fermentas, Lithuania) and 1 X restriction buffer made to a final volume of 20 to 30 μl with distilled H_2O. The reaction mixture was incubated at the appropriate temperature for a minimum of 1 h. Reactions were analysed by agarose gel electrophoresis, appropriate bands were excised prior to DNA extraction using the Qiagen Gel Extraction Kit (Qiagen, UK).

2.7.4 Alkaline dephosphorylation of DNA

For each pmol of DNA ends, 0.01 U of Calf Intestinal Alkaline Phosphatase (CIAP) was used. The reaction mix was incubated for 30 min at 37°C before another 0.001 U of CIAP was added and incubation is continued at 37°C for an additional 30 min. The DNA is then immediately cleaned with the Qiagen PCR Purification Kit (Qiagen, UK) before use.

2.7.5 Ligation of DNA fragments

The ratio of vector to insert was calculated using this formula:

$$\frac{\text{50 ng vector X size of insert (kb)}}{\text{size of vector (kb)}} \text{ X molar ratio of } \frac{\text{insert}}{\text{vector}} = \chi \text{ ng of insert needed}$$

The molar ratios used were 1:1, 1:3, and 3:1. Ligations were performed at room temperature for 16 h and the reaction mix contained 3 units of T4 ligase and 10X buffer (NEB).

2.7.6 DNA gel electrophoresis

DNA fragments were analysed on 0.8 to 1.5% submerged agarose gels prepared in 1 X TAE buffer (0.04 M Tris base, 0.114% glacial acetic acid and 0.001 M EDTA (pH 8.0) using analytical grade agarose containing 0.4 mg ml^{-1} ethidium bromide. The DNA was mixed 5:1 with loading buffer (30% glycerol, 70% TAE, bromophenol blue), before an appropriate amount was loaded into a well in the gel and electrophoresis was performed using a horizontal gel apparatus filled with 1 X TAE buffer at 70 to 90 V. The DNA was visualised using a UV transilluminator. To establish the size of DNA

fragments, 1 μg of a 1 kb or 100 bp ladder (Promega, US) in DNA loading buffer was loaded on agarose gels.

2.8 Polymerase chain reaction (PCR) protocol for DNA amplification

PCR amplification was carried out with either a small quantity of a bacterial colony or 120 ng of template DNA, 5 units of Taq polymerase (Promega, US), 1 μg of each primer (Sigma-Genoys), 2.5 mM of each dinucleotide triphosphate (dNTP) and 10X reaction buffer. For colony PCR the reaction mix was incubated at 94°C for 10 min, or for template DNA PCR the reaction mix was incubated at 94°C for 5 min before Taq polymerase was added at 80°C. PCR was then carried out using 30 cycles of 94°C for 30 s (denaturation of template), 55°C for 30 s (annealing of primers), 74°C for 1 min (elongation of PCR product), followed by a final elongation of 74°C for 10 min. As needed, the annealing temperature were altered to account for the predicted melting temperature (Tm) of the primers and elongation time were altered to account for the predicted size of the PCR product (1 kilobase of PCR product require 1 min of elongation time).

2.8.1 Extraction and purification of DNA from agarose gels

DNA fragments were purified from agarose gel slices using a Qiagen Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. DNA was routinely eluted in 50 μl sterile distilled H_2O.

2.8.2 Blue/white selection

Recombinant plasmids containing the *E. coli lacZα* multicloning site were screened by blue/white selection where X-Gal (50 μg ml^{-1}) and IPTG (40 μg ml^{-1}) were added to LB agar. This allows screening for β-galactosidase production (*E. coli* DH5α has a *lacZα* deletion). Blue colonies indicate active β-galactosidase and therefore no insert DNA. Colonies lacking β-galactosidase activity, due to disruption of the *lacZα* gene by insert DNA, were identified by their white colour and selected for further analysis.

2.8.3 DNA sequence analysis

Analysis of DNA sequences was performed using the Lasergene computer package (DNAstar Ltd) in combination with the BLAST programs available from the NCBI web site (http://www.ncbi.nlm.nih.gov/).

2.9 Transformation of bacteria

2.9.1 Electrocompetent E. coli *and* Y. enterocolitica

E. coli DH5α and *Y. enterocolitica* cells were made into competent cells as previously described (Sambrook *et al.*, 1989). Briefly, 40 ml of appropriate media were inoculated with an overnight culture to give an initial OD_{600} of 0.1. The cells were then incubated at 26°C or 37°C until the culture reached an OD_{600} of 0.6 to 0.8. Then, the cells were centrifuged at 10,000 g for 5 min, washed in 40 ml, 20 ml, and 5 ml of pre-chilled 10% glycerol, before being resuspended in 100 to 500 µl of 10% glycerol.

2.9.2 Electroporation of E. coli *and* Y. enterocolitica

Prior to electroporation and to reduce arcing, the ligation mix was dialysed against distilled water for 15 min using 0.025 µm dialysis membranes (Millipore). Electroporation was performed essentially as previously described (Sambrook *et al.*, 1989) in 0.2 cm pre-chilled electroporation cuvettes containing the ligation mix and competent cells. After an electroporation pulse of 2.5 kV (25 µF, 200 Ω) was delivered with a micropulser (BioRad), the competent cells were recovered in 1 ml of LB and incubated for 1 h at 37°C before plating onto agar plates with appropriate antibiotics.

2.9.3 Conjugation of plasmid DNA into Y. enterocolitica

Plasmid transfers from *E. coli* donor (S17-1) to *Y. enterocolitica* recipient cells were carried out by bacterial matings. Both donor and recipient cells were grown by inoculating 10 ml of LB with 1% (v/v) overnight culture and incubated overnight. *Y. enterocolitica* recipient strains were grown at 30°C whilst *E. coli* donor strains were grown at 37°C.

Conjugations were achieved by mixing 50 µl of each from overnight cultures. The mixture was then spotted onto a non-selective agar plate and incubated for 16 hours at 30°C. The resulting colony was removed from the plate and resuspended in

500 µl LB broth. Suitable volumes were plated onto LB agar containing antibiotics to select for *Y. enterocolitica* transconjugants and incubated at 30°C for 24 to 48 h.

2.10 Purification and characterization of *N*-acylhomoserine lactones (AHLs)

2.10.1 Extraction of AHLs

Y. enterocolitica wildtype, and its QS mutants were grown at 30°C respectively in YLB buffered with 50 mM MOPS pH 6.8 (YLB_{MOPS}), to avoid the pH of the supernatant to rise above 7.0, thereby preventing lactonolysis (Yates *et al.*, 2002). Late-exponential-phase, cell-free culture supernatants were extracted twice with dichloromethane as described before (Throup *et al.*, 1995; Atkinson *et al.*, 1999).

2.10.2 AHLs detection by thin layer chromatography (TLC)

The AHLs extracts were assayed for AHLs using the biosensors *Chromobacterium violaceum* CV026 which detects short-chain AHLs (C6 and C8) and *E. coli* pSB1075 which detects long chain AHLs (C10 to C14), by producing the purple pigment violacein or bioluminescence, respectively. Bioluminescence was detected using a Berthold LB980 photon video camera (E.G. and G Berthold U.K. Ltd., Milton Keynes, U.K). AHLs were detected by using the biosensors as agar overlays either on LB agar plates or on thin-layer chromatograms as described before (Latifi *et al.*, 1996; McClean *et al.*, 1997; Winson *et al.*, 1998a).

2.10.3 Liquid chromatography (LC) coupled to hybrid quadrupole–linear ion trap (QqQLIT) mass spectrometry

A comprehensive profiling of the AHLs in *Y. enterocolitica* wildtype and QS mutants was done using the liquid chromatography (LC) coupled to hybrid quadrupole–linear ion trap (QqQLIT) mass spectrometry method developed by Dr. Catherine Ortori (Centre for Analytical Bioscience, University of Nottingham). This method can simultaneously screen, quantify and identify multiple AHLs in a single sample. This MS method uses common AHL fragment ions attributed to the homoserine moiety and

the 3-oxo-, 3-hydroxy- or unsubstituted acyl side chains, to identify unknown AHLs in cell-free culture supernatants in an unbiased manner.

2.11 Southern blot analysis

Following electrophoresis (see Section 2.7.6) and photography, agarose gels were submerged in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min at room temperature with gentle agitation. After denaturation the gel was rinsed in distilled water and neutralised in sufficient neutralisation buffer (1 M Tris base, 1.5 M NaCl, pH 7.5) for 30 min at room temperature with gentle agitation (Sambrook *et al.*, 1989). DNA was transferred to nylon membranes (Hybond N+, Amersham Life Sciences, UK) by capillary transfer using nucleic acid transfer buffer (20 x SSC: 3M NaCl, 300 mM Na-citrate, pH 7.0) overnight at room temperature as described by Southern (1975). Following DNA transfer, the DNA was fixed to the membrane by exposing to UV light for 10 min using a transilluminator (Ultra-Violet Products Inc., US)

Digoxigenin (DIG)-labelled DNA fragment probes were used for DNA hybridisation with DIG-Easy Hyb (Roche, UK) solution as hybridisation buffer. DIG labelling, hybridisation and detection were performed using the DIG DNA labelling and detection kit supplied by Roche according to the manufacturer's instructions. The hybridisation temperature and the stringency conditions were altered to account for the predicted melting temperature (Tm) (Sambrook *et al.*, 1989) of the particular probe used. Membranes were washed for 15 min in 2 X SSC, 0.1% (w/v) SDS and 0.1 X SSC, 0.1% (w/v) SDS after hybridisation. Colourimetric detection methods were employed to visualise bands on the nylon membranes as detailed in the DIG user handbook (Roche, UK).

2.12 Construction of QS mutants

2.12.1 The λ red recombinase method

QS mutants were constructed using the λ red recombinase method, which was a modified method of Datsenko & Wanner (2000) adapted for *Y. enterocolitica* (Maxson & Darwin, 2004). This method allows *in vivo* recombination of constructed mutations with the insertion of an antibiotic cassette onto the *Y. enterocolitica* chromosome by using PCR products. In this procedure, recombination requires the expression plasmid, pAJD434 which encodes the phage λ red recombinase. This plasmid also encodes trimethoprim (Tp) resistance for easy selection.

Briefly, the λ red recombinase plasmid, pAJD434, was introduced into *Y. enterocolitica* 8081 by electroporation and positive transformants were selected on Tp^{100} agar plates. Meanwhile, an appropriate antibiotic cassette was amplified *via* PCR from a plasmid source with the addition of flanking homologous sequences for the target site of interest. The resultant PCR product was then transformed into competent *Y. enterocolitica* 8081 containing plasmid pAJD434. Mutants were selected with appropriate antibiotics and cured of the temperature sensitive pAJD434 plasmid by culturing them at 37°C. Mutation was then confirmed by PCR analysis, Southern hybridization and sequencing. Table 2.6 shows the antibiotic resistance cassettes used in this mutagenesis protocol.

Table 2.5. Antibiotic resistance cassettes used in λ red recombinase mutagenesis

Target gene	**Antibiotic resistance cassette**	**Plasmid source of cassette**
yenR	Kanamycin	pUC4K
yenI	Chloramphenicol	pACYC184
ycoR	Streptomycin	pHP45Ω

2.13 Protein purification

2.13.1 Protein purification from Y. enterocolitica

2.13.1.1 Extraction of exoproteins from bacterial culture supernatant

Proteins from the spent supernatant of *Y. enterocolitica* wildtype and its QS mutants were extracted using the trichloroacetic acid (TCA) precipitation method (Atkinson *et al.*, 1999).

Briefly, 1 ml of overnight culture was centrifuged at top speed for 5 min and the supernatant was removed to a clean tube. In a fume hood, 0.1 volume of 100% TCA was added to the supernatant and left on ice for 30 min. The samples were then centrifuged at 15, 600 x g for 5 min and the supernatant discarded. 500 µl of acetone was then added and the tubes were placed in a sonicating waterbath for approximately 20 min to disrupt the pellets and wash the proteins. The centrifugation, addition of acetone and sonication in the water bath was repeated. All acetone was removed and the samples are air-dried for a few minutes. 30 µl of loading buffer was added to samples. Finally, the samples are denatured by boiling for 3 min. Boiled samples was allowed to cool to room temperature and then they are ready to use in SDS-PAGE (refer to Section 2.13.2). Alternatively, samples can be kept at -20°C for use at a later date.

2.13.1.2 Extraction of cytoplasmic protein from bacterial culture

Proteins from the cytoplasm of *Y. enterocolitica* wildtype and its QS mutants were extracted using a method adapted from Carlone *et al.* (1986). Briefly, 10 ml of overnight culture was centrifuged at 5,000 x g for 10 min at 4°C and the supernatant discarded. The pellet was suspended in 1.5 ml of cold 10 mM sodium phosphate buffer (pH7.2) and the cell suspension was transferred to a 1.5 ml centrifuge tube. The cells were then washed once by centrifugation at 15,600 x g for 2 min at 4°C in a microcentrifuge, and the supernatant fluid was decanted. The cell pellets were used immediately or stored at -80°C.

Cell pellets were resuspended in 1 ml of 10 mM sodium phosphate buffer (pH7.2) and, while kept on ice, sonicated (six bursts, 10 s each at 40 W) with a cell disruptor; Soniprep 150 (Sanyo, Japan) fitted with a microtip. The unbroken cells and debris were removed by centrifugation at 15,600 x g for 2 min at 4°C. The supernatant fluid was transferred to a 1.5-ml centrifuge tube and centrifuged again for 15,600 x g for 30 min at 4°C. The supernatant contains the cytoplasmic proteins and was extracted

using the trichloroacetic acid (TCA) precipitation method (Atkinson *et al.*, 1999) as discussed in the previous section 2.13.1.1. The cell membrane pellet meanwhile was used to extract the inner membrane proteins (IMP) and outer membrane proteins (Perez-Gutierrez *et al.*, 2007) which is discussed in the following section (Section 2.13.1.3).

2.13.1.3 Extraction of inner membrane proteins (IMP) and outer membrane proteins (OMP) from bacterial culture

As explained in the previous section, the cell membrane pellet was thoroughly suspended in 0.2 ml of 10 mM sodium phosphate buffer (pH 7.2) by repeated pipetting. The OMP were solubilised by addition of an equal volume of 2% Sodium *N*-lauroyl sarcosinate (Sarkosyl) solution (Teknova Inc., USA) and incubated at room temperature for 30 min with intermittent mixing. The OMP were then pelleted by centrifugation at 15,600 x g for 30 min at 4°C. The supernatant fluid contains the IMP and was removed to a clean tube. The OMP were washed once (without resuspending the pellet) with 0.5 ml of 10 mM sodium phosphate buffer (pH7.2). 50 to 100 µl of loading buffer was added to the IMP supernatant fluid and also the OMP pellets to resuspend the proteins. Finally, the samples are denatured by boiling for 3 min. Boiled samples was allowed to cool on ice and they were then ready to use in SDS-PAGE (refer to Section 2.13.2). Alternatively, samples can be kept at -20°C for use at a later date.

2.13.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the method of Laemmli (1970). 12.5% gels were prepared with 2 ml acrylamide:bisacrylamide (37.1:1) (Severn Biotech, UK), 1.65 ml gel buffer (3 M Tris-HCl pH 8.45, 0.5% (w/v) SDS), 25 µl 10% (w/v) ammonium persulphate (APS), 5 µl *N,N,N,N*, tetramethyl ethylenediamine (TEMED) and 1.35 ml distilled H_2O. The gel solution was poured into a Mini-Protean II casting tray (Biorad, UK) with space for a stacking gel and overlaid with 1 ml of isopropanol. Once polymerised, a 5% (w/v) acrylamide stacking gel was prepared from 0.335 ml acrylamide:bisacrylamide (37.1:1), 0.625 ml gel buffer, 25 µl 10% (w/v) ammonium persulphate, 5 µl TEMED and 1.515 ml distilled H_2O. After removing the isopropanol, the stacking gel was poured and a comb inserted.

The samples and SDS sample buffer (200 mM Tris HCl pH 6.8; 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 100 mM DTT; 40% glycerol, 0.02% β-mercaptoethanol) were mixed in a 4:1 ratio and boiled for 5 min. 15 µl of the sample was loaded onto the gel. Electrophoresis was performed in a Cathode (0.1 M Tris-base, 0.1 M tricine and 0.1% SDS) /Anode (0.1 M Tris base pH8.9) buffer at 100 to 150 V until the dye had migrated to the bottom of the separating gel. Precision Plus Protein All Blue Standard (BioRad, UK) was used as the molecular weight marker.

The gel was stained with Coomassie Blue solution (25% (v/v) isopropanol, 10% (v/v) acetic acid, 0.025% (w/v) Coomassie Brilliant Blue R250) for 1 h and washed with destaining solution (25% (v/v) isopropanol, 10% (v/v) acetic acid, 1% (v/v) glycerol) then fixed with distilled water.

2.14 *Y. enterocolitica* phenotypic assays

2.14.1 Swarming/Swimming assay

Minimal swarm motility agar and semi solid swimming motility agar plates were prepared as previously described (See Section 2.5.4). 1 µl of overnight seed cultures grown at 30°C were inoculated into (swim) or onto (swarm) agar plates respectively and incubated at 22°C or 37°C. The ability to swarm or swim was assessed by the distance of swarming/swimming from the central inoculation site.

2.14.2 Congo Red binding assay

A modified magnesium oxalate agar which incorporated Congo Red (Riley & Toma, 1989) was used so that the presence of the pYVe virulence plasmid in pathogenic strains may be more easily detected by simultaneous demonstration of calcium dependency and Congo Red absorption. The Congo Red-magnesium oxalate agar (CRMOX) was prepared as follows. Tryptic soy agar (40 g) (Oxoid Ltd., UK) was mixed in 825 ml of distilled water and autoclaved for 15 min at 121°C. The molten medium was cooled to 55°C, and the following solutions were added aseptically: 80 ml of 0.25 M sodium oxalate (Sigma Ltd.), 80 ml of 0.25 M magnesium chloride (Sigma Ltd.), 10 ml of 20% *D*-galactose (Sigma Ltd.), and 5 ml of 1% Congo Red (Sigma Ltd.).

Test strains were streaked onto CRMOX plates and incubated for 24 h at 37°C. Strains were CRMOX negative (CRMOX-) if only large colourless colonies were

present. Positive strains (CRMOX+) always produced small red colonies (CRMOX+) and large colourless colonies (CRMOX-). The presence of CRMOX- colonies was due to a rapid loss of pYVe plasmid *in vitro*.

2.14.3 Agglutination assay

A modified agglutination assay was performed according to Laird and Cavanaugh (1980). Briefly, the autoagglutination test was performed using 1 ml of RPMI-1640 medium (Gibco, UK) supplemented with 10% calf serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) in sterile 2.0 ml microfuge tubes. Strains were streaked on Tryptic soy agar plates and grown for 2 to 3 days at 30°C. Cells from an isolated colony were inoculated into a pair of tubes. One tube was incubated for 18 h at 37°C, whereas the other was incubated for the same time period at 26°C. Routinely, ten colonies of each strain were tested to insure an adequate sample size. The growth in each tube was examined for evidence of bacterial agglutination.

The growth of autoagglutination-positive (Ag+) strains consisted of an irregularly edged layer of agglutinated bacteria which formed a flocculate covering the bottom of the tube. Usually, the medium in such tubes was clear. The growth of non-agglutinating (Ag-) strains was distinctly different. Although some bacteria had settled out to form a smooth round pellet in the centre of the bottom of the tube, the majority of the bacteria remained in suspension, therefore creating a turbid medium. When the various tubes were gently shaken, the agglutinated bacteria remained clumped whereas the non-agglutinated bacteria in the pellet formed a smooth turbid suspension. This agglutination phenomenon was found to be dependent on the temperature of growth. Strains were scored as Ag+ if the 37°C tube was positive and the 22°C tube was negative. Bacteria which agglutinated at both temperatures were considered to be false-positives.

2.14.4 Haemagglutination assay

Using a V-well 96 well plate (Ratiolab, Germany), 50 µl of overnight cultures of *Y. enterocolitica* wildtype and its QS mutants grown at 22°C or 37°C were added to the top wells. 50 µl of Phosphate Buffered Saline (PBS) was added to all wells to double dilute the sample. Two drops of a 1% sheep blood suspension (Oxoid Ltd., UK) in PBS were added to each well. PBS solution was used as a negative control. All samples were tested in triplicate. The plate was then incubated at 4°C and examined after 3 h or overnight. The negative control should appear as a pellet of blood cells settled in the V-shaped bottom of the well. Any agglutination occurring will inhibit the formation of this pellet and the blood cells appear to be coating, or partially coating, the bottom of the well.

2.14.5 Plasmid loss study

To study the rate of loss for the pYVe plasmid *in vivo* at 37°C, 10 ml of YLB broth was seeded from plate cultures for the wildtype and 5 QS mutants and left to grow overnight with agitation. These overnight cultures (day 1 cultures) were serially diluted to 10^{-6}. 100 µl of this diluted culture was plated onto a CRMOX plate (refer to Section 2.14.2) and incubated at 37°C for 24 h to obtain single colonies. The number of white colonies (colonies which have lost pYVe) and red colonies (colonies still harbouring pYVe) was determined for each strain. Then, the percentages of white colonies were calculated. Rates of plasmid loss were observed by subculturing the seed cultures over 10 days, plating serially diluted cultures at 10^{-6} and the percentages of white colonies were determined for each day.

2.15 Bioluminescence and optical density assay

Overnight seed cultures were diluted 1:10 (v/v) in fresh LB, and incubated at 30°C with agitation for 3 h. These cultures were then diluted again to an OD_{600} of 0.01 and 200 µl of the diluted cultures were pipetted into a 96 well microtitre plate (Corning Inc., US) in triplicates. Bioluminescence and optical density were monitored simultaneously using the Anthos LUCY1 combined photometer and luminometer controlled by the Stingray software (Dazdaq). Assays were performed at optimum temperature depending on the bacterial strain used. The program measures optical density at 405 nm (OD_{405}) and luminescence from every well every 30 min for 24 h.

2.16 Tissue culture

2.16.1 Passage of cell lines

Cells were routinely grown in 75 cm^3 flasks until they were approximately 85 to 90% confluent. To passage the cells, they were first washed three times with PBS before 2 ml of 0.05% (w/v) trypsin, 0.2 g L^{-1} EDTA solution (Gibco, UK) was added. The flask was incubated at 37°C, 5% CO_2 for 2 to 5 min until the cells had detached from the surface of the flask. 10 ml of fresh culture medium was added to resuspend the cells and inactivate the trypsin. The cells were centrifuged at 100 x g in a centrifuge (Avanti 30, Beckman, UK) for 5 min before removing the supernatant and resuspending in 10 ml media of fresh media. The cell suspension was added to new sterile flasks containing 20 to 30 ml of complete growth medium at a ratio of 1:3 to 1:10 (Davies, 1994).

2.16.2 Bacterial adhesion assay with Caco-2

Caco-2 cell semi-confluent monolayers (2 X 10^5 cells ml^{-1}) were grown in 24-well Nunc culture dishes without antibiotics. Before infection, bacteria were subcultured for 1 h at 37°C in BHI, pH 5.5, according to Pepe *et al.* (1994).

For adhesion assays, a method previously described by Di Biase *et al.* (2000) was used. Caco-2 monolayer cells were infected at a MOI (Multiplicity of Infection) of 100 cfu/cell for 3 h at 4°C. After infection, cell monolayers were carefully washed five times with MEM to remove unattached bacteria, lysed by the addition of cold Triton X-100 0.1% and plated on TSA to determine the number of bound bacteria by performing cfu counts. Adherence was expressed as the number of associated bacteria/100 Caco-2 cells.

2.16.3 Bacterial invasion assay with Caco-2

For invasion assays, a method previously described by Di Biase *et al.* (2000) was used. Caco-2 monolayer cells were infected for 3 h at 37°C at a MOI of 100 cfu/cell. After infection, monolayer cells were washed five times with Eagle's MEM. One ml of fresh medium containing 50 μg ml^{-1} gentamicin was added to each well. After incubation for 1 h at 37°C, the cells were washed in MEM, tryptinised, lysed by addition of cold Triton X-100 0.1% and plated on TSA to determine the number of viable intracellular bacteria. Invasiveness was expressed as the number of internalised bacteria/100 Caco-2 cells.

2.17 Whole genome expression analysis using microarray method

The gene expression profiles of the wildtype and 5 of the QS mutants (Δ*yenI*, Δ*yenR*, Δ*ycoR*, Δ*yenRycoR* and Δ*yenIyenRycoR*) were studied. Each strain was subjected to growth at 22°C and 37°C. RNA was isolated at the late-log phase of growth (OD_{600} 0.75). All experiments were done in triplicates. All strains were analysed for changes in gene expression *via* quantification of RNA transcripts.

To extract the RNA, 4 ml of culture was added to 8 ml RNAprotect™ (Qiagen, UK). Cells were then pelleted and RNA was extracted using RNeasy (Qiagen, UK) midi columns with on column DNaseI digestion as per manufacturer's instructions. RNA was then quantified using the NanoDrop ND-1000 (Nanodrop Technologies) and samples were checked for integrity using the Agilent Bioanalyzer 2100 with 2100 Expert software (Agilent technologies).

The YP v3 Pan Yersinia array chips produced by the Bacterial Microarray Group at St George's (BμG@S, University of London) were designed to contain all *Y. enterocolitica* 8081 genes. Probe labelling, hybridization and signal scanning were conducted using standard procedures. 3 of these chips were hybridised for each culture condition. In this experiment, the two-colour system was used. Cy3-labelled genomic DNA extracted from *Y. enterocolitica* 8081 wildtype which acts as a normalisation control and Cy5-labelled cDNA generated from RNA were hybridised to the same microarray chip. Cy3 has a fluorescence emission wavelength of 570 nm (green) while Cy5 (red) has a fluorescence emission wavelength of 670 nm.

In brief, 2 μg of genomic DNA was labelled with Cy3 (Amersham Biosciences) by adding 1μl of random primers (3μg/μl) and H_2O to 41.5μl. This mixture was then heated at 95°C for 5 min, snap-cooled on ice and centrifuged briefly. Then, 5μl of 10× reaction 2 buffer, 1μl of dNTPs (5mM dA/G/TTP, 2mM dCTP), 1.5μl of Cy3 dCTP and 1μl of Klenow fragment (Promega Corporation) (3-9 U/μl) were added before incubating the mixture at 37°C in dark for 90min. 10 μg total RNA was used in a reverse transcription reaction to synthesize Cy5-labelled (Amersham Biosciences) complementary DNA (cDNA) probes by adding 1μl of random primers (3μg/μl) and H_2O to 11μl. Then, this mixture was heated at 95°C for 5min, snap-cooled on ice and centrifuged briefly. Then, 5μl of 5× first strand buffer, 2.5μl of DTT (100mM), 2.3μl of dNTPs (5mM dA/G/TTP, 2mM dCTP), 1.7μl of Cy5 dCTP and 2.5μl of SuperScript II

(200U/μl) (Invitrogen) were added before incubating the mixture at 25°C in dark for 10 min followed by 42°C in the dark for 90 min. The labelled cDNA probes were purified with QIAquick PCR purification system (Qiagen, UK). The microarrays were prehybridised at 65°C for 20 min and further hybridised at 65°C for 16 h in the dark with the mixed Cy3- and Cy5-labelled cDNA probes that had been denatured at 95°C for 2 min. After hybridization, the microarrays were washed and scanned using the GenePix 4000B (Axon Instruments Inc., USA) to visualize fluorescence of the two fluorophores after excitation at 570 nm and 670 nm. Relative intensities of each fluorophore were used in ratio-based analysis to identify up-regulated and down-regulated genes.

The raw data was analysed by Ms. Victoria Wright (Genomics Lab, Centre of Biomolecular Sciences). The scanned output files (.gpr) were imported into the microarray analysis program GeneSpring GX 7.3.1 (Agilent Technologies Inc). The BμG@S group (University of London) supplied the *Y. enterocolitica* 8081 microarray genome. The Cy5 (RNA) probe was set as the signal with the Cy3 (DNA) probe set as the control. The signal intensities for each spot were obtained by using the median background-corrected values. A global normalisation strategy was used to centre the data around 1. For this, values below 0.01 were set to 0.01. The intensity of each gene was divided by its control channel value in each sample; if the control channel value was below 4 then 4 was used instead. If the control channel and the signal channel were both below 4 then no data was reported. Each measurement was divided by the 50.0^{th} percentile of all measurements in that sample. Normalisation strategy and array replicate reproducibility was checked by box plot and clustering analyses. Mean values for each condition (from the replicate data) were then used in downstream analysis. Genes were filtered to find up and down regulated genes according to fold change. The up or down-regulated genes list then underwent a Student's T-test to find statistically changed genes ($p \leq 0.05$). For the purpose of this study, only the 4,291 CDS of the *Y. emterocolitica* 8081 were analysed and the rest of the CDS on the Pan-*Yersinia* array were disregarded.

2.18 Relative quantification PCR assay (RQ-PCR)

The total RNA isolated for the microarray experiment explained in the previous section were used in this experiment.

2.18.1 Removal of DNA from RNA samples

RQ-PCR is very sensitive to any residual DNA left in the RNA samples during extraction. Thus all RNA samples are treated with DNAseI for 1 h to remove residual DNA. The remaining RNA was washed and eluted in 50 µl elution buffer. Samples were stored at -20°C.

2.18.2 1st strand cDNA synthesis

1st strand cDNA synthesis was performed on the RNA samples using the 1st strand cDNA synthesis kit (GE Healthcare) according to manufacturer's instructions. Briefly, 1 to 5 µg of total RNA was diluted to 10 µl using RNAse-free H_2O. 1 µl of Random Primers and 1 µl of dNTP mix were added before the solution was heated to 65°C for 5 min and then chilled on ice. This was then added to a separate mixture containing 4 µl of 5X first strand buffer and 2 µl of 0.1M DTT solution. This is then incubated for 2 min at 25°C. Next, 1 µl of SuperscriptII and 1 µl of dH_2O were added to make a final volume of 20 µl. This mixture was pipetted gently to mix before being incubated at 25°C for 10 min, then at 42°C for 50 min and finally at 70°C for 10 min to inactivate the enzyme. The cDNA were purified with MinElute PCR purification system (Qiagen, UK) and quantified using the Nanodrop.

2.18.3 SYBR® Green PCR protocol

The expressions of seven genes of interest (*virF*, *yscF*, *yadA*, *invA*, *repA*, *spyA* and *tyeA*) were studied. The *dnaE* gene was used as an endogenous control gene (See Table 2.7). The software Primer Express was used to design 8 pairs of primers (refer to Table 2.5) for this experiment. All primer pairs were optimised for efficiency by varying the primer ratios. Once the correct primer ratios are selected for each primer pair, the PCR efficiencies were assessed by performing standard curves using serial fold dilutions of the template. The slope of the standard curve indicated the efficiency.

The RQ-PCR was performed using the SYBR Green PCR Master Mix according to manufacturer's protocol (Applied Biosystems, USA). This master mix contains SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimised buffer components. Direct detection of polymerase chain reaction (PCR) product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to cDNA. The 25 ml reaction mixture contained 1 ng cDNA, 12.5 ml SYBR Green PCR Master Mix, and 10 μM of each primer. The enzyme was activated at 50°C for 2 min, and then at 95°C for 10 min. Reactions were performed in triplicate at 95°C for 15 s, 60°C for 1 min (40 x).

This comparative cycle threshold method (C_T) determines the changes in steady-state mRNA levels of a gene in the wildtype compared to QS mutants and expresses it relative to the levels of an internal control gene. C_T value is the number of cycles it takes for the first significant increase in the amount of PCR product. The more cycles it takes mean the less initial concentration of target template, and hence mean less expression. The C_T values were used to calculate fold changes in gene expression using the following equations.

$\Delta C_T = C_T$ (target gene) – C_T (reference gene)

$\Delta\Delta C_T$ = Difference of ΔC_T (QS mutant) and ΔC_T (wildtype)
= ΔC_T (QS mutant) - ΔC_T (wildtype)

Fold change = $2^{-\Delta\Delta CT}$

Table 2.6. Endogenous gene control and target genes chosen for RQ-PCR experiment

Gene	Function	Location
dnaE (control gene)	DNA polymerase III, ά chain, housekeeping gene.	chromosome
tyeA (target gene)	negative regulator of type III targeting pathway in the bacterial cytoplasm by preventing the export of YopN	pYVe plasmid
virF (target gene)	transcriptional activator of *yop*, *ysc* (injectisome), *yadA* and *yplA*	pYVe plasmid
yscF (target gene)	forms the 'needle end' part of the injectisome	pYVe plasmid
yadA (target gene)	virulence factor, mediates low rate invasion to tissue culture cells	pYVe plasmid
invA (target gene)	primary invasion factor	chromosome
repA (target gene)	putative replication initiator protein, controls replication of the virulence plasmid, pYVe	pYVe plasmid
spyA (target gene)	controls partition of the virulence plasmid, pYVe	pYVe plasmid

CHAPTER 3

GENOME ANALYSIS AND SPECIES WIDE STUDY OF QUORUM SENSING IN *Y. ENTEROCOLITICA*

3.1 Introduction

A recently published study on the comparative phylogenomics (whole-genome comparisons of microbes with DNA microarrays combined with Bayesian phylogenies) of a diverse collection of *Y. enterocolitica* strains found that the species is highly heterogeneous (Howard *et al.*, 2006). Phylogenomics is microarray technology coupled with complex mathematical analysis to determine phylogeny and is used as a sensitive and robust method to examine the genetic relatedness of bacterial populations. The genetic relationships described by Bayesian phylogeny of a DNA-DNA microarray data set can then be correlated against the known phenotypes and ecological behaviour of each bacterial strain in the analysis; this is particularly useful when studying the epidemiology and host association of pathogens (Champion *et al.*, 2005). However, one disadvantage of using single-genome microarrays is that it is only possible to detect predicted coding sequences (CDSs) that are present in the strain used to make the array.

Howard *et al.* (2006) utilised a whole-genome microarray which is based on the sequenced genome of *Y. enterocolitica* 8081, biotype 1B serotype O:8 representing 4, 208 predicted CDSs from the *Y. enterocolitica* 8081 chromosome and 83 from the virulence plasmid, pYVe. Comparative phylogenomics were applied to investigate a diverse collection of 94 strains of *Y. enterocolitica.* These strains consisted of 35 human (12 non-pathogenic, 15 low-pathogenicity, and 8 highly pathogenic), 35 pig (10 non-pathogenic and 25 low-pathogenicity), 15 sheep (8 non-pathogenic and 7 low-pathogenicity), and 9 bovine (7 non-pathogenic and 2 low-pathogenicity) isolates (McNally *et al.*, 2004). The strains isolated from animals were from an abattoir survey in the United Kingdom from 1999 to 2000. Twenty-six of the strains isolated from humans were from patients presenting to their general practitioners (GPs) with diarrhoea or were collected during the Infectious Intestinal Diseases (IID) study (Wheeler *et al.*, 2005). A further eight human biotype 1B isolates of different serotypes originating from the United States were also included for analysis (Beer & Miller, 1992). No biotype 5 strains were tested, as they were absent from the study samples, and environmental biotype 1A isolates were not included in this study.

Statistical analyses by Howard *et al.* (2006) confirmed that three distinct clusters exist; composed of a non-pathogenic group, a low-pathogenicity group, and a highly pathogenic group. 125 CDSs are present exclusively in all highly pathogenic strains but are absent from the other groups, including a haemolysin, a metalloprotease, and a type

III secretion effector protein which are previously uncharacterised virulence determinants. 27 CDSs were identified to be present only in the 8081 strain and all low-pathogenicity strains. Only 20.8% of the genes were shared by all of the *Y. enterocolitica* strains. This low percentage of core gene set confirms that this species is highly heterogeneous. Further analysis also revealed that *Y. enterocolitica* does not cluster according to source (host).

This chapter reveals how an additional LuxR homologue, named YcoR was identified in *Y. enterocolitica* and describes how, using the data and strains provided by Howard *et al.* (2006), a species-wide study of the QS systems of *Y. enterocolitica* revealed that the presence of YcoR is strain dependent and associated with hypervirulent strains found in the USA.

3.2 Results

3.2.1 Y. enterocolitica *8081 possesses an additional* luxR *homologue*

Since two other *Yersinia* spp. i.e. *Y. pseudotuberculosis* and *Y. pestis* both have two pairs of *luxIR* homologues, *in silico* analysis of the 8081 strain was performed to determine if additional *luxR* or *luxI* homologues were present in the 8081 genome. Previously, in a study on another *Y. enterocolitica* strain, strain 90/54, efforts failed to clone a second AHL synthase in using PCR with primers designed to amplify the *ytbI/R* locus from *Y. pseudotuberculosis* or by complementing the AHL biosensor *E. coli*[pSB401] with both plasmid and cosmid gene libraries (Atkinson *et al.*, 2006).

In this study, small sequence sections of LuxR homologues (LuxR, YenR, YpsR, YtbR) of between 20 to 30 amino acids were entered into a BLAST (Basic Local Alignment Search Tool, at www.ncbi.nlm.nih.gov/BLAST) search against the published *Y. enterocolitica* 8081 genome. Candidate genes from the genome were examined using pileup analysis to ensure key conserved residues were present when compared to known LuxR homologues. This analysis revealed an open reading frame (ORF), termed *ycoR,* of 723 bp was found. This ORF, originally named YE1026 in the published genome, begins at base pair 1,145,104 and terminates at base pair 1,144,382 (see http://www.sanger.ac.uk/Projects/Y_enterocolitica). When translated, it showed similarity to other members of the LuxR family of transcriptional regulators (Figure 3.1). However, no additional *luxI* homologues were found using a similar strategy.

YcoR is similar to the first characterised *Y. enterocolitica* transcriptional regulator protein YenR (43.2% similarity, E value: 6e-37) and many other LuxR homologues including SmaR (of *Serratia sp.* 58.6% similarity, E value: 4e-56), EchR (of *Erwinia chrysanthemi*, 50.6% similarity, E value: 1e-45), ExpR (of *Erwinia caratovora*, 45.0% similarity) and EsaR (of *Erwinia stewartii*, 41.0% similarity, E value: 5e-49). YcoR is also similar to other *Yersinia* LuxR homologue proteins; YpsR (of *Y. pseudotuberculosis*, 44.0% similarity, E value: 1e-53), YtbR (of *Y. pseudotuberculosis*, 42.0% similarity), YpeR (of *Y. pestis*, 44.0% similarity, E value: 7e-54) and YukR (of *Y. ruckeri*, 44% similarity, E value: 3e-49).

Protein motif analysis revealed that, when compared to known LuxR homologues, YcoR has highly conserved regions in the AHL binding domain (N terminal, residues 30 to 155) and DNA binding domain (C terminal, residues 175 to 231) (Figure 3.1). YcoR has 7 out of 10 residues that are known to be conserved in most members of the LuxR protein family (Fuqua & Greenberg, 2002), namely W53, Y57, D66, P67, G104, E179 and G200 (numbering relative to YcoR sequence).

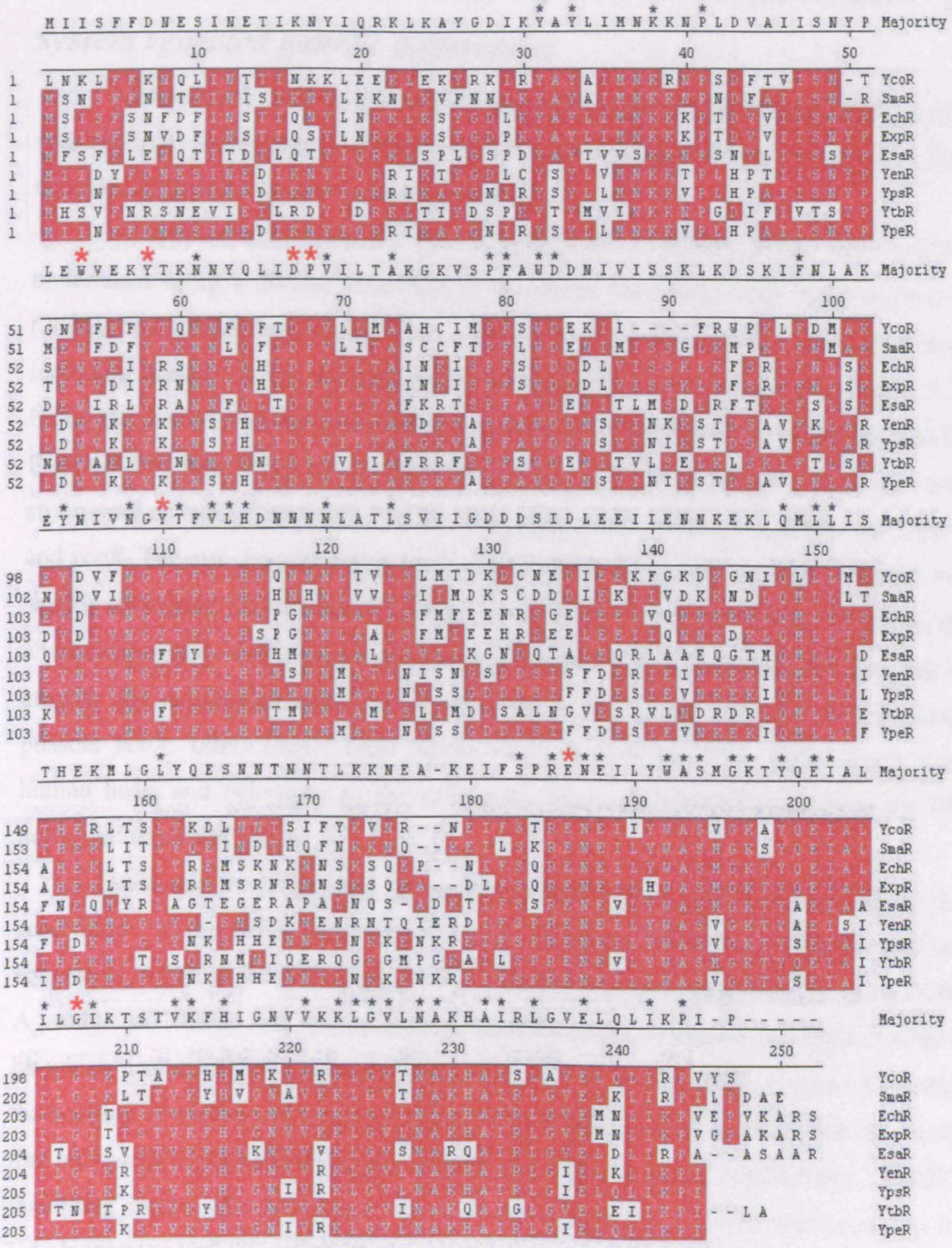


Figure 3.1. Pileup analysis of YcoR compared with SmaR (*Serratia sp.*), EchR (*Erwinia chrysanthemi*), ExpR (*Erwinia caratovora*), EsaR (*Erwinia stewarti*) and other *Yersinia* LuxR homologue proteins (YenR of *Y. enterocolitica*, YpsR and YtbR of *Y. pseudotuberculosis*, YpeR of *Y. pestis* and YukR of *Y. ruckeri*). Conserved residues are coloured red and shown as 'Majority' residues at that particular position. Within the N-terminal AHL binding domain (residues 30 to 155) and C-terminal DNA binding domain (residues 175 to 231), 25 and 35 residues are completely conserved (marked *) respectively. Red asterisks show residues known to be conserved in most members of the LuxR protein family.

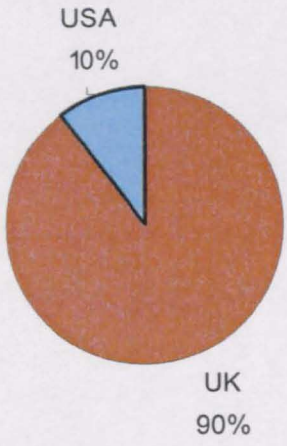
3.2.2 Species-wide study of the Y. enterocolitica *QS system revealed genetic differences*

Given that *Y. enterocolitica* 8081 possesses a second *luxR* homologue, *ycoR*, it was important to investigate whether the heterogeneity described by Howard *et al.* 2006 extended to include the newly discovered *ycoR*.

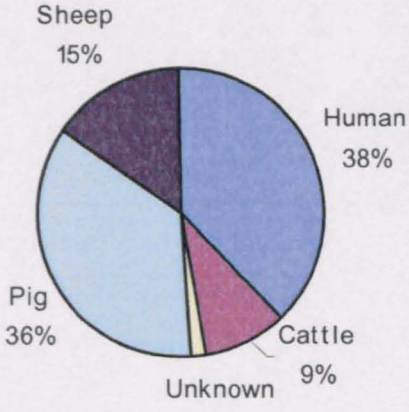
A species-wide study of the QS system of *Y. enterocolitica* was therefore undertaken using a diverse collection of 94 strains previously used by Howard *et al* (2006). The strains were isolated in the UK and USA from a number of different hosts including human, sheep, pig and cattle, belonging to several biogroups (Figure 3.2). Using the data of Howard *et al* (2006) deposited in the BμG@Sbase (accession number E-BUGS-36; http://bugs.sgul.ac.uk/E-BUGS-36), the raw microarray data for each strain was examined manually for the absence/presence of the QS genes: *yenI*, *yenR* and *ycoR*. The raw data for each strain is divided into three lists; 'present', 'absent' and 'highly divergent'. In the case of a QS gene appearing on the 'highly divergent' list, the absence/presence of that gene was then determined by PCR using primers designed to amplify the appropriate gene (see Table 2.4). It was found that 10% of the strains possess *ycoR*. Interestingly, only strains isolated from the USA (all isolated from human hosts and belonging to the pathogenic biogroup 1B) but none from the UK possessed this *ycoR* locus.

Since only the pathogenic US strains possessed the second *luxR* homologue, the *ycoR* locus of the 8081 strain (a US strain, biogroup 1B) was compared with the equivalent region in the pathogenic 90/54 strain (a UK strain, biogroup 1B) using PCR. A product of 1799bp was amplified from the 8081 strain compared to a product of 638 bp which was amplified from the 90/54 strain. A comparison of the two loci indicated the position at which *ycoR* is absent in the 90/54 strain (Figure 3.3a and 3.3b). It is clear that only *ycoR* is absent at this locus with the two adjacent genes, YE1025 and YE1027, remaining intact. The intergenic region is intact between YE1025 and *ycoR* but is largely missing between YE1027 and *ycoR*.

(i) Country of Origin



(ii) Host



(iii) Biogroup and Pathogenicity

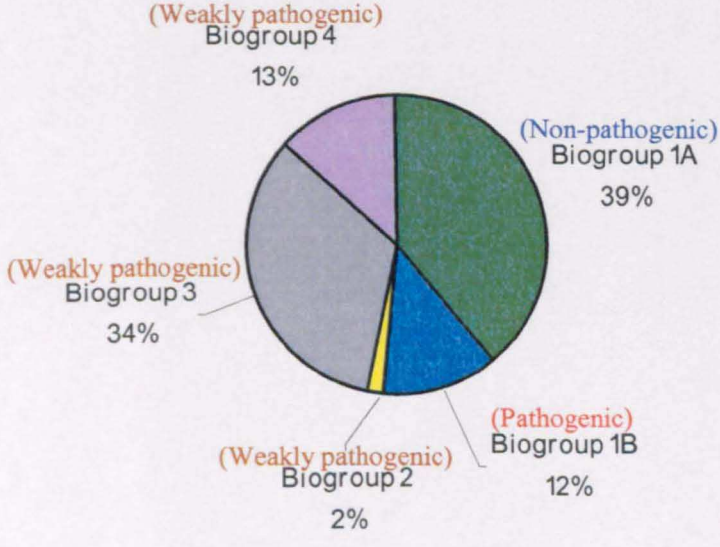


Figure 3.2. The collection of 94 strains of *Y. enterocolitica* were isolated from the UK or the USA (i), from different hosts (ii), and consisting of different biogroups and pathogenicity (iii).

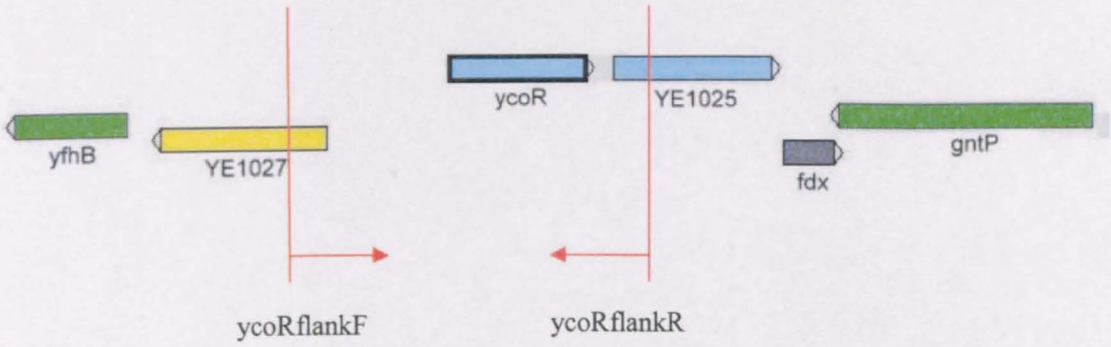


Figure 3.3a. The *ycoR* and its upstream and downstream genes in the strain 8081 genome. The ycoRflankF and ycoRflankR primers (red arrows) were used to amplify the flanking region of *ycoR* in the 8081 strain and the missing region in the UK strain. The different colours of genes represent different open reading frames for protein transcription.

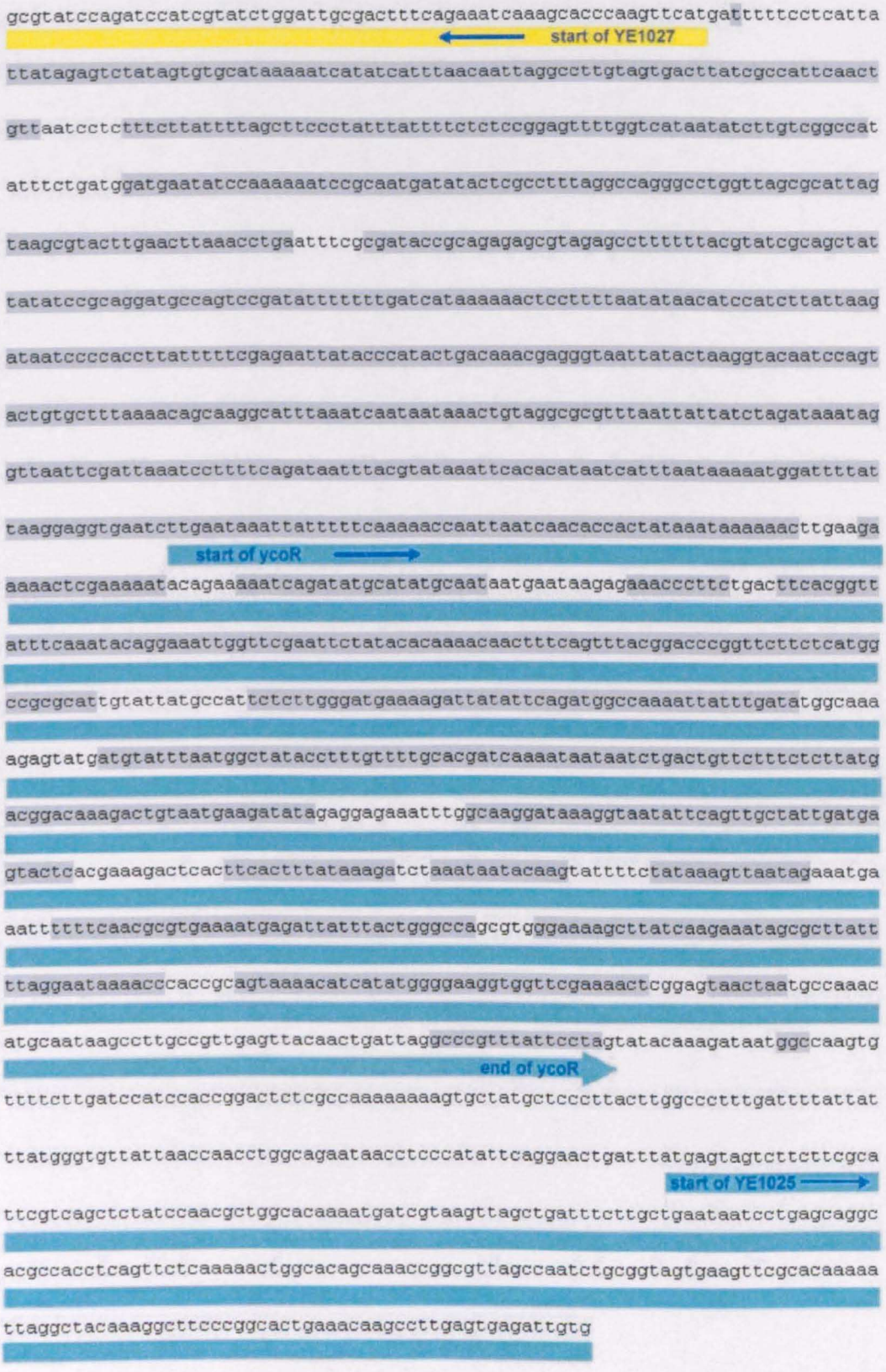


Figure 3.3b. Sequence of the amplified *ycoR* locus in the 8081 strain indicating the position at which *ycoR* is missing (shaded grey) in the UK strain.

3.3 Discussion

LuxR-type proteins can be subdivided into two functional domains, an AHL-binding domain (N-terminal) and a DNA binding domain (C-terminal). Protein motif analysis revealed that YcoR has highly conserved regions in the AHL binding domain and DNA binding domain when compared with known LuxR homologues, which strongly suggest that YcoR is a LuxR homologue.

The N-terminal domain of LuxR-type proteins forms an α–β–α sandwich that coordinates ligand interactions through the central β-sheet while the DNA-binding domain contains a helix-turn-helix motif and binds DNA (Fuqua & Greenberg, 2002). A crystal structure of TraR of *Agrobacterium tumefaciens*, associated with its cognate ligand (3-oxo-C8-HSL) has been published, providing insights about TraR and, by extension, other LuxR homologues (Zhang *et al.*, 2002). Many predictions based on genetic and biochemical studies of diverse LuxR-type proteins are confirmed by analysis of the TraR structure. Many LuxR homologues including LuxR, CarR, and TraR, form dimers or multimers upon binding to AHLs (reviewed by Whitehead *et al.*, 2001 and Pappas *et al.*, 2004). LuxR proteins are response regulators; some act as transcriptional activators, others as transcriptional repressors (reviewed by Smith *et al.*, 2006).

Often, members of the *luxR* family of transcriptional regulators are found adjacent to their corresponding *luxI*-type AHL synthases. However, a number of *luxR* 'orphans' i.e. not linked to or associated with an AHL synthase gene were discovered through genome interrogation (Fuqua, 2006). Therefore, *ycoR*, with no corresponding *luxI*-type AHL synthase is one such 'orphan'. In *A. tumefaciens*, there is also an orphan AHL receptor, TrlR, which is a homologue to TraR. TrlR has a functional amino-terminal domain but a mutation eliminates its DNA-binding region. TrlR works as a negative regulator of QS by forming inactive heterodimers or sequestering its cognate AHL signal molecule, 3-oxo-C8-HSL (Oger *et al.*, 1998; Zhu & Winans, 1998; Chai *et al.*, 2001).

P. aeruginosa also has an orphan LuxR homologue, named QscR. This is in addition to two QS signal receptor/generator pairs, LasR–LasI and RhlR–RhlI. QscR is an integral component of the *P. aeruginosa* QS system. QscR governs the timing of QS-controlled gene expression and it dampens virulence in an insect model, which suggests the primary role of QscR is repression of *lasI* (Chugani *et al.*, 2001). A *qscR* mutant produces the LasI-generated signal prematurely, and this results in premature

transcription of a number of QS-regulated genes including *phz* (required for phenazine synthesis) and *hcn* (required for hydrogen cyanide synthesis) (Chugani *et al.*, 2001). When fed to *Drosophila melanogaster*, the *qscR* mutant is hypervirulent and kills the animals more rapidly than parental *P. aeruginosa*. The repression of *lasI* by QscR could serve to ensure that QS-controlled genes are not activated in environments where they are not useful (Chugani *et al.*, 2001). A later study identified two other QscR targets, *lasB*, encoding the extracellular elastase, and a second phenazine gene cluster, both of which are downregulated by QscR. Using *in vivo* fluorescence anisotropy technology, it was shown that QscR are multimers in the absence of any AHLs, lower order oligomers when associated either with C4-HSL or 3-oxo-C12-HSL and heterodimers with LasR or RhlR (Ledgham *et al.*, 2003). In a further study on QscR, transcription profiling was used to identify a QscR-dependent regulon and revealed that QscR activates some genes and represses others. Some of the repressed genes are not regulated by the LasR-I or RhlR-I systems, while others are. QscR uses the LasI-generated 3-oxo-C12-HSL and controls a specific regulon that overlaps with the already overlapping LasR- and RhlR-dependent regulons. This transcriptome study confirmed previous studies that QscR repressed *hcnABC* and the two phenazine operons (*phzI* and *phzII*) in logarithmic phase. It was also revealed QscR induced *hcnA* in stationary phase and repressed *lasB* until early stationary phase (Lequette *et al.*, 2006).

There is a fourth LuxR homologue, designated VqsR (virulence and quorum-sensing regulator) in *P. aeruginosa* (Juhas *et al.*, 2004), adding to the level of complexity in the QS circuit. It was shown that inactivation of the *vqsR* gene abrogates the production of AHL signal molecules, decreases the production of virulence factors and reduces the pathogenicity of *P. aeruginosa* in a nematode infection model system. Transcriptomic analysis revealed that VqsR is involved in the regulation of the expression of some virulence and QS controlled genes in *P. aeruginosa* (Mario *et al.*, 2005). *vqsR* has a 20-bp *las* box upstream and the transcriptional start site for *vqsR* was determined (Li *et al.*, 2007). The *vqsR* promoter was identified and purified LasR protein binds directly to the *vqsR* promoter in the presence of 3-oxo-C12-HSL. The *las* system was demonstrated to be the dominant regulator for *vqsR* (Li *et al.*, 2007).

In *Erwinia carotovora* ssp. *carotovora* (*Ecc*), there are two orphan LuxR homologues. The first, CarR, controls the production of the antibiotic carbapenem. CarR occurs as dimers and AHL-binding converts the dimers to higher order multimers. The CarR-AHL complex binds DNA and activates the transcription of the *carA-H*

operon required for the biosynthesis of the antibiotic (McGowan *et al.*, 1995; 1996). The cognate LuxI homologue which generates the 3-oxo-C6-HSL to which CarR responds to is unlinked to *carR* although it is known, for historical reasons, as *carI* (Swift *et al.*, 1993; Pirhonen *et al.*, 1993). The second orphan, ExpR2 acts synergistically with the first ExpR1 (of the QS signal receptor/generator pair ExpR1/ExpI) to repress the production of plant cell wall degrading enzymes. This repression is released by the addition of AHLs and appears to be largely mediated *via* the negative regulator RsmA (Sjoblom *et al.*, 2006). Additionally, it was shown that ExpR2 has the novel property to sense AHLs with different acyl chain lengths. The first AHL receptor, ExpR1, has strict specificity for the cognate 3-oxo-C8-HSL but ExpR2 is able to act in response to both the non-cognate 3-oxo-C6-HSL and the cognate 3-oxo-C8-HSL. These results suggest that *Ecc* is able to react to both the cognate AHL signal and the signals produced by other bacterial species (Bell *et al.*, 2004; Sjoblom *et al.*, 2006).

In the closely related *Erwinia caratovora* ssp. *atroseptica* (*Eca*) (Wiley *et al.*, 2006), an equivalent to ExpR2, VirR, has been identified and characterised as a novel regulator of virulence. *Eca* virulence depends on the production of 3-oxo-C6-HSL. A *virR* mutation completely restores virulence factor production in an *Eca* mutant unable to synthesize 3-oxo-C6-HSL. This effect of the *virR* mutation translates to a restoration of virulence to wild-type levels and thus provides evidence that VirR acts to prevent the production of virulence factors at low cell density. It was show that transcription of virulence genes is controlled by 3-oxo-C6-HSL and that this control is mediated through the action of VirR (Bell *et al.*, 2004; Burr *et al.*, 2006).

More recently, an orphan LuxR homologue, CepR2 was found in *Burkholderia cenocepacia* strains (Malott *et al.*, 2009). In addition to CepR and CciR, the third LuxR homologue, CepR2, regulates gene expression and virulence factor production. Expression of *cepR2* was negatively autoregulated and was negatively regulated by CciR. CepR2 contributes to the QS regulatory network in two distinct strains of *B. cenocepacia*. In strain K56-2, CepR2 negatively regulated expression of several known QS-controlled genes, including genes encoding zinc metalloproteases. Meanwhile in strain H111, which lacks the CciIR QS system, CepR2 positively regulated pyochelin production by controlling transcription of one of the operons required for the

biosynthesis of the siderophore in an AHL-independent manner. CepR2 activation of a *luxI* promoter was demonstrated in a heterologous *E. coli* host (Malott *et al.*, 2009).

Both ExpR2 and QscR have broader signal specificities than ExpR (AHL regulator of *Erwinia carotovora* ssp. *carotovora*) and LasR (AHL regulator of *P. aeruginosa*) respectively. It has been suggested that bacteria which possess a receptor for non-cognate AHLs will expand the regulatory network or integrate additional environmental controls. This ability would confer on these bacteria the ability to identify neighbouring bacteria by sensing and possibly responding to the accumulation of different kinds of AHLs. Thus, eavesdropping on possible competitors would offer a biological advantage (Lazdunski *et al.*, 2004; Waters & Bassler, 2005). It would be interesting to determine the AHL specificities of YenR and YcoR and whether these specificities overlapped.

Given that *ycoR* in *Y. enterocolitica* 8081 is located only 165 bp upstream of an ORF encoding for a putative RpiR-family transcriptional regulatory protein in the same reading frame, it is possible that *ycoR* was acquired from a *Rhizobium* or closely related species. The RpiR family are known to be involved in host-bacteria interactions for example in *Rhizobium* species residing in the roots of leguminous plants (reviewed by Haine *et al.*, 2005). One example of host-bacteria interactions mediated by QS is the interactions between *Sinorhizobium meliloti* and its *Medicago* legume hosts. It has been shown that higher plants such as *Medicago* synthesize and secrete a diversity of compounds that effectively mimic AHLs in their ability to activate (or inhibit) QS-regulated gene expression in reporter bacteria (Qin *et al.*, 2000; Pellock *et al.*, 2002). *S. meliloti* QS mutants were significantly delayed in their ability to initiate nodules on the primary root of the host plant, *Medicago truncatula*, indicating that QS regulation and QS-regulated proteins contribute importantly to the rate or efficiency of nodule initiation. Thus, the AHL QS signals produced by *S. meliloti* could elicit many symbiotically relevant changes in the host root while concurrently regulating various QS-regulated activities in the bacterium (Gao *et al.*, 2005).

By extension, we speculate that YcoR may possibly play a role in the QS regulon as a modulator of interspecies communication with both pathogenic and non-pathogenic gut flora in the animal gut during infection. One example of a LuxR homologue playing a role in this type of communication is the SdiA of *E. coli* and *S. typhimurium*. Both of these bacteria do not have a *luxI* homologue, and do not produce AHLs (Wang *et al.*, 1991; Ahmer *et al.*, 1998; Michael *et al.*, 2001). Given that *S.*

typhimurium and *E. coli* are most often associated with the intestinal environment, it was hypothesised that instead of using LuxR homologues to gauge the population density of its own species, *S. typhimurium* and *E. coli* appear to have dispensed with any AHL synthase genes they may have had in the past and instead use SdiA to detect the AHLs produced by other species of bacteria (Michael *et al.*, 2001). SdiA regulates a few genes in *Salmonella* including one gene potentially involved in resistance to human complement, *rck* (Ahmer *et al.*, 1998). However, mutation of the *sdiA* gene had no effect on virulence of *Salmonella* in mouse, chicken or bovine models of disease (Ahmer, 2004).

Another example of interspecies communication is the AI-2 signalling in co-cultures of *E. coli* and *V. cholerae*, two bacteria that co-colonize the human intestine during *V. cholerae* infection (Xavier & Bassler, 2005). AI-2 (product of the LuxS enzyme) is a type of QS molecule which is produced and detected by a wide variety of bacteria and is proposed to allow interspecies communication (Bassler *et al.*, 1997; Chen *et al.*, 2002). In the co-culture, AI-2 production by either *E. coli* and *V. harveyi* can regulate light production in *V. harveyi* and trigger *lsr* (LuxS-regulated transporter) induction in *E. coli*. Thus, AI-2 production allows each species to include the other in the 'census' (Xavier & Bassler, 2005). It was also suggested that when enteric bacteria supply AI-2, a nearby species could use the information to count the enteric cells in the mixed-species community or prematurely activate the expression of QS-regulated genes. However, when enteric bacteria remove AI-2, a neighbouring species could underestimate cell number and fail to initiate or incorrectly terminate QS. Thus, consuming AI-2 could allow enteric cells to interfere with AI-2-mediated communication in other bacteria (Xavier & Bassler, 2005). This type of relationship may exist between *Y. enterocolitica* and other bacteria in the proximity of the gut in which YcoR may play a part in recognising non-cognate AHLs. Alternatively, YcoR may also act as a receptor for non-cognate AHLs from other bacteria in the soil/water environment.

It has been long known that *Yersinia* have some form of communication with the human gut which results in their tropism for lymphoid tissues and their capacity to resist the non-specific immune response. Anatomo-pathological examinations of artificially infected mice show that *Yersinia* form extracellular microcolonies. In accordance with these *in vivo* observations, *Yersinia* is resistant to phagocytosis *in vitro* by macrophages and polymorphonuclear leukocytes. Once they are phagocytosed, *Y.*

pseudotuberculosis and *Y. enterocolitica* are generally killed (Cornelis & Wolf-Watz, 1997). It is likely that the human gut has co-evolved concurrently to interact with bacteria for the maintenance of normal gut microflora and the prevention of bacterial diseases (Xavier & Bassler, 2005). Therefore it is possible that these interactions occur *via* QS and YcoR may play a part in the process.

According to a recent review (von Bodman *et al.*, 2008), there is increasing evidence to suggest that gut microbial flora can modulate host physiology and immunity (Hooper & Gordon, 2001). Human intestinal microflora produces a putative QS signal, termed autoinducer-3 (AI-3) (Sperandio *et al.*, 2003). AI-3 has a tentative molecular weight of 213.1 Da but its structure has not been fully characterised. It may be structurally similar to epinephrine (adrenaline) and norepinephrine (noradrenaline), which can substitute for AI-3 and acts as adrenergic antagonists inhibiting AI-3-induced gene expression. This cross-functionality is highly suggestive of interkingdom communication (Walters & Sperandio, 2006). Epinephrine and norepinephrine are adrenergic hormones which are vital in maintaining intestinal homeostasis in the mammalian gut. AI-3 produced by commensal intestinal microbiota may have a similar function. Enterohaemorrhagic *E. coli* (EHEC) can sense both host adrenergic hormones and AI-3. AI-3 binding in EHEC triggers a complex regulatory cascade which subsequently promotes growth, motility, cellular adherence, and virulence factor expression (Hughes & Sperandio, 2008).

The presence/absence of *ycoR* in the USA and UK strains indicate that there are genetic differences in the QS systems of 'New World' (North America) and 'Old World' (Europe and Japan) strains of *Y. enterocolitica*. There are two possibilities, UK strains may have lost *ycoR* or the USA strains may have gained *ycoR* through lateral gene transfer. Examination the *ycoR* locus of the 8081 strain and the equivalent region in the 90/54 strain (Figure 3.3a and 3.3b) shows that only *ycoR* is absent at this locus with the two adjacent genes, YE1025 and YE1027, remaining intact. The intergenic region is intact between YE1025 and *ycoR* but is largely missing between YE1027 and *ycoR*. There are small remnants of bases in the missing zone and this shows that the possibility of the UK strains having lost the *ycoR* is more plausible than the possibility of the USA strains having gained the *ycoR*. It is therefore possible that the *Y. enterocolitica* common ancestor acquired *ycoR* from a *Rhizobium* or closely related species. This common ancestor subsequently split and evolved independently into the 'New World' and 'Old World' strains and the ancestor of 'Old World' strains

eventually lost the *ycoR*. This loss of genetic material is not uncommon in *Yersinia*. *Y. pestis*, the causative agent of plague, is a clone that diverged recently from the enteric pathogen *Y. pseudotuberculosis* (Achtman *et al.*, 1999). Despite their close genetic relationship, they differ radically in their pathogenicity and transmission. A detailed genome comparison of *Y. pestis* and *Y. pseudotuberculosis* revealed that 13% of *Y. pseudotuberculosis* genes have been lost or no longer function in *Y. pestis* (Chain *et al.*, 2004). Genome rearrangements through sequence insertion and gene deletion resulted in the abolition and alteration of pre-existing gene expression pathways is instrumental in the evolution of *Y. pestis* to become a more virulent pathogen compared to *Y. pseudotuberculosis* (Chain *et al.*, 2004). Thus, the loss of the *ycoR* loci in UK strains of *Y. enterocolitica* maybe part of the evolutionary process.

In the following chapters, we attempt to elucidate how *ycoR* fits into the QS network of *Y. enterocolitica* 8081. As DNA microarrays are becoming more readily available for analysis of genome-wide gene expression patterns in many different microbial systems, an investigative approach using *Y. enterocolitica* DNA microarrays would provide insight into a possible discrete regulon for *ycoR*. Transcriptional profiling studies may also provide clues as to how YcoR function, whether by formation of heterodimers with YenR or by sequestration of the AHL signal molecules.

CHAPTER 4

MUTAGENESIS OF QS GENES AND TRANSCRIPTOMIC ANALYSIS OF QS IN *Y. ENTEROCOLITICA* 8081

4.1 Introduction

4.1.1 Mutagenesis

Previously, to disrupt or mutate bacterial chromosomal genes, the mutation needed to be created on a suitable suicide vector before recombining with the chromosome (Datsenko & Wanner, 2000). In contrast, genes can be directly disrupted in *Saccharomyces cerevisiae* by transformation with PCR fragments (with 35 to 60 nucleotides homology flanking the gene to be mutated). In both *S. cerevisiae* and *Candida albicans*, PCR-mediated gene replacement has greatly facilitated the generation of specific mutants. This method relies on the high efficiency of mitotic recombination in *S. cerevisiae*. However, except for a few naturally competent bacteria, most bacteria are not readily transformable with linear DNA because of the presence of intracellular exonucleases that degrade linear DNA (Datsenko & Wanner, 2000).

Datsenko and Wanner (2000) developed a mutagenesis method for *E. coli* based on a bacteriophage recombination system: the λ Red (γ, β, *exo*) system. The λ Red system includes three genes: γ, β, and *exo* (products, Gam, Bet, and Exo, respectively) (Murphy, 1998). Gam inhibits the host RecBCD exonuclease V so that Bet and Exo can gain access to DNA ends to promote recombination. The basic strategy is to replace a chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR using primers with 36 to 50 nucleotides extensions that are homologous to regions adjacent to the gene to be inactivated. The replacement is accomplished by Red-mediated recombination in these flanking homologies using the phage λ Red recombinase. This is encoded and synthesised under the control of an inducible promoter on a low copy number plasmid named pKD46, which also has an ampicillin resistance gene for selection. After mutagenesis, the λ Red helper plasmid can simply be cured by growth at 37°C because it has a temperature-sensitive replicon (Datsenko & Wanner, 2000). The λ Red recombinase system was adapted for use in *Y. enterocolitica* (Maxson & Darwin, 2004), which is naturally ampicillin resistant by replacing the ampicillin resistance of pKD46 with trimethoprim resistance at the unique PstI site to give pAJD434.

AHL synthesis is impaired in several *Y. pseudotuberculosis* and *Y. enterocolitica* QS mutants (Throup *et al.*, 1995; Atkinson *et al.*, 1999). Mutagenesis has also revealed a number of other important phenotypes. For example, in *Y. pseudotuberculosis*, QS represses clumping and swimming motility at 22°C (Atkinson *et al.*, 1999) while in *Y.*

enterocolitica strain 90/54 (serotype O:9) Δ*yenI*, swimming motility is temporally delayed while swarming motility is abolished. This impairment could be restored by complementation with a plasmid-borne copy of *yenI*. In contrast, an isogenic *yenI* mutant of the avirulent pYVe negative strain 10460 is not affected in swimming or swarming motility (Atkinson *et al.*, 2006).

4.1.2 Microarray technology

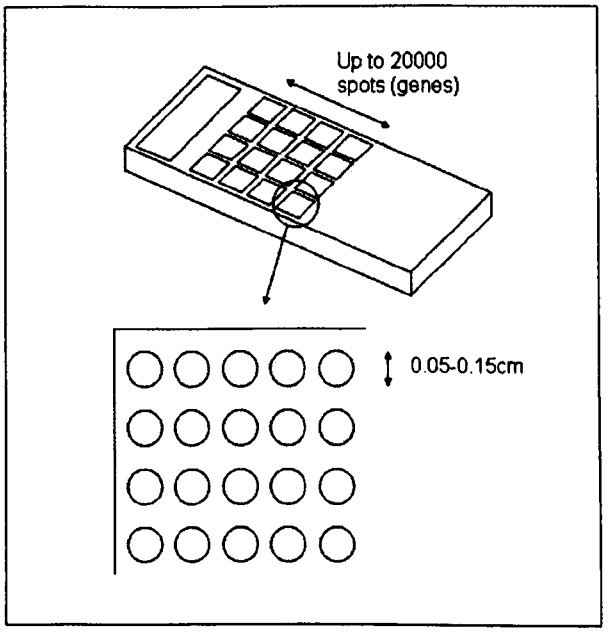
In the field of molecular biology, microarray technology has transformed the way biological systems are analysed. The use of microarrays allowed global investigation of cellular activity which would otherwise be limited to analysis of the individual functions of a few related genes or proteins. Microarray was initially developed in the 1990s for large-scale DNA mapping and sequencing (Hoheisel, 2006) with the first publication describing the application of DNA microarray technology for gene expression analysis in 1995 by Schena *et al.* Since the start of the millennium, microarray has been successfully developed as a tool for transcriptional profiling and has gained wide acceptance.

A DNA microarray is an impermeable solid support, usually glass, silicon chips or nylon membrane, on which PCR products or synthesised oligonucleotides which represent a single identified sequenced gene are systematically arranged and printed. The DNA microarrays used in this study were the YP v3 Pan *Yersinia* microarray which were printed on glass slides [produced by the Bacterial Microarray Group (BμG@S) at St George's, University of London] and were designed to contain a total of 20, 000 genes including all *Y. enterocolitica* 8081 genes, as well as *Y. pestis* CO92 and KIM genes.

Glass microarrays are produced by using a high-speed precision robot. This type of DNA microarray can have between 10,000 to 20,000 spots (genes) on an area of 3.6 cm^2 (http://www.lshtm.ac.uk/itd/grf/microarrayoverview.htm.). Each spot represents the product of a specific gene and is generated by depositing a few nanolitres of PCR product or oligonucleotides representing that specific gene usually at a concentration of 100 to 500 μg ml^{-1}. The diameter of each spot is typically 50 to 150 μm. Figure 4.1 shows a schematic representation of a glass (cDNA) microarray. For transcriptional profiling, RNA samples are isolated, from which cDNA is then generated, followed by fluorescent labelling of the cDNA probes and hybridisation of the sample to the immobilised target DNA. Finally, the hybridised microarray is scanned, and the image

analysed using sophisticated software programs to quantify and interpret the data. Microarray technology has continued to flourish in recent years and many different new applications have been developed. However, this type of transcriptional profiling may soon be falling out of favour because it focuses on a biological intermediate, RNA, which is highly unstable and prone to producing artefacts.

Peptide/antibody-based and protein microarrays are now being used for translational profiling (termed 'proteomics'). The proteome of an organism represents its entire complement of proteins (Wilkins *et al.*, 1996), including the modifications made to a particular set of proteins, as conditions such as time and or stresses vary. Hence, proteomics gives a much better understanding of an organism than genomics as the level of transcription of a gene is not an accurate estimate of its level of translation into a protein (Dhingra *et al.*, 2005; Rogers *et al.*, 2008). Another exciting advance in microarray technology is the development of cell microarrays. Cell microarrays transform microarray technology from an *in vitro* technology to an *in vivo* functional analysis tool where the microarray chip is spotted with varying materials, such as antibodies, proteins, or lipids, which allows for the multiplex interrogation of living cells, leading to their capture on specific spots (Sobek *et al.*, 2006; Chen & Davis, 2006).



(Marenne, 2003 #603).1. Glass cDNA microarray produced by using high-speed precision robot. Each microarray typically has between 10,000 to 20,000 spots (genes) on an area of 3.6 cm^2. Each spot (ranging from 50 to 150 μm) corresponds to the PCR product or oligonucleotide of a specific gene which is usually at a concentration of 100 to 500 μg ml^{-1} (Reproduced from http://www.lshtm.ac.uk/itd/grf/microarrayoverview.htm.).

4.1.3 Relative quantification PCR (RQ-PCR)

RQ-PCR measures PCR amplification of the cDNA template (which is derived from an RNA transcript) as it occurs, cycle-by-cycle, allowing quantitative measurements to be made in the highly reproducible exponential phase of PCR. This enables extremely accurate and precise quantification of the transcript concentration over a large dynamic range compared to traditional techniques. RQ-PCR assays using the SYBR® Green system, together with the gene expression analysis software with powerful data-viewing capabilities, allows for simultaneous analysis of up to ten 96-well plates (Applied Biosystems, USA).

SYBR Green binds to the minor groove of dsDNA and once bound to dsDNA it emits a signal 50 times greater than when unbound. In the RQ-PCR reaction, there is a point where the fluorescence signals becomes detectable (Figure 4.3). At this point in the reaction and when all other reagents are in excess, the limiting factor is the amount of template. Therefore, the cycle at which the fluorescence signal becomes detectable is determined by the amount of starting template. Thus, when comparing the amount of cDNA present in one sample with respect to another, the relative amount of starting template can be determined by comparing the cycle number at which the same amount of SYBR Green is detected when all of the PCR reactions are in exponential phase. Figure 4.2 shows the mechanisms which underlay RQ-PCR with SYBR Green.

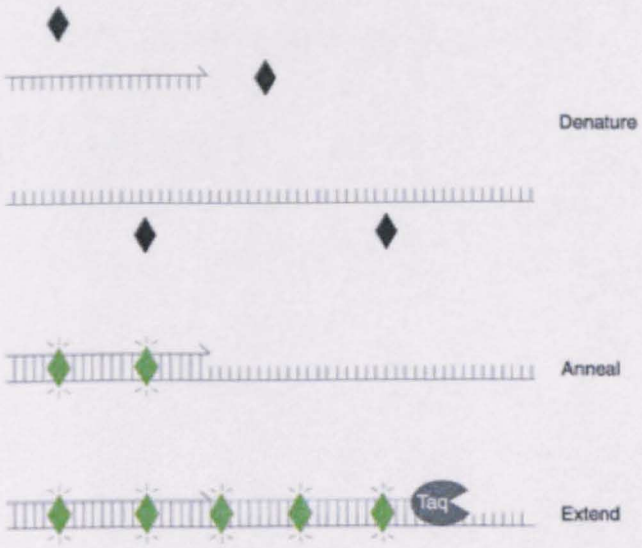


Figure 4.2. RQ-PCR with SYBR® Green. At the beginning of amplification, the reaction mixture contains the denatured single-stranded DNA, the primers, and the dye. The unbound dye molecules fluoresce weakly, producing a minimal background fluorescence signal which is subtracted during computer analysis. After annealing of the primers, dye molecules bind to the double stranded DNA resulting in a dramatic increase of the SYBR® Green molecules to emit light upon excitation. During elongation, more dye molecules bind to the newly synthesised DNA. As the reaction is monitored continuously in real-time, an increase in fluorescence is observed. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

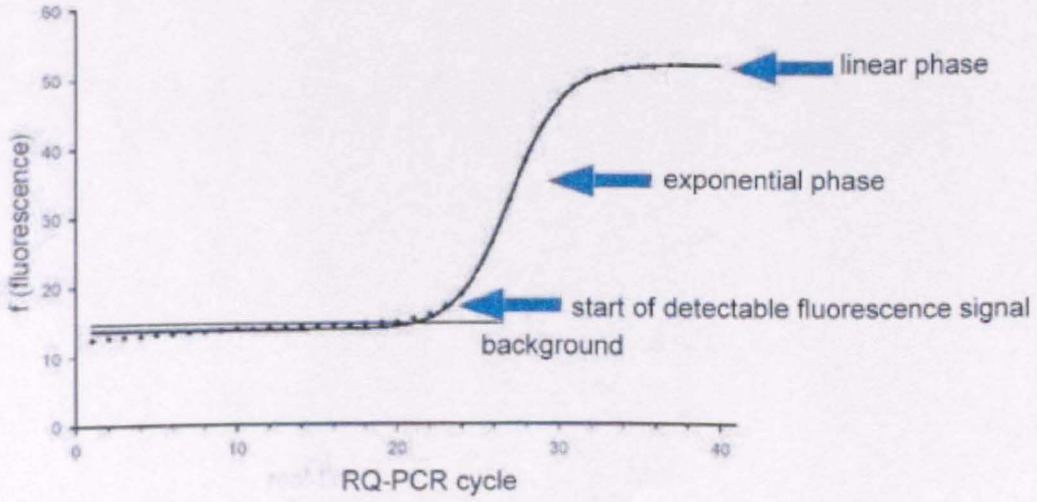


Figure 4.3. Graph showing the start of detectable fluorescence signal, the exponential and the linear phases of a RQ-PCR cycle (Adapted from www.appliedbiosystems.com).

RQ-PCR relies upon the dynamics of the PCR reaction; so it is essential to perform RQ-PCR when the PCR reactions are highly efficient. Also, as RQ-PCR relies upon the comparison of a Control Gene (CG) to the Gene Of Interest (GOI) (Bourgois *et al.*, 2001), the PCR efficiencies of CG and GOI need to be similar. The control gene is usually an endogenous control gene whose expression is expected to remain constant throughout the variety of conditions being tested. In this study, the *dnaE* gene was used as an endogenous control gene. *dnaE* is a housekeeping gene which encodes for the α chain of DNA polymerase III and is one of several genes which are routinely used in bacterial microarray experiments as a control gene (Townsend *et al.*, 2008). Other frequently used control genes are the 16S RNA and other house keeping genes including *polA* (DNA polymerase I), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *glnA* (glutamine synthetase, *recA* (DNA repair and recombination), *gyrB* (DNA gyrase B subunit) and *trpE* (anthranilate synthase component I). These genes are generally perceived as constitutively expressed in bacteria (Townsend *et al.*, 2008). Any inhibitors of the PCR reaction in one sample compared to another can be clearly seen and accounted for by the comparison to the endogenous control.

Standard curves have to be generated for each CG/GOI which entails serial dilution of the sample, leading to 5 or 6 different concentrations, covering 5 Logs, to ensure the sample detection is within the standard curve range. Another issue with SYBR Green detection is that although the primers in the PCR reaction should be specific to the gene, SYBR Green itself is not specific, so it could bind to potential primer dimers or non-specific product.

This chapter describes a microarray-based gene expression profile study of QS mutants of *Y. enterocolitica* 8081 compared with wildtype and the use of RQ-PCR assay to verify some of the key microarray results.

4.2 Results

4.2.1 Construction of QS mutants in Y. enterocolitica *8081*

Deletion insertion mutants were constructed in the QS genes of *Y. enterocolitica* 8081 in order to assess their contribution to the QS phenotype in the bacterium. Each deletion was designed to preserve the existing reading frame to avoid any polar effects. Using the λ Red recombinase method (See Section 2.12), seven QS mutants were successfully constructed. 3 of them were single mutants (Δ*yenR*, Δ*yenI* and Δ*ycoR*), 3 were double mutants (Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenRycoR*) and 1 was a triple mutant (Δ*yenIyenRycoR*). The Δ*yenR*, Δ*yenI* and Δ*ycoR* mutations were constructed by using PCR products generated from primer pairs yenICm-r/yenICm-r; yenRKan-f/yenRKan-r and ycoRSm-f/ycoRSm-r, respectively (refer to Table 2.5). Deletions of 660 bp (from position 10 at the beginning of the start codon to position 670) in *yenR*; 627 bp (from position 10 at the beginning of the start codon to position 637) in *yenI*; and 597 bp (from position 12 at the beginning of the start codon to position 606) in *ycoR* were made and kanamycin, chloramphenicol or streptomycin resistance cassettes were inserted, respectively. Double mutants were then constructed using the appropriate single mutants and PCR products for the second gene of interest. Likewise, a triple mutant was constructed using the double Δ*yenIyenR* mutant and PCR product for mutating *ycoR*. Colony PCR was used as a screen for *yenR*, *yenI* and *ycoR* mutation using primer pairs yenRf/yenRr; yenIf/yenIr and ycoRf/ycoRr respectively (refer to Table 2.5). The primer pairs gave products of 1,152 bp (mutant) and 885 bp (wildtype); 917 bp (mutant) and 779 bp (wildtype); and 1,182 bp (mutant) and 827 bp (wildtype), respectively.

All mutants were confirmed by Southern hybridization and sequencing. An example of mutant confirmation is shown in Appendix 1 where the sequencing results and alignment studies with the predicted sequence of the cloned Δ*yenR* mutant showed exact matches at the start and end of the insertion of the kanamycin resistance cassette is shown. Figure 4.4 is a diagrammatic illustration of the 7 QS mutants constructed.

Figure 4.4. The seven QS mutants of *Y. enterocolitica* 8081 constructed for this study.

4.2.2 Microarray-based gene expression profile study in QS mutants

The RNA expression profiles of five *Y. enterocolitica* 8081 QS mutants (Δ*yenI*; Δ*yenR*; Δ*ycoR*; Δ*yenRycoR* and Δ*yenIyenRycoR*) were compared with the wildtype in this microarray study (see Section 2.17). Each strain was grown at 22°C or 37°C and RNA isolation was performed at the late-log phase of growth (~OD_{600} 0.75). Figures 4.5 and 4.6 shows the growth curves of *Y. enterocolitica* 8081 wildtype and 5 QS mutants at 22°C or 37°C, respectively. The point when RNA extractions were done is also shown. All experiments were done in triplicate and statistically significant up or down-regulated genes list were generated using a Student's T-test ($p \leq 0.05$).

Figure 4.7 is a representative gel image of RNA samples analysed with a bioanalyser showing the distinctive 3 bands profile of whole cell RNA extracted from *Y. enterocolitica* 8081 wildtype and Δ*yenI* mutant. Figure 4.8 shows a representative electropherogram of RNA samples extracted from the wildtype grown at 22°C. The additional peak is probably a 23S RNA fragment which is characteristic for a number of *Yersinia* spp., including *Y. enterocolitica* and *Y. pseudotuberculosis* (Hinds, J., personal communication). Figure 4.9 shows a representative microarray chip hybridised with Cy3-labelled DNA (green) and Cy5-labelled RNA (red).

Figure 4.10 shows a Venn diagram illustrating the general overview of the QS regulon in *Y. enterocolitica* 8081. It shows the number of genes which are regulated (up- or down-regulated with a cut off value of 1.5 fold) exclusively by each of the single mutants (Δ*yenI*, Δ*yenR* and Δ*ycoR*) and the number of their overlapping sets of genes. For simplicity, data from both the 22°C and 37°C microarray were combined. In addition, the double and triple mutants (Δ*yenRycoR* and Δ*yenIyenRycoR*) were not included in this overview. The microarray contained oligonucleotides representing a total of 4,291 CDS from the *Y. enterocolitica* 8081 genome, of which 4,208 are from the chromosome and 83 are from the pYVe plasmid. Number of genes which are regulated by *yenI*, *yenR* and *ycoR* is 2,427 (56.6% of the total number of CDS). The number of genes exclusively regulated by *yenI*, *yenR* and *ycoR* is 322 (13.3%), 510 (21.0%) and 460 (19.0%), respectively. Of the 2,427 genes, 330 genes (13.6%) are regulated by all three genes. The *yenI* and *yenR* regulon shares 169 genes (7.0%), the *yenI* and *ycoR* shares 148 genes (6.1%); and the *yenR* and *ycoR* shares 488 genes (20.1%).

Table 4.2 shows a selection of genes which are up or downregulated in the 5 QS mutants compared to the wildtype at 22°C or 37°C. The RNA expression profiles of *Y. enterocolitica* 8081 QS mutants (Δ*yenI*; Δ*yenR*; Δ*ycoR*; Δ*yenRycoR* and Δ*yenIyenRycoR*) revealed some clues on the regulation within the QS system of *Y. enterocolitica* 8081 (See Table 4.2, no. 209-211 and 458-460) which is summarised as follows:

In the Δ*yenI* microarray at 22°C or 37°C, *yenR* expression was upregulated by 4.8 and 5.2 fold, suggesting that the AHLs associated with YenI repress *yenR* expression at both temperatures. In the Δ*yenR* microarray at 22°C, *ycoR* expression was upregulated by 3.1 fold, suggesting that YenR represses *ycoR* at 22°C. In the Δ*ycoR* microarray at 22°C or 37°C, *yenI* expression was upregulated by 2.3 fold and downregulated by 2.3 fold, respectively, suggesting that YcoR represses *yenI* at 22°C and activates *yenI* at 37°C.

Table 4.2 lists many of the key genes whose expression was altered in the QS mutants and include genes related to flagellar, type III secretion (T3SS), methylation, histidine transport and tryptophan metabolism, as well as genes in the high-pathogenicity island (HPI). For ease of reference in the following discussion, the gene cluster names and numbers for selected groups of genes corresponding to Table 4.2 are given below:

Table 4.1. Gene cluster names and numbers for selected groups of genes corresponding to Table 4.2.

Group of genes	Gene cluster name	Temperature	
		22°C	37°C
Flagellar-related	*flg, flh, fli*	95-137	344-386
Type III secretion system	*yop, ysc, syc, vir*	36-66, 73-80	285-315, 322-329
Plasmid partition and replication	*spy, rep*	67-72	316-321
DNA adenine methylation-related	*dam*	5-6	254-255
Tryptophan metabolism-related	-	1-4	250-253
High Pathogenicity Island	*irp, ybt*	197-208	446-457
Histidine transport-related	*his*	9-12	258-261

* numbers in the last two columns correspond to selected groups of genes in Table 4.2

The expression trend of flagellar-related genes is predominantly downwards in all the QS mutants at 22°C but in contrast, is upwards at 37°C. The trend of expression for T3SS-related genes is upwards at 22°C for all mutants except for the Δ*yenRycoR* where the trend is downwards. At 37°C, all mutants show a downward trend in expression for T3SS-related genes. Expressions of pYVe plasmid partition and replication genes are predominantly downwards at 22°C. At 37°C, plasmid replication genes expressions are upwards in the single mutants (Δ*yenI*, Δ*yenR* and Δ*ycoR*) but are downwards in the Δ*yenRycoR* and Δ*yenIyenRycoR* mutants. Expressions of plasmid partition genes at 37°C are in a downward trend in all QS mutants. DNA Methylation and tryptophan metabolism-related genes, as well as HPI genes are mostly up-regulated at both temperatures in the QS mutants. Histidine tramsport-related genes are all upregulated in expression at both temperatures in the QS mutants.

From the compiled list in Table 4.2, seven genes of particular interest were chosen as targets in further experiments because of their involvement of in Type III secretion and/or virulence. Six of these genes, *virF*, *yscF*, *yadA*, *repA*, *spyA* and *tyeA*, are encoded on the pYVe plasmid whereas *invA* is encoded chromosomally.

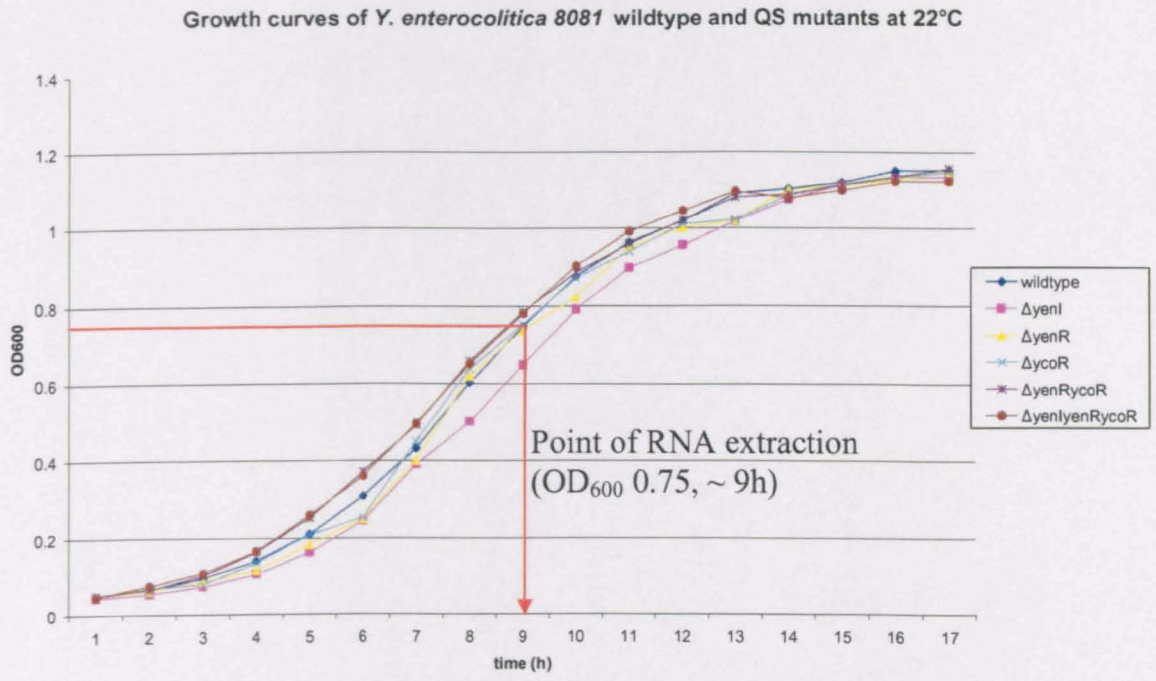


Figure 4.5. Growth curves of *Y. enterocolitica* 8081 wildtype and 5 QS mutants at 22°C. Red arrow denotes the point where RNA extractions were performed.

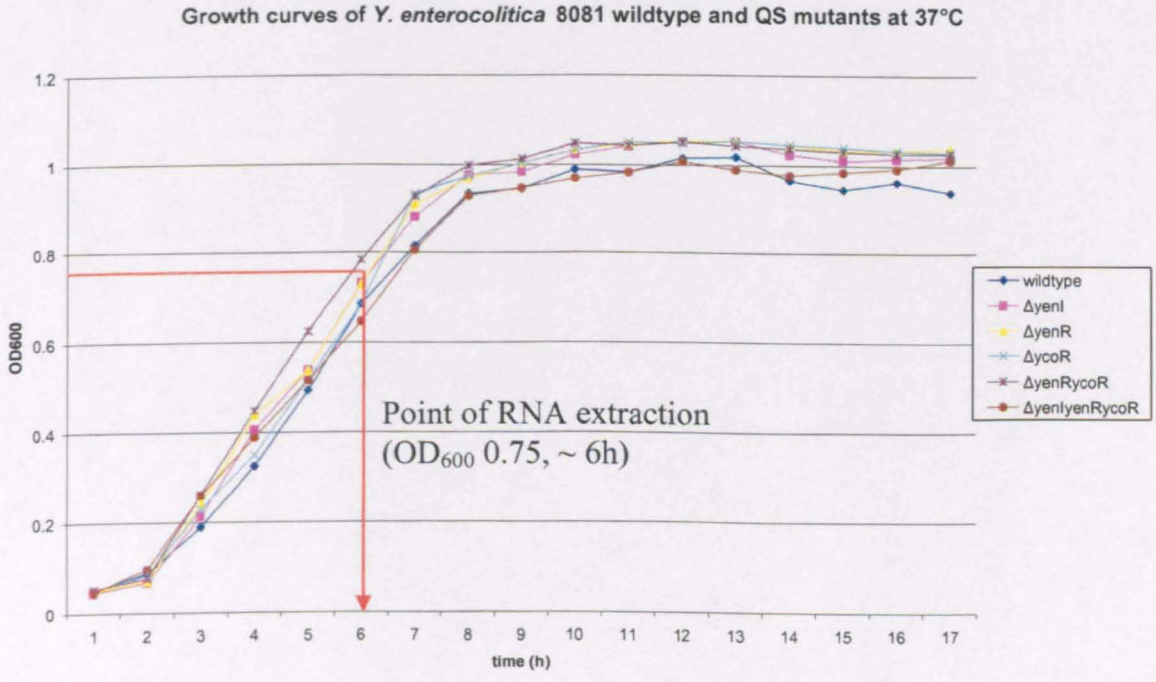


Figure 4.6. Growth curves of *Y. enterocolitica* 8081 wildtype and 5 QS mutants at 37°C. Red arrow denotes the point where RNA extractions were performed.

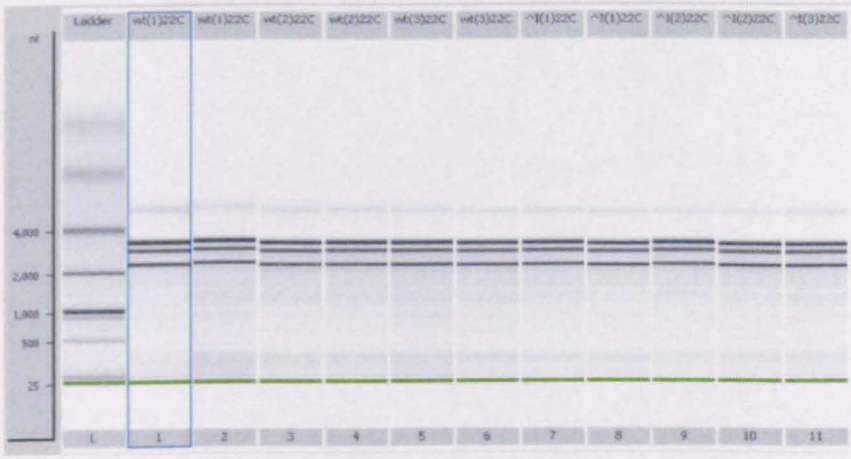


Figure 4.7. The characteristic 3 bands profile of RNA extracted from *Y. enterocolitica* 8081 on a representative gel image, analysed with a bioanalyser. Whole cell RNA was extracted from *Y. enterocolitica* 8081 wildtype and Δ*yenI* mutant. Lane L: RNA ladder. Lanes 1 to 6: RNA from wildtype grown at 22°C. Lanes 7 to 11: Δ*yenI* mutant grown at 22°C.

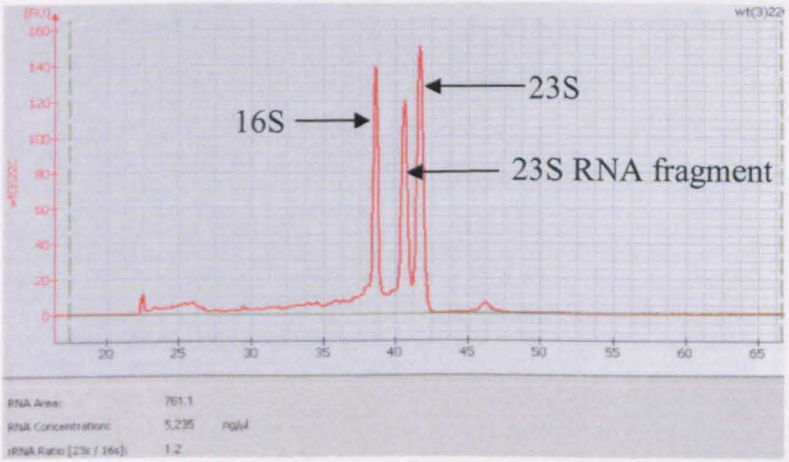


Figure 4.8. The characteristic 3 bands profile of RNA on a representative electropherogram, extracted from *Y. enterocolitica* 8081 wildtype grown at 22°C. The additional peak is probably a 23S RNA fragment which is characteristic for a number of *Yersinia* spp., including *Y. enterocolitica* and *Y. pseudotuberculosis* (Hinds, J., personal communication).

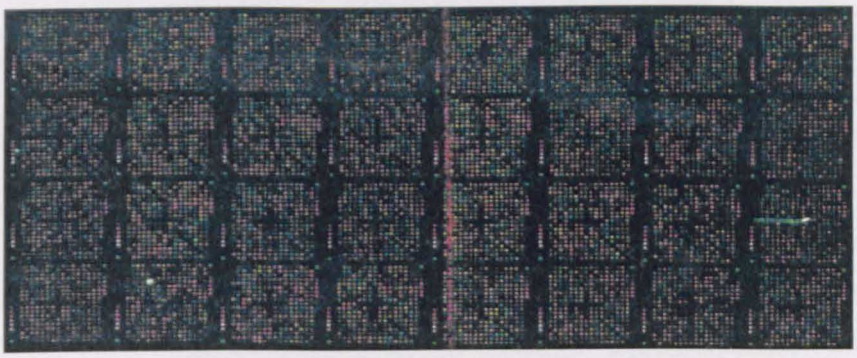


Figure 4.9. A representative microarray chip hybridised with Cy3-labelled DNA (green) and Cy5-labelled RNA (red).

Figure 4.10. Venn diagram illustrating the general overview of the QS regulon in *Y. enterocolitica* 8081. It shows the number of genes which are regulated (up- or down-regulated with a cut off value of 1.5 fold) exclusively by each of the single mutants (Δ*yenI*, Δ*yenR* and Δ*ycoR*) and the number of their overlapping sets of genes.

Table 4.2. Results of the microarray experiment. A selection of genes with altered expression at 22°C and 37°C, expressed as fold changes with a cut off value of 1.5.

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
1	Ye8081-0650	tnaA	tryptophanase	97.8 ↑	20.8 ↑	47.3 ↑	18.4↑	3.9 ↑
2	Ye8081-0649	mtr	tryptophan-specific transport protein	84.2 ↑	7.5 ↑	15.3↑	5.8 ↑	3.1 ↑
3	Ye8081-0743	rumA	23s rRNA (uracil-5-)-methyltransferase (tryptophan metab		1.5 ↓	1.7 ↓		
4	Ye8081-1222	ipdC	indole-3-pyruvate decarboxylase (tryptophan metabolism)	1.7 ↓				2.1 ↑
5	Ye8081-3972	dam	DNA adenine methylase	4.1 ↓	2.4 ↑		17.7 ↓	
6	Ye8081-2361	dam	putative adenine-specific modification methylase		2.6 ↑	1.8 ↑	3.5 ↑	8.5↑
7	Ye8081-0893	clpB	Clp ATPase					4.3 ↓
8	Ye8081-2696	clpB5	Clp ATPase				3.4 ↑	8.0 ↑
9	Ye8081-1318	hisJ	histidine-binding periplasmic protein	1.7 ↑	2.0 ↑	1.5 ↑		2.2 ↑
10	Ye8081-1319	hisQ	histidine transport system permease protein HisQ	2.9 ↑	2.0 ↑	1.9 ↑	2.9 ↑	4.5 ↑
11	Ye8081-1320	hisM	histidine transport system permease protein HisM	3.3 ↑	2.8 ↑	2.5 ↑	3.5 ↑	4.5 ↑
12	Ye8081-1321	hisP	histidine transport ATP-binding protein HisP		1.9 ↑	1.7 ↑	4.5 ↑	5.7 ↑
13	Ye8081-1393	nrdA	ribonucleoside-diphosphate reductase 1 alpha chain					
14	Ye8081-1392	nrdB	ribonucleoside-diphosphate reductase 1 beta chain					2.4 ↓
15	Ye8081-0482	nrdD	anaerobic ribonucleoside-triphosphate reductase				1.6 ↑	3.2 ↑
16	Ye8081-0481	nrdG	anaerobic ribonucleoside-triphosphate reductase activating					2.4 ↑
17	Ye8081-0927	nrdE	ribonucleoside-diphosphate reductase 2 alpha chain				2.0 ↑	3.1 ↑
18	Ye8081-0926	nrdF	ribonucleoside-diphosphate reductase 2 beta chain				4.1 ↑	6.1 ↑
19	Ye8081-2092	acpD	acyl carrier protein phosphodiesterase				5071.0 ↓	2.3 ↓
20	Ye8081-1636	acpP	acyl carrier protein					112.6 ↓
21	Ye8081-0309	acs	acetyl-coenzyme A synthetase	4.5 ↑	3.2 ↑	2.1 ↑		
22	Ye8081-2001	araA	L-arabinose isomerase		1.9 ↓			
23	Ye8081-2002	araB	L-ribulokinase				4.1 ↑	
24	Ye8081-2006	araC	arabinose operon regulatory protein				1.7 ↑	
25	Ye8081-1976	araD	L-ribulose-5-phosphate 4-epimerase			1.6 ↓	2.7 ↑	
26	Ye8081-2003	araF	L-arabinose-binding periplasmic protein precursor				4.8 ↑	1.9 ↑
27	Ye8081-2004	araG	L-arabinose transport ATP-binding protein				3.0 ↑	2.0 ↑
28	Ye8081-2005	araH	L-arabinose transport system permease protein				2.9 ↑	

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
29	Ye8081-2238	adhE	aldehyde-alcohol dehydrogenase		1.5 ↓			1.7 ↓
30	Ye8081-2460	hutC	putative GntR-family transcriptional regulatory protein	1.7 ↑		1.5 ↑	4.2 ↓	3.1 ↓
31	Ye8081-2461	hutI	imidazolonepropionase				28.3 ↓	16.0 ↓
32	Ye8081-2462	hutG	putative N-formylglutamate amidohydrolase				48.6 ↓	21.1 ↓
33	Ye8081-4095	hutU	urocanate hydratase		2.2 ↑	1.7 ↑	7.9 ↓	6.5 ↓
34	Ye8081-4094	hutH	histidine ammonia-lyase	2.4 ↑	2.4 ↑	1.8 ↑	9.9 ↓	3.0 ↓
35	YepYV-51c	yomA	YomA			1.7 ↑		11.6 ↑
36	YepYV-07c	yopB	translocator YopB					1.5 ↑
37	YepYV-44	yopE	Yop effector YopE					2.4 ↑
38	YepYV-41	yopH	Yop effector YopH					2.5 ↑
39	YepYV-04c	yopM	Yop effector YopM	1.5 ↑				2.9 ↑
40	YepYV-17c	yopN	YopN					
41	YepYV-60	yopO	protein kinase YopO					
42	YepYV-61	yopP	Yop effector YopP				2.2 ↓	
43	YepYV-01c	yopQ	YopQ					
44	YepYV-02	yopT	Yop effector YopT					
45	YepYV-19	yscO	YscO	1.7 ↑				
46	YepYV-20	yscP	YscP					
47	YepYV-14c	yscX	YscX					
48	YepYV-30	yscC	YscC					1.8 ↑
49	YepYV-0003	yscw	VirG	1.5 ↑	1.5 ↑			2.9 ↑
50	YepYV-37	yscJ	YscJ				1.9 ↓	1.5 ↓
51	YepYV-25	yscU	YscU		1.7 ↓		1.7 ↑	11.7 ↓
52	YepYV-24	yscT	YscT					
53	YepYV-23	yscS	YscS				3.5 ↓	

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
54	YepYV-21	yscQ	YscQ	1.5 ↑				1.6 ↑
55	YepYV-18	yscN	YscN	2.4 ↑	1.6 ↑		1.7 ↑	3.8 ↑
56	YepYV-29	yscB	YscB					2.3 ↑
57	YepYV-31	yscD	YscD					
58	YepYV-32	yscE	YscE					40764.0 ↓
59	YepYV-34	yscG	YscG					
60	YepYV-35	yscH	YscH					
61	YepYV-36	yscI	YscI				1.5 ↓	
62	YepYV-38	yscK	YscK				1.8 ↓	1.7 ↓
63	YepYV-39	yscL	YscL			1.5 ↓		
64	YepYV-40	yscM1	regulatory protein YscM1		2.0 ↑			6.1 ↑
65	YepYV-0010	yscM2	regulatory protein YscM2					
66	YepYV-13c	yscY	YscY				2.6 ↓	
67	YepYV-67	repA	putative replication initiator protein RepA				1.5 ↓	
68	YepYV-66	repB	putative leader peptide RepB	3.5 ↑			10.8 ↓	40638.0 ↓
69	YepYV-0012	repC	putative replication protein RepC				2.41 ↓	2.6 ↓
70	YepYV-65	repC	putative replication protein RepC				1.8 ↓	1.7 ↓
71	YepYV-49c	spyA	SpyA (partition)	2.3 ↓			4.0 ↓	
72	YepYV-48c	spyB	SpyB (partition)				1.7 ↓	
73	Ye8081-3538	sycA	putative type III secretion apparatus protein					
74	YepYV-08c	sycD	YopB/D chaperone SycD					
75	YepYV-43c	sycE	YopE chaperone SycE		1.6 ↑			
76	YepYV-42c	sycH	YopH chaperone SycH				2.7 ↓	
77	YepYV-03	sycT	YopT chaperone SycT					
78	YepYV-16c	tyeA	TyeA (control of Yop release; required for delivery of YopE and YopH)				2.1 ↓	
79	YepYV-27	virF	transcriptional activator VirF	1.8 ↑	1.7 ↑		2.5 ↑	4.5 ↑
80	YepYV-26	virG	VirG (required for localization of YscC)					2.7 ↑

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
81	YepYV-52c	yadA	adhesin YadA	1.5 ↑				2.4 ↑
82	Ye8081-3548	ysaC	possible type III secretion system protein		4.0 ↑		5.8 ↑	18.9 ↑
83	Ye8081-3549	ysaE	AraC family regulatory protein		1.6 ↑		2.7 ↑	5.2 ↑
84	Ye8081-3557	ysaH	possible type III secretion system effector protein					3.2 ↑
85	Ye8081-3552	ysaI	putative virulence associated protein				2.1 ↑	3.0 ↑
86	Ye8081-3553	ysaJ	type III secretion system apparatus lipoprotein	1.5 ↑			2.8 ↑	2.7 ↑
87	Ye8081-3545	ysaK	type III secretion sytem protein				1.8 ↑	3.1 ↑
88	Ye8081-3544	ysaN	type III secretion sytem protein		1.9 ↑		3.6 ↑	8.5 ↑
89	Ye8081-3542	ysaR	type III secretion apparatus protein		2.2 ↑		3.7 ↑	8.4 ↑
90	Ye8081-3540	ysaT	putative type III secretion apparatus protein		2.2 ↑		4.6 ↑	8.8 ↑
91	Ye8081-3539	ysaU	putative type III secretion apparatus protein		1.8 ↑		2.9 ↑	4.7 ↑
92	Ye8081-3546	ysaV	type III secretion system apparatus protein		2.2 ↑		2.9 ↑	7.2 ↑
93	Ye8081-3547	ysaW	type III secreted effector protein		2.1 ↑		2.6 ↑	5.6 ↑
94	Ye8081-3537	yspB	putative type III secretion system effector protein		1.5 ↑			
95	Ye8081-2561	flgA	flagella basal body P-ring formation protein FlgA					
96	Ye8081-2560	flgB	flagellar basal-body rod protein FlgB		2.3 ↓			15.3 ↓
97	Ye8081-2559	flgC	flagellar basal-body rod protein FlgC					
98	Ye8081-2558	flgD	basal-body rod modification protein FlgD					
99	Ye8081-2557	flgE	flagellar hook protein FlgE		1.8 ↓			5.0 ↓
100	Ye8081-2556	flgF	flagellar basal-body rod protein FlgF					3.2 ↓
101	Ye8081-2555	flgG	flagellar basal-body rod protein FlgG		2.1 ↓		1.9 ↓	4.3 ↓
102	Ye8081-2554	flgH	flagellar L-ring protein precursor					
103	Ye8081-2553	flgI	flagellar P-ring protein precursor				1226 ↓	
104	Ye8081-2552	flgJ	flagellar protein FlgJ					
105	Ye8081-2551	flgK	flagellar hook-associated protein 1				1.5 ↓	
106	Ye8081-2550	flgL	flagellar hook-associated protein 3					
107	Ye8081-2562	flgM	negative regulator of flagellin synthesis					3.6 ↓
108	Ye8081-2563	flgN	flagella synthesis protein FlgN					2.9 ↓
109	Ye8081-2567	flhB	flagellar biosynthetic protein FlhB	1.9 ↑				4.5 ↑
110	Ye8081-2580	flhC	flagellum biosynthesis transcription activator				1.7 ↑	

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
111	Ye8081-2581	flhD	flagellar transcriptional activator				2.2 ↓	
112	Ye8081-2565	flhE	flagellar protein FlhE precursor					
113	Ye8081-2519	fliA	RNA polymerase sigma factor for flagellar operon				1.9 ↓	
114	Ye8081-2520	fliB	flagellin lysine-N-methylase					
115	Ye8081-2522	fliC	thermoregulated motility protein					
116	Ye8081-2521	fliC2	flagellin					1.8 ↓
117	Ye8081-2523	fliC3	flagellin					1.9 ↓
118	Ye8081-2524	fliD	flagellar hook-associated protein 2	2.3 ↑				1.7 ↑
119	Ye8081-2536	fliE	flagellar hook-basal body complex protein FliE		2.0 ↓		1.9 ↓	2.6 ↓
120	Ye8081-2537	fliF	flagellar M-ring protein					
121	Ye8081-2538	fliG	flagellar motor switch protein FliG	1.7 ↓	1.7 ↓			2.2 ↓
122	Ye8081-2539	fliH	flagellar assembly protein		1.9 ↓			
123	Ye8081-2540	fliI	flagellum-specific ATP synthase					
124	Ye8081-2541	fliJ	flagellar protein FliJ		1.4 ↓		1.8 ↓	2.3 ↓
125	Ye8081-2542	fliK	flagellar hook-length control protein FliK		1.6 ↓			1.3 ↓
126	Ye8081-2543	fliL	flagellar protein FliL	1.9 ↓			1.9 ↓	2.8 ↓
127	Ye8081-2544	fliM	flagellar motor switch protein FliM		1.9↓		2.0 ↓	2.5 ↓
128	Ye8081-2545	fliN	flagellar motor switch protein FliN		2.2 ↓			2.1 ↓
129	Ye8081-2546	fliO	flagellar protein FliO					
130	Ye8081-2547	fliP	flagellar biosynthetic protein FliP				1.6 ↓	
131	Ye8081-2548	fliQ	flagellar biosynthetic protein FliQ				3.7 ↓	
132	Ye8081-2549	fliR	flagellar biosynthetic protein FliR					4.0 ↑
133	Ye8081-2525	fliS	flagellar protein FliS				1.8 ↓	2.2 ↓
134	Ye8081-2526	fliT	FliT				2.1 ↓	3.0 ↓
135	Ye8081-2516	fliY	putative cystine-binding periplasmic protein			3.2 ↑	2.7 ↑	
136	Ye8081-2518	fliZ	putative alternative sigma factor regulatory protein		1.8 ↓			3.9 ↓
137	Ye8081-1298	flk	putative flagellar assembly regulatory protein, Flk					2.2 ↑
138	Ye8081-2579	motA	chemotaxis MotA protein				1.7 ↓	
139	Ye8081-2578	motB	chemotaxis MotB protein				2.5 ↓	1.8 ↓

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
140	Ye8081-2564	invA	invasin				4.3 ↑	3.9 ↑
141	Ye8081-2949	gltA	citrate synthase GltA					
142	Ye8081-3735	gltB	glutamate synthase [NADPH] large chain precursor					
143	Ye8081-3736	gltD	glutamate synthase [NADPH] small chain					
144	Ye8081-2992	gltJ	putative glutamate/aspartate transport system permease	3.6 ↑	2.7 ↑	2.4 ↑	3.8 ↑	5.0 ↑
145	Ye8081-2993	gltK	putative glutamate/aspartate transport system permease	2.5 ↑	2.3 ↑	2.1 ↑	3.0 ↑	3.7 ↑
146	Ye8081-2994	gltL	putative glutamate/aspartate transport ATP-binding protein					
147	Ye8081-0310	gltP	proton glutamate symport protein		1.4 ↓	1.5 ↓	1.8 ↓	
148	Ye8081-1879	gltP	putative transport protein		4.3 ↑	5.7 ↑	2.5 ↑	
149	Ye8081-0042	gltS	sodium/glutamate symport carrier protein			1.3 ↑		
150	Ye8081-1215	gltX	glutamyl-tRNA synthetase		1.4 ↓	1.7 ↓		2.0 ↓
151	Ye8081-0027	glnA	glutamine synthetase					3.0 ↓
152	Ye8081-1039	glnB	nitrogen regulatory protein P-II	3.4 ↓	1.9 ↓	2.1 ↓		1.6 ↓
153	Ye8081-3674	glnE	glutamate-ammonia-ligase adenylyltransferase					
154	Ye8081-2991	glnH	putative amino acid-binding protein precursor	2.4 ↑	1.6 ↑	1.5 ↑		
155	Ye8081-2845	glnH	putative glutamine-binding periplasmic protein	2.3 ↑	1.5 ↑			
156	Ye8081-2846	glnP	putative glutamine transport system permease	1.8 ↑	1.6 ↑		2.8 ↑	2.7 ↑
157	Ye8081-2847	glnQ	putative glutamine transport ATP-binding protein	1.9 ↑			3.1 ↑	2.4 ↑
158	Ye8081-2977	glnS	glutaminyl-tRNA synthetase			1.6 ↓		
159	Ye8081-3300	gcvA	glycine cleavage system transcriptional activator					1.6 ↑
160	Ye8081-3391	gcvP	glycine dehydrogenase					
161	Ye8081-1136	gcvR	glycine cleavage system transcriptional repressor					
162	Ye8081-3393	gcvT	aminomethyltransferase					
163	Ye8081-2792	cdd	cytidine deaminase					1.8 ↑
164	Ye8081-4067	dctA	C4-dicarboxylate transport protein		1.7 ↓	1.5 ↓		2.6 ↓
165	Ye8081-1533	focA	putative formate transporter 1	1.5 ↑				
166	Ye8081-2812	focA	putative formate transporter		1.6 ↑		2.3 ↑	4.1 ↑

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
167	Ye8081-1975	osmB	osmotically inducible lipoprotein B precursor					1.8 ↑
168	Ye8081-0565	osmY	osmotically inducible protein Y					
169	Ye8081-0655	leuO	LysR-family transcriptional regulator LeuO					1.6 ↑
170	Ye8081-1581	ompA	putative outer membrane porin A protein					
171	Ye8081-1401	ompC	outer membrane protein C, porin	1.5 ↓		1.7 ↓		2.0 ↓
172	Ye8081-2856	ompC2	outer membrane protein C2	1.6 ↓				
173	Ye8081-1563	ompF	putative outer membrane porin F protein					
174	Ye8081-3275	ompH	cationic 19 kDa outer membrane protein precursor				2.0 ↓	2.0 ↓
175	Ye8081-4004	ompR	transcriptional regulatory protein				1.7 ↓	2.0 ↓
176	Ye8081-2216	ompW	putative exported protein	1.5 ↓		1.6 ↓		
177	Ye8081-2835	ompX	putative outer membrane protein				1.7 ↓	2.9 ↓
178	Ye8081-3776	mgtA	Mg2+ transporter ATPase					2.7 ↑
179	Ye8081-2585	mgtB	Mg(2+) transport ATPase protein B				3.1 ↑	3.2 ↑
180	Ye8081-2586	mgtC	Mg(2+) transport ATPase protein C				1.7 ↑	
181	Ye8081-1532	pflB	formate acetyltransferase 1 (SAM)					
182	Ye8081-1681	YE1681	prophage encoded DNA modification methylase (biosynthesis					
183	Ye8081-0710	speE	spermidine synthase (SAM)					1.5 ↓
184	Ye8081-3228	mtrK	possible 5-methylthioribose kinase (SAM)					
185	Ye8081-1470	uptG	cystathionine beta-lyase (biosynthesis of methionine, which	3.0 ↑	28.8 ↑	63.2 ↑	45.0 ↑	16.4 ↑
186	Ye8081-1469	uptH	putative cystathionine gamma-synthase (SAM)	1.5 ↑		30.6↑	18.7 ↑	5.6 ↑
187	Ye8081-4122	xylA	xylose isomerase	3.0 ↑		1.7 ↑		
188	Ye8081-4123	xylB	xylulose kinase	2.5 ↑		1.5 ↑		
189	Ye8081-2908	bioA	adenosylmethionine-8-amino-7-oxononanoate aminotransfera					3.5 ↑
190	Ye8081-2907	bioB	biotin synthase		1.8 ↑		1.9 ↑	3.7 ↑
191	Ye8081-2905	bioC	biotin synthesis protein BioC		1.7 ↑		2.1 ↑	4.8 ↑
192	Ye8081-2904	bioD	dethiobiotin synthetase				1.8 ↑	4.9 ↑
193	Ye8081-2024	bioD	putative dethiobiotin synthetase					
194	Ye8081-2906(	bioF	8-amino-7-oxononanoate synthase		6.1 ↑		6.3 ↑	23.5 ↑
195	Ye8081-3995	bioH	putative biotin biosynthesis protein					2.5 ↑

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
196	Ye8081-0478	phnF	probable gntR-family transcriptional regulatory protein		3.151 ↑		2.7 ↑	7.9 ↑
197	Ye8081-2618	irp1	yersiniabactin biosynthetic protein			2.0 ↑	3.8 ↑	5.1 ↑
198	Ye8081-2617	irp2	yersiniabactin biosynthetic protein	1.7 ↑		2.5 ↑	4.6 ↑	3.9 ↑
199	Ye8081-2616	ybtA	transcriptional regulator YbtA	1.7 ↑	2.3 ↑		4.6 ↑	6.2 ↑
200	Ye8081-2621	ybtE	yersiniabactin siderophore biosynthetic protein					
201	Ye8081-2615	ybtP	lipoprotein inner membrane ABC-transporter	2.2 ↑	2.5 ↑	2.3 ↑	3.1 ↑	5.8 ↑
202	Ye8081-2614	ybtQ	inner membrane ABC-transporter YbtQ	2.8 ↑	5.3 ↑	1.7 ↑	6.7 ↑	16.2 ↑
203	Ye8081-2612	ybtS	putative salicylate synthetase	1.5 ↑			3.1 ↑	
204	Ye8081-2620	ybtT	yersiniabactin biosynthetic protein YbtT					
205	Ye8081-2619	ybtU	yersiniabactin biosynthetic protein YbtU	1.3 ↑	1.6 ↑	2.1 ↑	2.6 ↑	
206	Ye8081-2613	ybtX	putative signal transducer	2.0 ↑	3.2 ↑	1.9 ↑	5.4 ↑	13.0 ↑
207	Ye8081-2622	fyuA	pesticin/yersiniabactin receptor protein		1.6 ↓	1.9 ↑	3.5 ↑	3.3 ↓
208	Ye8081-2611	int	integrase					
209	Ye8081-1600	yenI	N-acylhomoserine lactone synthase	10.5 ↓		2.3 ↓		
210	Ye8081-1599	yenR	quorum-sensing transcriptional activator	4.9 ↑	5.3 ↓		6.7 ↓	3.5 ↓
211	Ye8081-1026	ycoR	LuxR-family transcriptional regulator		3.1 ↑			
212	Ye8081-2577	cheA	chemotaxis protein CheA				2.1 ↓	6.4 ↓
213	Ye8081-2571	cheB	protein-glutamate methylesterase			1.7 ↓		
214	Ye8081-3338	cheD	putative methyl-accepting chemotaxis protein				1.6 ↑	2.7 ↑
215	Ye8081-2575	cheD	methyl-accepting chemotaxis protein					2.0 ↓
216	Ye8081-2572	cheR	chemotaxis protein methyltransferase			1.7 ↓		
217	Ye8081-2576	cheW	chemotaxis protein CheW					
218	Ye8081-2570	cheY	chemotaxis protein CheY			1.9 ↓		2.2 ↓
219	Ye8081-2569	cheZ	chemotaxis protein CheZ					1.9 ↓
220	Ye8081-2468	astA	arginine N-succinyltransferase	4.5 ↑	5.8 ↑	5.4 ↑	8.6 ↑	18.0 ↑
221	Ye8081-2466	astB	succinylarginine dihydrolase	2.8 ↑	3.5 ↑	3.4 ↑	6.0 ↑	14.0 ↑
222	Ye8081-2469	astC	succinylornithine aminotransferase	4.7 ↑	4.6 ↑	4.5 ↑	10.6 ↑	36.7 ↑
223	Ye8081-2467	astD	succinylglutamic semialdehyde dehydrogenase	4.0 ↑	3.8 ↑	3.6 ↑	12.3 ↑	41.2 ↑
224	Ye8081-2465	astE	succinylglutamate desuccinylase	2.3 ↑	2.2 ↑	2.1 ↑	5.41 ↑	14.2 ↑

				22°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
225	Ye8081-2654	citA	sensor kinase protein					1.6 ↑
226	Ye8081-2653	citC	[citrate [pro-3s]-lyase] ligase				3.6 ↓	2.2 ↓
227	Ye8081-2652	citD	citrate lyase acyl carrier protein	2.3 ↓	3.0 ↓	1.9 ↓	28.3 ↓	22.4 ↓
228	Ye8081-2651	citE	putative citrate lyase beta chain	2.4 ↓	2.8 ↓	2.3 ↓	8.5 ↓	3.9 ↓
229	Ye8081-2650	citF	citrate lyase alpha chain	2.1 ↓	2.9 ↓	2.6 ↓	6.5 ↓	3.4 ↓
230	Ye8081-2648	citG	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase					
231	Ye8081-2647	citT	citrate carrier	2.6 ↓	3.4 ↓	2.6 ↓	10.8 ↓	7.2 ↓
232	Ye8081-2649	citX	putative apo-citrate lyase phosphoribosyl-dephospho-CoA tra		2.0 ↓	1.8 ↓	6.9 ↓	4.0 ↓
233	Ye8081-4208	atpA	ATP synthase alpha subunit protein				1.6 ↓	2.3 ↓
234	Ye8081-4212	atpB	ATP synthase subunit B protein					1.5 ↓
235	Ye8081-4205	atpC	ATP synthase epsilon subunit protein	2.9 ↓				
236	Ye8081-4206	atpD	ATP synthase beta subunit protein					6.6 ↓
237	Ye8081-4211	atpE	ATP synthase subunit C protein					3.1 ↓
238	Ye8081-4210	atpF	ATP synthase subunit B protein				2.2 ↓	
239	Ye8081-4207	atpG	ATP synthase gamma subunit protein				2.0 ↓	
240	Ye8081-4209	atpH	ATP synthase delta subunit protein				2.6 ↓	3.0 ↓
241	Ye8081-4213	atpI	ATP synthase protein I					
242	Ye8081-3766	pyrB	aspartate carbamoyltransferase catalytic subunit					
243	Ye8081-1625	pyrC	dihydroorotase					14.0 ↓
244	Ye8081-1568	pyrD	dihydroorotate dehydrogenase				1.7 ↑	
245	Ye8081-0059	pyrE	orotate phosphoribosyltransferase		1.7 ↓	1.5 ↓		2.2 ↓
246	Ye8081-1973	pyrF	orotidine 5'-phosphate decarboxylase				2.2 ↑	
247	Ye8081-0746	pyrG	CTP synthase		1.6 ↓			
248	Ye8081-3282	pyrH	uridylate kinase					
249	Ye8081-3767	pyrI	aspartate carbamoyltransferase regulatory chain					2.1 ↓

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
250	Ye8081-0650	tnaA	tryptophanase	6.3 ↑	3.6 ↑	15.6 ↑	6.4 ↑	2.1 ↑
251	Ye8081-0649	mtr	tryptophan-specific transport protein	4.8 ↑	2.7 ↑	2.0 ↓	4.3 ↑	2.4 ↑
252	Ye8081-0743	rumA	23s rRNA (uracil-5-)-methyltransferase (tryptophan metab		1.8 ↓	2.0 ↓	1.4 ↓	1.5 ↓
253	Ye8081-1222	ipdC	indole-3-pyruvate decarboxylase (tryptophan metabolism)				4.0 ↑	3.8 ↑
254	Ye8081-3972	dam	DNA adenine methylase					
255	Ye8081-2361	dam	putative adenine-specific modification methylase				6.5 ↑	6.1 ↑
256	Ye8081-0893	clpB	Clp ATPase			2.2 ↓	4.3 ↓	4.9↓
257	Ye8081-2696	clpB5	Clp ATPase				3.4 ↑	3.0 ↑
258	Ye8081-1318	hisJ	histidine-binding periplasmic protein	2.9 ↑				2.7 ↑
259	Ye8081-1319	hisQ	histidine transport system permease protein HisQ	4.4 ↑			3.7 ↑	5.0 ↑
260	Ye8081-1320	hisM	histidine transport system permease protein HisM	3.4 ↑			3.3 ↑	4.4 ↑
261	Ye8081-1321	hisP	histidine transport ATP-binding protein HisP	2.2 ↑			4.8 ↑	3.8 ↑
262	Ye8081-1393	nrdA	ribonucleoside-diphosphate reductase 1 alpha chain		1.6 ↓	2.08 ↓		1.9 ↓
263	Ye8081-1392	nrdB	ribonucleoside-diphosphate reductase 1 beta chain	1.6 ↓			3.1 ↓	3.0 ↓
264	Ye8081-0482	nrdD	anaerobic ribonucleoside-triphosphate reductase			1.9 ↓	1.9 ↓	2.1 ↓
265	Ye8081-0481	nrdG	anaerobic ribonucleoside-triphosphate reductase activating		1.7 ↑		2.2 ↑	
266	Ye8081-0927	nrdE	ribonucleoside-diphosphate reductase 2 alpha chain					
267	Ye8081-0926	nrdF	ribonucleoside-diphosphate reductase 2 beta chain			1.7 ↓	2.4 ↑	2.3 ↑
268	Ye8081-2092	acpD	acyl carrier protein phosphodiesterase		1.9 ↓		5.3 ↓	
269	Ye8081-1636	acpP	acyl carrier protein		3.2 ↓		484.7 ↓	136.3 ↓
270	Ye8081-0309	acs	acetyl-coenzyme A synthetase					
271	Ye8081-2001	araA	L-arabinose isomerase	4.0 ↓				
272	Ye8081-2002	araB	L-ribulokinase		2.3 ↓		3.6 ↑	2.7 ↑
273	Ye8081-2006	araC	arabinose operon regulatory protein					
274	Ye8081-1976	araD	L-ribulose-5-phosphate 4-epimerase	1.7 ↑			4.1 ↑	3.7 ↑
275	Ye8081-2003	araF	L-arabinose-binding periplasmic protein precursor				3.0 ↑	2.9 ↑
276	Ye8081-2004	araG	L-arabinose transport ATP-binding protein				2.0 ↑	2.3 ↑
277	Ye8081-2005	araH	L-arabinose transport system permease protein		1.5 ↓			

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
278	Ye8081-2238	adhE	aldehyde-alcohol dehydrogenase					
279	Ye8081-2460	hutC	putative GntR-family transcriptional regulatory protein				1.9 ↑	2.0 ↑
280	Ye8081-2461	hutI	imidazolonepropionase				2.9 ↑	3.7 ↑
281	Ye8081-2462	hutG	putative N-formylglutamate amidohydrolase				2.1 ↑	2.4 ↑
282	Ye8081-4095	hutU	urocanate hydratase				2.4 ↑	
283	Ye8081-4094	hutH	histidine ammonia-lyase				2.4 ↑	2.4 ↑
284	YepYV-51c	yomA	YomA				3.4 ↓	
285	YepYV-07c	yopB	translocator YopB				1.5 ↓	
286	YepYV-44	yopE	Yop effector YopE					
287	YepYV-41	yopH	Yop effector YopH	1.6 ↓			2.0 ↓	
288	YepYV-04c	yopM	Yop effector YopM				1.8 ↓	
289	YepYV-17c	yopN	YopN	2.7 ↓	3.1 ↓		28.7 ↓	
290	YepYV-60	yopO	protein kinase YopO				2.9 ↓	
291	YepYV-61	yopP	Yop effector YopP				3.2 ↓	1.9 ↓
292	YepYV-01c	yopQ	YopQ				4.1 ↓	
293	YepYV-02	yopT	Yop effector YopT				7.8 ↓	2.3 ↓
294	YepYV-19	yscO	YscO				3.6 ↓	
295	YepYV-20	yscP	YscP		1.7 ↑		1.9 ↓	
296	YepYV-14c	yscX	YscX				1.8 ↓	
297	YepYV-30	yscC	YscC					2.1 ↑
298	YepYV-0003	yscw	VirG					1.6 ↑
299	YepYV-37	yscJ	YscJ				5.1 ↓	2.6 ↓
300	YepYV-25	yscU	YscU					
301	YepYV-24	yscT	YscT		1.8 ↓	1.6 ↓	2.0 ↓	
302	YepYV-23	yscS	YscS				7.2 ↓	2.9 ↓

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
303	YepYV-21	yscQ	YscQ				1.5 ↓	
304	YepYV-18	yscN	YscN					
305	YepYV-29	yscB	YscB				6.3 ↓	
306	YepYV-31	yscD	YscD				1.8 ↓	
307	YepYV-32	yscE	YscE				3.1 ↓	
308	YepYV-34	yscG	YscG				8.0 ↓	
309	YepYV-35	yscH	YscH				1.9 ↓	
310	YepYV-36	yscI	YscI				3.2 ↓	
311	YepYV-38	yscK	YscK				9.8 ↓	3.5 ↓
312	YepYV-39	yscL	YscL					
313	YepYV-40	yscM1	regulatory protein YscM1				1.9 ↓	
314	YepYV-0010	yscM2	regulatory protein YscM2			1.6 ↓	2.5 ↓	1.8 ↓
315	YepYV-13c	yscY	YscY				4.1 ↓	
316	YepYV-67	repA	putative replication initiator protein RepA	1.81 ↑			1.7 ↓	
317	YepYV-68	repB	putative leader peptide RepB					
318	YepYV-0012	repC	putative replication protein RepC	1.9 ↑		1.5 ↑	6.4 ↓	
319	YepYV-66	repC	putative replication protein RepC	1.9 ↑			2.2 ↓	1.9 ↓
320	YepYV-49c	spyA	SpyA (partition)	2.4 ↓	2.0 ↓	1.8 ↓	11.8 ↓	5.0 ↓
321	YepYV-48c	spyB	SpyB (partition)				2.9 ↓	
322	Ye8081-3538	sycA	putative type III secretion apparatus protein				1.9 ↑	
323	YepYV-08c	sycD	YopB/D chaperone SycD					1.4 ↓
324	YepYV-43c	sycE	YopE chaperone SycE					
325	YepYV-42c	sycH	YopH chaperone SycH					
326	YepYV-03	sycT	YopT chaperone SycT		1.5 ↓			3.4 ↓
327	YepYV-16c	tyeA	TyeA (regulator of Yop release; required for delivery of Yo			1.6 ↓	3.1 ↓	
328	YepYV-27	virF	transcriptional activator VirF					
329	YepYV-26	virG	VirG (required for localization of YscC)					

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
330	YepYV-52c	yadA	adhesin YadA			1.7 ↓	3.7 ↓	3.5 ↓
331	Ye8081-3548	ysaC	possible type III secretion system protein				6.1 ↑	5.3 ↑
332	Ye8081-3549	ysaE	AraC family regulatory protein				4.2 ↑	2.9 ↑
333	Ye8081-3557	ysaH	possible type III secretion system effector protein					
334	Ye8081-3552	ysaI	putative virulence associated protein					
335	Ye8081-3553	ysaJ	type III secretion system apparatus lipoprotein				3.5 ↑	2.0 ↑
336	Ye8081-3545	ysaK	type III secretion sytem protein			1.4 ↓	1.7 ↑	1.8 ↑
337	Ye8081-3544	ysaN	type III secretion sytem protein				5.5 ↑	3.9 ↑
338	Ye8081-3542	ysaR	type III secretion apparatus protein					2.3 ↑
339	Ye8081-3540	ysaT	putative type III secretion apparatus protein					
340	Ye8081-3539	ysaU	putative type III secretion apparatus protein				2.7 ↑	2.4 ↑
341	Ye8081-3546	ysaV	type III secretion system apparatus protein			1.9 ↓		2.0 ↑
342	Ye8081-3547	ysaW	type III secreted effector protein				2.8 ↑	2.2 ↑
343	Ye8081-3537	yspB	putative type III secretion system effector protein				3.3 ↑	2.3 ↑
344	Ye8081-2561	flgA	flagella basal body P-ring formation protein FlgA				4.0 ↑	2.9 ↑
345	Ye8081-2560	flgB	flagellar basal-body rod protein FlgB					
346	Ye8081-2559	flgC	flagellar basal-body rod protein FlgC				3.1 ↑	4.0 ↑
347	Ye8081-2558	flgD	basal-body rod modification protein FlgD				2.3 ↑	2.0 ↑
348	Ye8081-2557	flgE	flagellar hook protein FlgE					
349	Ye8081-2556	flgF	flagellar basal-body rod protein FlgF					
350	Ye8081-2555	flgG	flagellar basal-body rod protein FlgG					
351	Ye8081-2554	flgH	flagellar L-ring protein precursor	2.1 ↑	1.7 ↑			2.7 ↑
352	Ye8081-2553	flgI	flagellar P-ring protein precursor					
353	Ye8081-2552	flgJ	flagellar protein FlgJ				4.3 ↑	3.6 ↑
354	Ye8081-2551	flgK	flagellar hook-associated protein 1					1.7 ↑
355	Ye8081-2550	flgL	flagellar hook-associated protein 3			1.6 ↓	2.2 ↑	
356	Ye8081-2562	flgM	negative regulator of flagellin synthesis	4.3 ↑		1.7 ↑		
357	Ye8081-2563	flgN	flagella synthesis protein FlgN					
358	Ye8081-2567	flhB	flagellar biosynthetic protein FlhB					
359	Ye8081-2580	flhC	flagellum biosynthesis transcription activator			1.8 ↓		

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
360	Ye8081-2581	flhD	flagellar transcriptional activator					
361	Ye8081-2565	flhE	flagellar protein FlhE precursor					1.8 ↑
362	Ye8081-2519	fliA	RNA polymerase sigma factor for flagellar operon				4.6 ↑	5.8 ↑
363	Ye8081-2520	fliB	flagellin lysine-N-methylase				4.1 ↑	3.4 ↑
364	Ye8081-2522	fliC	thermoregulated motility protein			1.7 ↓	4.2 ↑	2.3 ↑
365	Ye8081-2521	fliC2	flagellin		1.6 ↓	1.6 ↓	4.4 ↑	2.3 ↑
366	Ye8081-2523	fliC3	flagellin		1.5 ↓	1.5 ↓	4.3 ↑	2.2 ↑
367	Ye8081-2524	fliD	flagellar hook-associated protein 2				7.1 ↑	5.0 ↑
368	Ye8081-2536	fliE	flagellar hook-basal body complex protein FliE	2.3 ↑				
369	Ye8081-2537	fliF	flagellar M-ring protein				6.2 ↑	3.6 ↑
370	Ye8081-2538	fliG	flagellar motor switch protein FliG				2.4 ↑	2.2 ↑
371	Ye8081-2539	fliH	flagellar assembly protein					4.0 ↑
372	Ye8081-2540	fliI	flagellum-specific ATP synthase				3.1 ↑	2.7 ↑
373	Ye8081-2541	fliJ	flagellar protein FliJ					1.7 ↑
374	Ye8081-2542	fliK	flagellar hook-length control protein FliK				3.3 ↑	2.1 ↑
375	Ye8081-2543	fliL	flagellar protein FliL					
376	Ye8081-2544	fliM	flagellar motor switch protein FliM					
377	Ye8081-2545	fliN	flagellar motor switch protein FliN				2.3 ↑	1.6 ↑
378	Ye8081-2546	fliO	flagellar protein FliO				2.5 ↑	2.1 ↑
379	Ye8081-2547	fliP	flagellar biosynthetic protein FliP				2.6 ↑	2.8 ↑
380	Ye8081-2548	fliQ	flagellar biosynthetic protein FliQ					
381	Ye8081-2549	fliR	flagellar biosynthetic protein FliR				4.5 ↑	5.3 ↑
382	Ye8081-2525	fliS	flagellar protein FliS	1.7 ↑			2.9 ↑	2.7 ↑
383	Ye8081-2526	fliT	FliT	2.5 ↑				2.2 ↑
384	Ye8081-2516	fliY	putative cystine-binding periplasmic protein	2.7 ↓	1.9 ↓	3.1 ↓	1.5 ↓	2.3 ↓
385	Ye8081-2518	fliZ	putative alternative sigma factor regulatory protein					
386	Ye8081-1298	flk	putative flagellar assembly regulatory protein, Flk			1.4 ↓	3.1 ↑	2.1 ↑
387	Ye8081-2579	motA	chemotaxis MotA protein					
388	Ye8081-2578	motB	chemotaxis MotB protein					

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
389	Ye8081-2564	invA	invasin				4.7 ↑	3.3 ↑
390	Ye8081-2949	gltA	citrate synthase GltA		1.7 ↑			
391	Ye8081-3735	gltB	glutamate synthase [NADPH] large chain precursor				3.3 ↓	
392	Ye8081-3736	gltD	glutamate synthase [NADPH] small chain		1.5 ↓	1.6 ↓		3.6 ↓
393	Ye8081-2992	gltJ	putative glutamate/aspartate transport system permease		1.5 ↓		2.6 ↑	2.1 ↑
394	Ye8081-2993	gltK	putative glutamate/aspartate transport system permease	2.5 ↓				
395	Ye8081-2994	gltL	putative glutamate/aspartate transport ATP-binding protein				6.5 ↓	
396	Ye8081-0310	gltP	proton glutamate symport protein	2.6 ↓	2.0 ↓	2.1 ↓	4.6 ↓	3.1 ↓
397	Ye8081-1879	gltP	putative transport protein	2.3 ↓	1.7 ↓	2.7 ↓		1.9 ↓
398	Ye8081-0042	gltS	sodium/glutamate symport carrier protein		1.8 ↓	2.1 ↓	1.8 ↓	2.7 ↓
399	Ye8081-1215	gltX	glutamyl-tRNA synthetase		2.1 ↓	2.7 ↓	2.9 ↓	4.9 ↓
400	Ye8081-0027	glnA	glutamine synthetase	4.0 ↓		2.1 ↓	9.9 ↓	9.3 ↓
401	Ye8081-1039	glnB	nitrogen regulatory protein P-II					
402	Ye8081-3674	glnE	glutamate-ammonia-ligase adenylyltransferase					1.7 ↓
403	Ye8081-2991	glnH	putative amino acid-binding protein precursor					
404	Ye8081-2845	glnH	putative glutamine-binding periplasmic protein	2.3 ↑		2.1 ↑	2.6 ↑	2.3 ↑
405	Ye8081-2846	glnP	putative glutamine transport system permease	2.0 ↑			1.9 ↑	2.1 ↑
406	Ye8081-2847	glnQ	putative glutamine transport ATP-binding protein					2.2 ↑
407	Ye8081-2977	glnS	glutaminyl-tRNA synthetase	2.6 ↓	1.8 ↓	3.0 ↓		1.7 ↓
408	Ye8081-3300	gcvA	glycine cleavage system transcriptional activator	1.7 ↑			3.7 ↑	2.6 ↑
409	Ye8081-3391	gcvP	glycine dehydrogenase				1.9 ↓	2.1 ↓
410	Ye8081-1136	gcvR	glycine cleavage system transcriptional repressor			1.5 ↓		1.7 ↓
411	Ye8081-3393	gcvT	aminomethyltransferase				1.5 ↓	
412	Ye8081-2792	cdd	cytidine deaminase	1.5 ↓			2.3 ↑	1.7 ↑
413	Ye8081-4067	dctA	C4-dicarboxylate transport protein	1.8 ↓	2.3 ↓		2.6 ↓	2.9 ↓
414	Ye8081-1533	focA	putative formate transporter 1					
415	Ye8081-2812	focA	putative formate transporter	1.8 ↑			2.8 ↑	3.1 ↑

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
416	Ye8081-1975	osmB	osmotically inducible lipoprotein B precursor	1.7 ↑			2.0 ↑	2.0 ↑
417	Ye8081-0565	osmY	osmotically inducible protein Y			2.3 ↓		
418	Ye8081-0655	leuO	LysR-family transcriptional regulator LeuO	1.8 ↑				
419	Ye8081-1581	ompA	putative outer membrane porin A protein				53.0 ↓	9.0 ↓
420	Ye8081-1401	ompC	outer membrane protein C, porin	2.0 ↓		3.4 ↓	1.8 ↓	2.0 ↓
421	Ye8081-2856	ompC2	outer membrane protein C2		2.2 ↓	3.0 ↓	2.2 ↓	2.2 ↓
422	Ye8081-1563	ompF	putative outer membrane porin F protein			1.7 ↓		
423	Ye8081-3275	ompH	cationic 19 kDa outer membrane protein precursor		1.8 ↓	1.9 ↓	2.4 ↓	2.4 ↓
424	Ye8081-4004	ompR	transcriptional regulatory protein					
425	Ye8081-2216	ompW	putative exported protein	2.4 ↓			2.4 ↓	3.0 ↓
426	Ye8081-2835	ompX	putative outer membrane protein			1.7 ↓		1.7 ↓
427	Ye8081-3776	mgtA	Mg2+ transporter ATPase				1.6 ↑	
428	Ye8081-2585	mgtB	Mg(2+) transport ATPase protein B					
429	Ye8081-2586	mgtC	Mg(2+) transport ATPase protein C					
430	Ye8081-1532	pflB	formate acetyltransferase 1 (SAM)				11.0 ↓	
431	Ye8081-1681	YE1681	prophage encoded DNA modification methylase (biosynthesis		1.5 ↓	1.8 ↓	1.6 ↓	1.6 ↓
432	Ye8081-0710	speE	spermidine synthase (SAM)			1.5 ↓		1.9 ↓
433	Ye8081-3228	mtrK	possible 5-methylthioribose kinase (SAM)			1.6 ↓	3.0 ↑	1.5 ↑
434	Ye8081-1470	uptG	cystathionine beta-lyase (biosynthesis of methionine, which is	5.2 ↓	3.0 ↓	5.2↓		
435	Ye8081-1469	uptH	putative cystathionine gamma-synthase (SAM)	5.7 ↓	4.9↓	6.6 ↓		2.6 ↓
436	Ye8081-4122	xylA	xylose isomerase				3.7 ↑	3.2 ↑
437	Ye8081-4123	xylB	xylulose kinase				4.6 ↑	2.6 ↓
438	Ye8081-2908	bioA	adenosylmethionine-8-amino-7-oxononanoate aminotransfera	1.7 ↑			3.8 ↑	3.7 ↑
439	Ye8081-2907	bioB	biotin synthase					2.0 ↑
440	Ye8081-2905	bioC	biotin synthesis protein BioC				2.7 ↑	3.3 ↑
441	Ye8081-2904	bioD	dethiobiotin synthetase				2.3 ↑	3.0 ↑
442	Ye8081-2024	bioD	putative dethiobiotin synthetase			2.4 ↓	1.7 ↓	2.0 ↓
443	Ye8081-2906	bioF	8-amino-7-oxononanoate synthase				8.0 ↑	8.2 ↑
444	Ye8081-3995	bioH	putative biotin biosynthesis protein			1.5 ↓	2.0 ↑	1.5 ↑

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
445	Ye8081-0478	phnF	probable gntR-family transcriptional regulatory protein				3.8 ↑	3.8 ↑
446	Ye8081-2618	irp1	yersiniabactin biosynthetic protein				3.6 ↑	3.8 ↑
447	Ye8081-2617	irp2	yersiniabactin biosynthetic protein					
448	Ye8081-2616	ybtA	transcriptional regulator YbtA					
449	Ye8081-2621	ybtE	yersiniabactin siderophore biosynthetic protein				3.3 ↑	2.9 ↑
450	Ye8081-2615	ybtP	lipoprotein inner membrane ABC-transporter					
451	Ye8081-2614	ybtQ	inner membrane ABC-transporter YbtQ				4.0 ↑	3.7 ↑
452	Ye8081-2612	ybtS	putative salicylate synthetase					
453	Ye8081-2620	ybtT	yersiniabactin biosynthetic protein YbtT				2.5 ↑	
454	Ye8081-2619	ybtU	yersiniabactin biosynthetic protein YbtU				3.1 ↑	3.1 ↑
455	Ye8081-2613	ybtX	putative signal transducer				4.9 ↑	5.0 ↑
456	Ye8081-2622	fyuA	pesticin/yersiniabactin receptor protein	1.6 ↓	2.4 ↓	2.3 ↑		
457	Ye8081-2611	int	integrase			1.6 ↑	1.8 ↑	
458	Ye8081-1600	yenI	N-acylhomoserine lactone synthase	12648.0 ↓		2.3 ↓	5.6 ↓	50.7 ↓
459	Ye8081-1599	yenR	quorum-sensing transcriptional activator	5.2 ↑	10.5 ↓		30.1 ↓	6.5 ↓
460	Ye8081-1026	ycoR	LuxR-family transcriptional regulator					
461	Ye8081-2577	cheA	chemotaxis protein CheA					
462	Ye8081-2571	cheB	protein-glutamate methylesterase				5.2 ↑	3.1 ↑
463	Ye8081-3338	cheD	putative methyl-accepting chemotaxis protein				3.6 ↑	3.2 ↑
464	Ye8081-2575	cheD	methyl-accepting chemotaxis protein					
465	Ye8081-2572	cheR	chemotaxis protein methyltransferase				2.3 ↑	2.6 ↑
466	Ye8081-2576	cheW	chemotaxis protein CheW					742.7 ↓
467	Ye8081-2570	cheY	chemotaxis protein CheY				1.8 ↑	
468	Ye8081-2569	cheZ	chemotaxis protein CheZ				1.9 ↑	
469	Ye8081-2468	astA	arginine N-succinyltransferase	4.7 ↑			13.6 ↑	11.4 ↑
470	Ye8081-2466	astB	succinylarginine dihydrolase				2.9 ↑	5.3 ↑
471	Ye8081-2469	astC	succinylornithine aminotransferase	5.7 ↑			51.1 ↑	34.6 ↑
472	Ye8081-2467	astD	succinylglutamic semialdehyde dehydrogenase	2.8 ↑			10.5 ↑	10.5 ↑
473	Ye8081-2465	astE	succinylglutamate desuccinylase	2.2 ↑			2.9 ↑	7.5 ↑

				37°C				
No.	Systematic	Common	Product	Δ*yenI*	Δ*yenR*	Δ*ycoR*	Δ*yenRycoR*	Δ*yenIyenRycoR*
474	Ye8081-2654	citA	sensor kinase protein					3.1 ↑
475	Ye8081-2653	citC	[citrate [pro-3s]-lyase] ligase				3.6 ↓	2.7 ↑
476	Ye8081-2652	citD	citrate lyase acyl carrier protein				28.3 ↓	
477	Ye8081-2651	citE	putative citrate lyase beta chain				8.5 ↓	
478	Ye8081-2650	citF	citrate lyase alpha chain				6.5 ↓	3.7 ↑
479	Ye8081-2648	citG	2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase	1.5 ↑				
480	Ye8081-2647	citT	citrate carrier		1.5 ↓		10.8 ↓	2.1 ↑
481	Ye8081-2649	citX	putative apo-citrate lyase phosphoribosyl-dephospho-CoA tra				6.9 ↓	
482	Ye8081-4208	atpA	ATP synthase alpha subunit protein				4.7 ↓	4.2 ↓
483	Ye8081-4212	atpB	ATP synthase subunit B protein				1.9 ↓	1.9 ↓
484	Ye8081-4205	atpC	ATP synthase epsilon subunit protein	7.1 ↓				
485	Ye8081-4206	atpD	ATP synthase beta subunit protein					
486	Ye8081-4211	atpE	ATP synthase subunit C protein				4.3 ↓	3.0 ↓
487	Ye8081-4210	atpF	ATP synthase subunit B protein				3.8 ↓	3.9 ↓
488	Ye8081-4207	atpG	ATP synthase gamma subunit protein				8.1 ↓	4.5 ↓
489	Ye8081-4209	atpH	ATP synthase delta subunit protein				3.9 ↓	3.1 ↓
490	Ye8081-4213	atpI	ATP synthase protein I	3.1 ↑				
491	Ye8081-3766	pyrB	aspartate carbamoyltransferase catalytic subunit			1.9 ↓	2.5 ↓	4.1 ↓
492	Ye8081-1625	pyrC	dihydroorotase		1.7 ↓			19.3 ↓
493	Ye8081-1568	pyrD	dihydroorotate dehydrogenase					
494	Ye8081-0059	pyrE	orotate phosphoribosyltransferase	3.2 ↓			12.0 ↓	6.1 ↓
495	Ye8081-1973	pyrF	orotidine 5'-phosphate decarboxylase			1.5 ↓		1.6 ↓
496	Ye8081-0746	pyrG	CTP synthase			1.8 ↓		2.0 ↓
497	Ye8081-3282	pyrH	uridylate kinase	1.9 ↓				1.7 ↓
498	Ye8081-3767	pyrI	aspartate carbamoyltransferase regulatory chain				2.9 ↓	4.1 ↓

4.2.3 Relative quantification-PCR assay

To validate the microarray data for *virF*, *yscF*, *yadA*, *invA*, *repA*, *spyA* and *tyeA*, RQ-PCR was performed. TyeA is a negative regulator of type III targeting pathway in the bacterial cytoplasm (Cheng & Schneewind, 2000; Cheng *et al.*, 2001). VirF is a key transcription activator encoded on the pYVe plasmid which is only transcribed at 37°C (de Rouvroit *et al.*, 1992). YscF forms the 'needle end' part of the injectisome (Hoiczyk & Blobel, 2001). YadA is a virulence factor which promotes adhesion of enteropathogenic *Yersiniae* to eukaryotic cell membranes (Heesemann & Grüter, 1987; Isberg, 1989; Miller, 1992). InvA is the primary invasion factor of *Y. enterocolitica* and mediates binding to host epithelial cells (Isberg *et al.*, 1987; Miller & Falkow, 1988; Miller, 1988; Pepe & Miller, 1990; Ellison *et al.*, 2004). RepA and SpyA controls the replication and partition of the pYVe plasmid, respectively (Snellings *et al.*, 2001).

To prepare for the RQ-PCR experiments, the PCR efficiencies were assessed by performing standard curves using serial 10-fold dilutions of the template (Figures 4.9 and 4.10). The cycle value at which the threshold line hits the curve for each sample is called the 'cycle threshold' (C_T). In a 10-fold dilution series the distance between each C_T should be 3.32 and thus the slope of the standard curve should be -3.32 if the reaction is 100 % efficient. The red line in Figure 4.11 denotes the threshold at which point the cycle number will be extrapolated for each sample. The *dnaE* gene was used as an endogenous control gene (See Table 2.7).

To test for non-specific product, a dissociation (melting) cycle is performed after the amplification steps are complete. During dissociation, the DNA products in the PCR reaction are slowly melted, by gradually increasing the temperature to 95°C. Because SYBR green only fluoresces in double stranded DNA, upon melting, the fluorescence rapidly drops as double-stranded DNA becomes single-stranded. Depending on size, different products will melt at different temperatures and if non-specific amplification or primer-dimer formation has occurred, then this should be detected by comparing the samples to a template free control. As quantitative PCR is based on determining the relative difference in copy number of the cDNA template in one sample compared to another, it is vital that the amount of starting template is carefully quantified prior to PCR. Each sample was checked for primer-dimer and non-specific amplification products by generating a melting curve. None of the samples

produced non-specific products and therefore were considered as being suitable for RQ-PCR analysis.

Tables 4.3 and 4.4 show the RQ-PCR results for expression of genes at 22°C and 37°C respectively. A matching set of results from the microarray experiments is included for comparison. Grey and green boxes denote agreements and disagreements in the two sets of data, respectively. The expression of *tyeA*, *virF* and *yscF* at 22°C are mostly unchanged. The only two changes are upregulation of *virF* and *yscF* in the Δ*yenIyenRycoR* mutant by 2.0 and 1.6 fold, respectively. At 37°C, changes are more pronounced. Expression of *tyeA* is downregulated in the Δ*yenI* and Δ*yenRycoR* mutants by 2.4 and 3.5 fold, respectively. *virF* expression is downregulated in the Δ*ycoR* (1.8 fold) and Δ*yenRycoR* (3.2 fold) mutants, but is upregulated by 1.5 fold in the Δ*yenIyenRycoR* mutant. For *yscF*, there were no changes except for the Δ*yenRycoR* mutant which was downregulated by 3.1 fold. *yadA* expression is downregulated more notably in the Δ*yenRycoR* mutant (10.1 fold) compared to the Δ*yenIyenRycoR* mutant (3.4 fold). Expression of *invA* is upregulated in the Δ*yenR* and Δ*yenRycoR* mutants by 1.6 and 2.5 fold, respectively. *repA* expression is upregulated in the Δ*yenR* mutant by 1.5 fold but is downregulated in the Δ*yenRycoR* mutant by 2.4 fold. The expression of *spyA* is downregulated in the Δ*yenI* (1.8 fold), Δ*yenRycoR* (6.3 fold) and Δ*yenIyenRycoR* (3.3 fold) mutants.

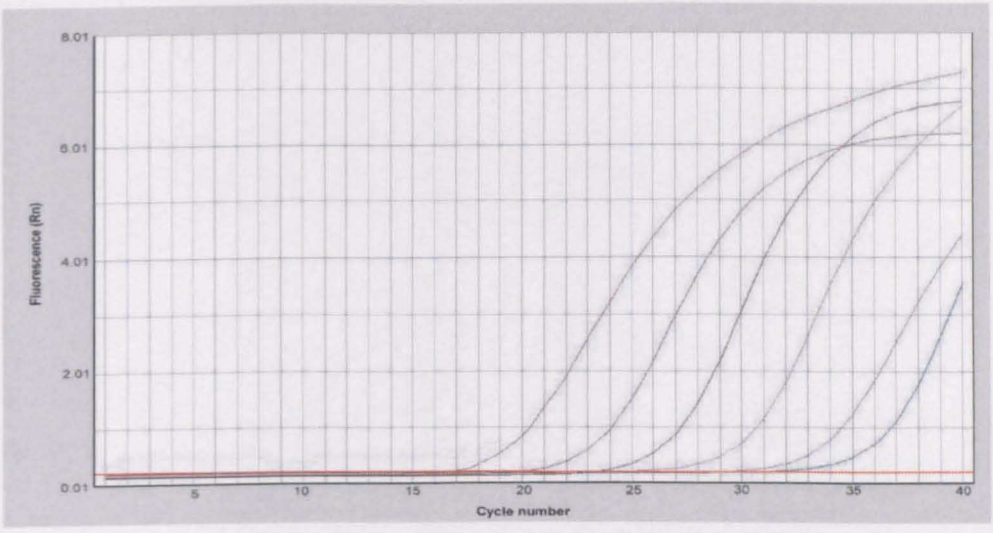


Figure 4.11. A representative graph showing PCR cycles for a standard curve where a cDNA sample has undergone 10-fold serial dilutions. The red line denotes the threshold at which point the cycle number will be extrapolated for each sample.

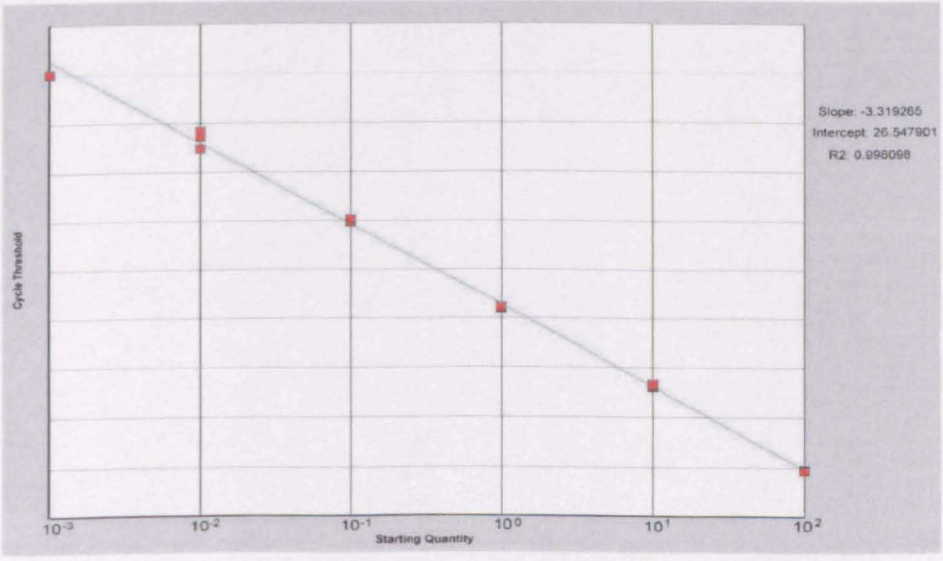


Figure 4.12. A representative graph showing a standard curve plotted from data obtained from PCR cycles in Figure 4.11. The slope of the standard curve is approximately -3.32, meaning the reaction is 100 % efficient.

Table 4.3. RQ-PCR results at 22°C. Fold changes in gene expression in QS mutants compared with wildtype (a). A matching set of results from the microarray data is shown for comparison (b). Shaded grey and green boxes show RQ-PCR data which agree (when both show the same up- or down regulation or no changes) and disagree (when both do not show the same up- or down regulation or no changes) with the microarray data respectively. Fold changes below 1.5 were defined as no changes.

(a) RQ-PCR results

	QS Mutants				
Genes	**Δ*yenI***	**Δ*yenR***	**Δ*ycoR***	**Δ*yenRycoR***	**Δ*yenIyenRycoR***
tyeA	no change	no change	no change	no change	no change
virF	no change	no change	no change	no change	2.0 ↑
yscF	no change	no change	no change	no change	1.6 ↑

(b) Microarray results

	QS Mutants				
Genes	**Δ*yenI***	**Δ*yenR***	**Δ*ycoR***	**Δ*yenRycoR***	**Δ*yenIyenRycoR***
tyeA	no change	no change	no change	2.1 ↓	no change
virF	1.8 ↑	1.7 ↑	no change	2.4 ↑	4.5 ↑
yscF	no change	no change	no change	no change	no change

Table 4.4. RQ-PCR results at 37°C. Fold changes in gene expression in QS mutants compared to wildtype (a). A matching set of results from the microarray data is shown for comparison (b). Shaded grey and green boxes show RQ-PCR data which agree (when both show the same up- or down regulation or no changes) and disagree (when both do not show the same up- or down regulation or no changes) with the microarray data respectively. Fold changes below 1.5 were defined as no changes.

(a) RQ-PCR results

	QS Mutants				
Genes	**Δ*yenI***	**Δ*yenR***	**Δ*ycoR***	**Δ*yenRycoR***	**Δ*yenIyenRycoR***
tyeA	2.4 ↓	no change	no change	3.5 ↓	no change
virF	no change	no change	1.8 ↓	3.2 ↓	1.5 ↑
yscF	no change	no change	no change	3.1 ↓	no change
yadA	no change	no change	no change	10.1 ↓	3.4 ↓
invA	no change	1.6 ↑	no change	2.5 ↑	no change
repA	no change	1.5 ↑	no change	2.4 ↓	no change
spyA	1.8 ↓	no change	no change	6.3 ↓	3.3 ↓

(b) Microarray results

	QS Mutants				
Genes	**Δ*yenI***	**Δ*yenR***	**Δ*ycoR***	**Δ*yenRycoR***	**Δ*yenIyenRycoR***
tyeA	no change	no change	1.6 ↓	3.1 ↓	no change
virF	no change	no change	no change	no change	no change
yscF	no change	no change	no change	no change	no change
yadA	no change	no change	1.7 ↓	3.7 ↓	3.5 ↓
invA	no change	no change	no change	4.7 ↑	3.3 ↑
repA	1.8 ↑	no change	no change	1.7 ↓	no change
spyA	2.4 ↓	2.0 ↓	1.8 ↓	11.8 ↓	5.0 ↓

4.3 Discussion

The λ Red recombinase method of mutagenesis greatly facilitated the rapid generation of QS mutants in *Y. enterocolitica* 8081 which has previously been considered as difficult to modify due to severe restriction of transformed or electroporated DNA (Kinder *et al.*, 1993). Thus, genetic analysis of the 8081 strain and other American serotype O:8 strains has been hampered. Kinder *et al.* (1993) constructed a mutant in the *yenIMR* locus (named JB580) encoding *Yen*I which is both a restriction endonuclease and methyltransferase (Miyahara *et al.*, 1988) (not to be confused with *yenI*, the AHL synthase gene). Subsequently, JB580 which has at least a 1000-fold increase in electroporation frequency compared to 8081 (Kinder *et al.*, 1993) has been routinely used as an experimental strain. In this study, we found that the robustness and efficiency of the λ Red recombinase allowed for mutagenesis of the 8081 strain. This chapter described how seven QS mutants in 8081 were constructed: Δ*yenR*, Δ*yenI*, Δ*ycoR*, Δ*yenIyenR*, Δ*yenIycoR*, Δ*yenRycoR* and Δ*yenIyenRycoR*. These mutants were then used to further study the regulatory network of the QS system of *Y. enterocolitica* 8081 *via* transcriptional studies.

The utilization of microarray technology to study gene expression in *Y. enterocolitica* 8081 wildtype and its QS mutants has provided a wealth of information on QS-related regulatory networks in this bacterium. It is important to note that this microarray analysis was performed with bacterial cultures at an OD_{600} of 0.75. These experiments therefore provide a 'snapshot' of what clearly is a dynamic process that varies as the population density changes in the growing bacterial culture. Differences in the expression of the QS controlled genes may occur as the culture goes through the various growth stages.

In an overview of the transcriptomic analysis, the total number of genes which appeared to be regulated (1.5 fold or greater) by *yenI*, *yenR* and *ycoR* is 2,427, which is 56.6% of the total genome (See Figure 4.10). This is an astonishingly high percentage. Most of these genes have not been documented previously as being regulated by QS in *Y. enterocolitica* and a large number of these ORFs encode putative and hypothetical proteins or proteins with unknown functions. For simplicity, the double and triple mutants (Δ*yenRycoR* and Δ*yenIyenRycoR*) were not included in this overview. The microarray contained 4,291 oligonucleotides representing a total of 4,291 CDS in *Y. enterocolitica* 8081, of which 4,208 are from the chromosome and 83 are from the

pYVe plasmid. The number of genes exclusively regulated by *yenI*, *yenR* and *ycoR* is 322 (13.3%), 510 (21.0%) and 460 (19.0%), respectively. Of the 2,427 genes, 330 genes (13.6%) are regulated by all three genes. The *yenI* and *yenR* regulon shares 169 genes (7.0%), the *yenI* and *ycoR* shares 148 genes (6.1%); and the *yenR* and *ycoR* shares 488 genes (20.1%).

For comparison, Wagner *et al.* (2003) reported that ~11% of the *P. aeruginosa* genome was found to be QS regulated (616 of 5,570 ORFs). The number of genes in the putative QS regulon of *Y. enterocolitica* 8081 appeared to be ~5 times that of *P. aeruginosa*. However, this is not a direct comparison and the immense difference in percentage is probably due to the differences in how the experiments were conducted and how data were interpreted. The conclusions reported by Wagner *et al.* (2003) were based on a comparison of the gene expression profiles of QS mutants cultivated with and without exogenous autoinducers which eliminated possible erroneous interpretations of QS regulation that might have been due to secondary mutations. Also, the cut-off value for fold changes were much more stringent (3 to 5 fold) compared to this study (1.5 fold). If more stringent cut-off values are applied to this study, the percentages of genes in the QS regulon would be reduced to 25.8% (2.0 fold or greater) and 6.1% (3.0 fold or greater) of the total genome. In conclusion, a high percentage of CDS in *Y. enterocolitica* 8081 are QS-regulated and it reflects the global level at which QS influences cellular behaviour.

One of the largest group of related genes which appeared in the microarray data were those of the motility/flagellar regulon. In *Y. enterocolitica* 8081 wildtype, temperature controls motility. The production of flagella and therefore motility, occurs in the laboratory at temperatures below 30°C and *Y. enterocolitica* is immotile at 37°C (Young, 2004). A total of 45 flagellar-related genes appeared at least once in the microarray data. At 22°C, almost all flagellar-related genes are downregulated in the QS mutants, implying that the QS system activates motility at this temperature. However, when examined under a microscope, the wildtype and all the QS mutants were not motile at the same point in the growth curve (OD_{600} 0.75, ~9 h) to that used to extract RNA for the microarray studies (data not shown). Only after 24 h in liquid culture the QS mutants appeared much less motile than the wildtype (data not shown). It is possible that at OD_{600} 0.75, the wildtype is flagellated but the flagella are not rotating, while the QS mutants have impaired or less flagella because of the downregulation of flagellar-related genes. This is because expression of two regulators

of Class III genes, *fliA* (σ factor) and *flgM* (anti-σ factor) as well as numerous Class II genes encoding the basal body and hook components are downregulated in the QS mutants. Consequently, defects in a functional basal body would result in repression of Class III genes which encodes flagellin (building blocks of the filament end of the flagellum), leading to impaired flagella.

In *Y. pseudotuberculosis,* the hierarchical QS systems, YpsRI and YtbRI, control swimming motility *via* regulation of *flhDC* and *fliA*. The AHLs synthesised *via* YtbI play a dual role, activating *flhDC*, in conjunction with YpsR but repressing *fliA* in conjunction with YtbR and YpsR. In liquid and plate assays, the early onset of motility observed in *ypsR* and *ypsI* mutants was abolished in *ytbI, ytbR ypsI/ytbI, ypsR/ytbR* mutants, indicating that QS regulates motility both positively (*via* YtbRI) and negatively (*via* YpsRI) (Atkinson *et al.*, 2008). However, for *Y. enterocolitica* 8081, the thermo sensitive regulator of pYVe-associated gene expression, *ymoA* (de Rouvroit *et al.*, 1992) and the master regulator of motility, *flhDC* (Class I) (Young, 2004) did not appear in the microarray data, suggesting that their expression is not governed by QS. In future work, flagellar extracts of wildtype and QS mutants can be compared using the method described by Atkinson *et al.* (2006). QS mutants can also be examined for flagella by electron microscopy and compared with the wildtype.

At 37°C, the flagellar-related genes show the opposite trend, where almost all of them are upregulated in the QS mutants compared to wildtype. This means that the QS system represses motility at 37°C. When examined under the microscope, the wildtype and all the QS mutants were not motile at the same point in the growth curve (OD_{600} 0.75, ~6 h) to that used to extract RNA for the microarray studies and remained non-motile after 24 h in liquid culture (data not shown). In agreement to the microarray data, swimming and swarming plate assays also showed that the QS mutants remained non-motile at 37°C. The observations that the QS mutants were non-motile despite the upregulation in expression of most flagellar-related genes compared to wildtype suggests that the QS system cannot override the temperature control of motility at 37°C. Since motility is controlled at multiple levels in a hierarchy, upregulation at the level of *fliA* and *fliC* expression (See Table 4.2) may not be sufficient to override *ymoA* and *flhDC* control.

Perhaps the most intriguing genes which appeared to be regulated by the QS system are Type III Secretion System (T3SS) genes. The expression of T3SS-related genes is generally upregulated at 22°C for all mutants except for the Δ*yenRycoR* mutant

where expression is mostly downregulated. At 37°C, all mutants show a generally downward trend in expression of T3SS-related genes. A total of 56 genes appeared at least once in the microarray data. Of these genes, 43 are genes from the archetypal T3SS (named the Ysc-Yop system) encoded on the pYVe plasmid including genes encoding the components of the injectisome, the Yops proteins, Yops chaperones and regulatory proteins (VirF and TyeA). 13 other genes are from a second T3SS system (named the Ysa-Ysp system) encoded on the chromosome (Haller *et al.*, 2000) including one transcriptional regulator, *ysaE*, a homologue of the AraC family of transcriptional regulators involved in the transcriptional regulation of secretion system components (Cornelis *et al.*, 1989).

RQ-PCR confirmed that the expression of *virF* in the Δ*yenIyenRycoR* mutant is upregulated by 2.0 fold at 22°C (Table 4.3), indicating that the QS system represses *virF* at this temperature. At 37°C, *virF* expression is downregulated by 1.8 and 3.2 fold in the Δ*ycoR* and Δ*yenRycoR* mutants, respectively. However, it is slightly upregulated in the Δ*yenIyenRycoR* mutant by 1.5 fold (Table 4.4). VirF is a transcriptional activator of many Yops, *ylpA yadA*, *sycE* (Skurnik & Toivanen, 1992; Wattiau & Cornelis, 1993) and the *virC* operon (Michiels & Cornelis, 1991) and since *virF* is transcribed strongly only at 37°C (de Rouvroit *et al.*, 1992), it is reasonable to conclude that through VirF, *yenR* and *ycoR* help to repress virulence determinants such as Yops, *ylpA yadA* and *sycE*, so that these resources are not produced prematurely when they are not needed. It is possible that *Y. enterocolitica* uses QS to suppress the release of virulence determinants to allow a growing population to remain in stealth as they make their way from the site of ingestion (the stomach) to their target sites, for examples gut lymphoid tissues and Peyer's patches, thereby avoiding the host's detection and defence mechanism. Once the population is quorate at their target site, the suppression of *virF* expression is relaxed and they can then mount an attack with the highest chance of success. However, this does not explain why downregulation of *virF* is not observed in the Δ*yenIyenRycoR* mutant. The upregulation of *virF* expression in the Δ*yenIyenRycoR* mutant against the downward trend in other QS mutants may indicate that *virF* is subject to additional levels of QS regulation which bypass VirF.

Another important regulatory gene studied in the RQ-PCR is *tyeA*. TyeA is a negative regulator of type III targeting pathway in the bacterial cytoplasm by preventing the export of YopN. The expression of *tyeA* is not affected at 22°C (Table 4.3). However, at 37°C, *tyeA* is downregulated in the Δ*yenI* and Δ*yenRycoR* mutants by

2.4 and 3.5 fold, respectively (Table 4.4). However, it was puzzling why this downregulation was not observed in the Δ*yenIyenRycoR* mutant. Another important component of the T3SS is YscF. YscF forms the tip of the injectisome and is essential for the delivery of specific Yops from the bacterial to the eukaryotic host cell cytosol. At 37°C, the expression of *yscF* is downregulated in Δ*yenRycoR* by 3.1 fold (Table 4.4) compared to no changes in the single Δ*yenR* and Δ*ycoR* mutants. These data suggest that *yenR* and *ycoR* works in tandem to activate the completion of the injectisome structure at 37°C, which is in agreement that Yops are only released *via* the injectisome at 37°C (Michiels & Cornelis, 1990). However, this does not explain why downregulation of *yscF* is not seen in the Δ*yenIyenRycoR* mutant.

According to the microarray data, QS appears to repress genes on the high-pathogenicity island (HPI) because they are mostly upregulated in the QS mutants at both 22°C and 37°C (See Table 4.2). The entire Yst locus which comprises of 11 genes divided into three functional groups: yersiniabactin biosynthesis, transport into the bacterial cell (outer membrane receptor and transporters) and regulation, appeared at least once in the microarray data. Genes on the HPI are involved in siderophore-mediated iron uptake and it endows *Y. enterocolitica* with the ability to multiply in the host and to cause systemic infections (Carniel *et al.*, 1996; Carniel, 2001). It is plausible that *Y. enterocolitica* couple expression of HPI genes to QS i.e. when population density is quorate, to ensure a successful infection of its host.

According to the microarray data, the expression of DNA adenine methylase gene (*dam*) at 22°C was downregulated in the Δ*yenI* and Δ*yenRycoR* mutants by 4.1 and 17.7 fold, respectively and upregulated in the Δ*yenR* mutant by 2.4 fold. A second putative *dam* is upregulated in the Δ*yenR*, Δ*ycoR*, Δ*yenRycoR* and Δ*yenIyenRycoR* mutants by 2.6, 1.8, 3.5 and 8.5 fold, respectively. At 37°C, the second putative *dam* is upregulated in the Δ*yenRycoR* and Δ*yenIyenRycoR* mutants by 6.5 and 6.0 fold, respectively. Dam catalyzes the methylation of adenine residues in GATC sequences, making them semi or fully methylated. Depending on their affinity, methylation-sensitive regulatory proteins either bind to hemimethylated DNA and prevent the subsequent methylation of GATC sequences or bind preferentially to fully methylated DNA (Falker *et al.*, 2007). Diverse virulence functions can be influenced by Dam such as secretion and translocation of type III effector proteins in *Y. pseudotuberculosis*, *S. enterica* and *A. hydrophila* (Garcia-Del Portillo *et al.*, 1999; Julio *et al.*, 2001; Julio *et al.*, 2002; Erova *et al.*, 2006); host cell invasion and adhesion in *Y. enterocolitica*, *S. enterica* and

Haemophilus influenzae (Garcia-Del Portillo *et al.*, 1999; Watson *et al.*, 2004; Falker *et al.*, 2007); pili switch of *S. enterica* and *E. coli* (Casadesus & Low, 2006) and swimming motility of *Y. enterocolitica*, *E. coli*, *A. hydrophila*, and *S. enterica* (Oshima *et al.*, 2002; Balbontin *et al.*, 2006; Erova *et al.*, 2006; Falker *et al.*, 2007; Badie *et al.*, 2007).

RQ-PCR results showed that the expression of *yadA* (adhesion factor) at 37°C was downregulated in the Δ*yenRycoR* (10.1 fold) and Δ*yenIyenRycoR* (3.4 fold) mutants (Table 4.4). *invA* (invasion factor) expression is upregulated in the Δ*yenR* (1.6 fold) and Δ*yenRycoR* (2.5 fold) mutants. Taken together, these data suggest that the QS system activates adhesion but represses invasion by *Y. enterocolitica* to eukaryotic cell membranes. This may reflect the situation where at the start of the temperature shift to 37°C, *Y. enterocolitica* is maximally expressing adhesion factors as it anticipates contact with host cells and repressing the generation of the invasion factor so that they are not prematurely released before actually adhering to its host.

It is of special interest that many genes associated with the Yop virulon are activated by QS at 37°C (Table 4.2). In QS mutants, genes encoding five of the six Yop effectors (YopE, YopH, YopM, YopO, and YopT) and one of three 'translocaters' (YopB) are downregulated. In addition, expression of Yop regulatory genes (*yopN*, *yopQ* and *yscM1/2*) and Yops chaperones genes (*sycD* and *sycT*) were also in a downward trend. Genes encoding 10 of 14 components of the Ysc injectisome (refer to Figure 1.4) also see a mostly downwards trend in expression. In contrast, the expression of genes in the Yops virulon at 22°C is generally upregulated, suggesting that the virulon is repressed by QS which is in agreement that Yops are only released *via* the injectisome at 37°C (Michiels *et al.*, 1990). It is plausible that *Y. enterocolitica* correlates QS with the Yops virulon to ensure that a sufficient population is present for a successful infection of its target eukaryotic host cells. The whole system is clearly tightly regulated by temperature (Cornelis *et al.*, 1989; de Rouvroit *et al.*, 1992) and at least partly by QS (this study). However, the exact mechanisms underlying the multilevel regulation remain to be elucidated.

CHAPTER 5

PHENOTYPIC STUDIES IN *Y. ENTEROCOLITICA* 8081 WILDTYPE AND ITS QS MUTANTS

5.1 Introduction

5.1.1 AHL detection and analysis

The earliest reported bacterial cell-to-cell communication system was identified in the bioluminescent symbiotic marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970) The LuxI-LuxR system of *V. fischeri* became the paradigm for AHL-based QS in which LuxI, the AHL synthase and LuxR, the transcriptional regulator couple the synthesis of 3-oxo-C6-HSL to the transcriptional regulation of bioluminescence (Eberhard *et al.*, 1981). As a population of *V. fischeri* cells grow, the concentration of the external AHL increases as a function of cell population density. When the AHL concentration reaches a threshold level, the AHL interacts with LuxR, and the LuxR-AHL complex binds to the luciferase promoter and activates transcription. This QS regulatory circuit allows light production to be tightly correlated with cell population density (Kolibachuk & Greenberg, 1993).

There are a number of biosensors based on the *lux* system that are able to detect and identify AHLs. For example, the pSB401 biosensor contains *V. fischeri luxR* and the promoter region of *luxI* (P_{luxI}) fused to *luxCDABE* from *Photorhabdus luminescens* (Winson *et al.*, 1998a). When introduced into an appropriate host, exogenous 3-oxo-C6-HSL or closely related compounds bind to LuxR and the LuxR-AHL complex activates transcription of *luxCDABE* from the promoter of *luxI*, resulting in the emission of light. Similarly, pSB1075 contains *lasR* and P_{lasI} from *P. aeruginosa* fused to *luxCDABE* and is used to detecting AHLs with acyl side chain lengths greater than 10 carbons (Winson *et al.*, 1998a). A third AHL reporter is based on *Chromobacterium violaceum* which produces C6-HSL signal molecule and QS mediated purple pigment, violacein (McClean *et al.*, 1997). Two transposon insertions into the *luxI* homologue, *cviI* and a QS negative regulatory RNA yielded a double mutant (CV026). This strain which is usually white produces the purple pigment violacein in the presence of an exogenous source of AHLs with *N*-acyl side chains of 6 to 8 carbons in length (McClean *et al.*, 1997).

Due to their hydrophobic nature, AHLs will readily partition from cell-free supernatant into organic solvents such as dichloromethane and ethyl acetate. Extracted AHLs can then be analysed using biosensors, either directly or they can be separated by Thin Layer Chromatography (TLC) and the component AHLs detected by overlaying

the TLC plate with the appropriate biosensor. (Latifi *et al.*, 1996; McClean *et al.*, 1997; Winson *et al.*, 1998a; Winson *et al.*, 1998b). By using this method, AHLs can be identified by comparison with synthetic standards but identification is presumptive especially if the sample contains multiple AHL derivatives. Furthermore, although AHL biosensors respond to a range of AHLs, their sensitivity to different types of AHLs can vary, therefore an intense positive signal does not necessarily correspond with the most abundant compound present (Ortori *et al.*, 2007).

pSB401 was used to detect AHLs in cell free supernatants of several strains *Y. enterocolitica* including serotypes O:3, O:8, O:9, O:10K and O:1(2a, 3) (Throup *et al.*, 1995). AHLs present in *Y. enterocolitica* strain 90/54 and 10460 were identified as an equimolar mix of 3-oxo-C6-HSL and C6-HSL by high-resolution tandem mass spectrometry (MS-MS). Atkinson *et al.* (2006) reexamined the *Y. enterocolitica* 90/54 AHL profile using AHL biosensors capable of detecting both short and long chain AHLs, discovered that in addition to 3-oxo-C6-HSL and C6-HSL, *Y. enterocolitica* synthesizes three long chain AHLs which were identified as 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL using HPLC, high resolution mass spectrometry and chemical synthesis. These long chain AHLs are present in the culture supernatants of the wildtype *Y. enterocolitica*, absent from the *yenI* mutant and were also produced by recombinant *E. coli* expressing *yenI*.

Ortori *et al.* (2007) developed a novel AHL profiling methodology which employs liquid chromatography (LC) coupled to hybrid quadrupole–linear ion trap mass spectrometry (LCQqQLIT) which is capable of unequivocal identification of AHLs. The simultaneous use of triple-quadrupole and linear ion trap modes in the same LC-MS/MS run enabled information-dependent acquisition (IDA) to screen, quantify and identify multiple AHLs in a single sample. This MS method identify AHLs by recognising the common AHL fragment ions attributed to the homoserine moiety and the 3-oxo-, 3-hydroxy- or unsubstituted acyl side chains. The identity of the unknown AHLs in cell-free culture supernatants can therefore be determined in an unbiased manner. The LC-QqQLIT methodology is broadly applicable to QS signal molecule analysis and can provide comprehensive AHL profiles and concentrations from a single sample and simultaneously collect confirmatory spectra for each AHL identified. This method was applied to wildtype *Y. pseudotuberculosis* YPIII and isogenic AHL synthase mutants (Δ*ypsI* and Δ*ytbI*) which revealed that *Y. pseudotuberculosis* YPIII produces at least twenty three AHLs including some types which had not previously

been considered to be synthesised naturally, for example, AHLs with an uneven number of carbons in the acyl chain such as 3-oxo-C7-HSL (Ortori *et al.*, 2007).

5.1.2 Use of bioluminescence promoter fusions as a measure of gene expression

The bioluminescence gene cluster of *V. fischeri* consists of eight *lux* genes (*luxA* to *E*, *luxG*, *luxI* and *luxR*) arranged in two genetically linked but divergent bi-directionally transcribed operons (Engebrecht & Silverman, 1984) (Figure 5.1). In the rightward transcriptional unit, *luxI* encodes the AHL synthase which is responsible for the production of 3-oxo-C6-HSL. Downstream of *luxI*, the *luxCDABE* operon encodes the proteins responsible for catalyzing the bioluminescence reaction. The *luxA* and *luxB* genes encode the α and β subunits of luciferase. The *luxC*, *luxD*, and *luxE* genes code for fatty acid reductase complex which converts tetradeconic acid (from the fatty acid biosynthesis pathway) into the fatty-aldehyde (RCHO). *luxG* encodes for LuxG, a flavin reductase which reduces flavin mononucleotide ($FMNH^-$) in the presence of NADH.

Luciferase catalyzes the oxidation of long-chain fatty aldehyde and reduced flavin mononucleotide ($FMNH^-$) by molecular oxygen, resulting in a long-chain fatty acid, oxidised flavin mononucleotide (FMN), and water. The release of a proton from the excited protonated flavin results in the emission of blue-green light at the wavelength of ~490 nm. The overall reaction is summarised below:

$$FMNH^- + H^+ + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + light$$

The leftward transcriptional unit consists of *luxR* which is the AHL dependent transcriptional regulator. The two operons are separated by a 219 bp intergenic region which contains a 20 nucleotide inverted repeat region termed the *lux* box shown to be necessary for *luxICDABE* transcription. This DNA element is conserved within binding sites of other LuxR-type proteins (Devine *et al.*, 1988; Hastings, 1996; Fuqua & Greenberg, 2002; Nijvipakul *et al.*, 2008).

This chapter describes the AHL profile of *Y. enterocolitica* 8081 wildtype using highly sensitive LC-QqQLIT and how mutations to the QS system affect AHL synthesis in the QS mutants. It also describes how the QS mutants respond in a number

of phenotypic assays related to virulence. Subsequently, bioluminescence promoter fusions were used to study gene expression in two selected genes of interest.

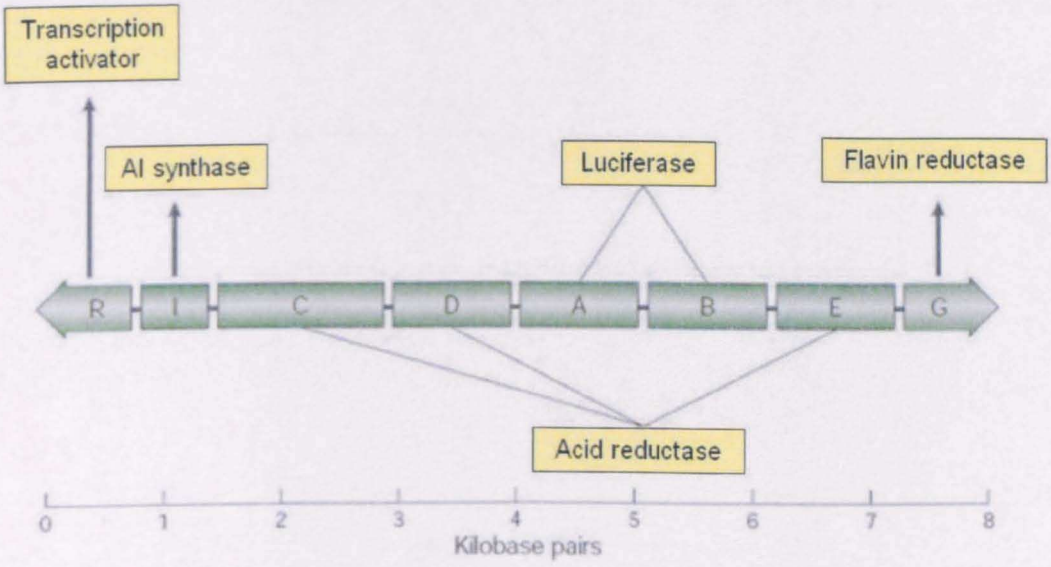


Figure 5.1. Organisation and function of the *lux* genes cloned from *Vibrio fischeri*. The *luxICDABEG* is transcribed as an operon and *luxR* is transcribed in the opposite direction. Their functions are also shown (Adapted from Fuqua & Greenberg, 2002).

5.2 Results

5.2.1 AHL profile of Y. enterocolitica 8081 *wildtype*

Preliminary studies of the AHL profile of the *Y. enterocolitica* 8081 wildtype were done using cross streak assays and Thin Layer Chromatography (TLC) analysis with AHL bioreporter strains CV026 and pSB1075 (not shown). These preliminary assays showed that the *Y. enterocolitica* 8081 wildtype produces short and long chain AHLs. In the TLC analysis, and based on previous work (Throup *et al.*, 1995; Atkinson *et al.*, 2006), the short chain AHLs produced by the wildtype include C6-HSL and 3-oxo-C6-HSL, while the long chain AHLs include 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL.

To obtain a comprehensive AHL profile for the *Y. enterocolitica* 8081 wildtype, similar to that previously produced for *Y. pseudotuberculosis* YPIII (Ortori *et al.*, 2007), cell-free supernatant extracts taken from cultures grown at 30°C in LB_{MOPS} and extracted twice with ethyl acetate. The extracts were then sent to Dr. Catherine Ortori (Centre for Analytical Bioscience, University of Nottingham) and analysed using LC-QqQLIT.

Y. enterocolitica 8081 wildtype produced at least 16 AHLs, 11 of which have not previously been documented for this bacterium. The 11 new AHLs include C4-HSL, C8-HSL, C12-HSL, C14-HSL, 3-oxo-C8-HSL, 3-oxo-C7-HSL, 3-OH-C4-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C12-HSL, and 3-OH-C14-HSL. Figure 5.2 illustrates the relative molar ratios of the major AHLs identified in the *Y. enterocolitica* 8081 wildtype. 62.4% of the total AHL is 3-oxo-C6-HSL, followed by C6-HSL (27.4%) while the uneven chain length 3-oxo-C7-HSL represents 5.1%. All percentages of the AHLs are summarised in Table 5.1.

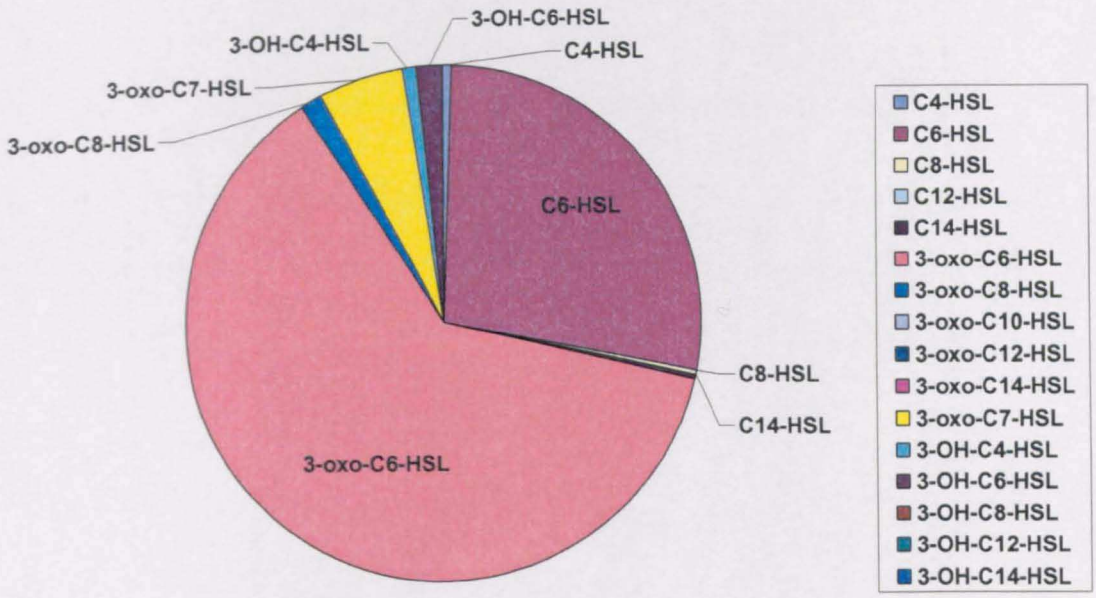


Figure 5.2. Pie chart illustrating the relative molar ratios of the AHLs identified in top *Y. enterocolitica* 8081 wildtype. The largest percentage of AHLs produced is 3-oxo-C6-HSL (62.4%), followed by C6-HSL (27.4%). The odd-numbered acyl chain AHL, 3-oxo-C7-HSL is the third highest (5.1%); 3-OH-C6-HSL is fourth (1.7%); followed by 3-oxo-C8-HSL(1.2%), 3-OH-C4-HSL (0.7%) and C4-HSL(0.6%).

Table 5.1. Percentages of AHLs produced by *Y. enterocolitica* 8081 wildtype

AHLs	Percentage
C4-HSL	0.6164
C6-HSL	27.4266
C8-HSL	0.2626
C12-HSL	0.0337
C14-HSL	0.2009
3-oxo-C6-HSL	62.4219
3-oxo-C8-HSL	1.2484
3-oxo-C10-HSL	0.0071
3-oxo-C12-HSL	0.0371
3-oxo-C14-HSL	0.0808
3-oxo-C7-HSL	5.1498
3-OH-C4-HSL	0.7140
3-OH-C6-HSL	1.7127
3-OH-C8-HSL	0.0417
3-OH-C12-HSL	0.0275
3-OH-C14-HSL	0.0188

5.2.2 AHL profiles of Y. enterocolitica 8081 *QS mutants*

The AHL profiles of the new 8081 QS mutants (Δ*yenR*, Δ*yenI*, Δ*ycoR*, Δ*yenIyenR*, Δ*yenIycoR*, Δ*yenRycoR* and Δ*yenIyenRycoR*) were examined using cross streak assays and Thin Layer Chromatography (TLC) with AHL bioreporter strains CV026 and pSB1075 (not shown) and with LC-QqQLIT analysis as described for the wildtype strain (See Section 5.2.1). The Δ*yenR*, Δ*ycoR* and Δ*yenRycoR* mutants showed similar total levels of AHL production to the wildtype (Figures 5.3, 5.4 and 5.5). The level of AHL synthesised by the Δ*yenI*, Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenIyenRycoR* mutants were greatly reduced compared to the wildtype but were not entirely abolished. As shown in Figure 5.6, Δ*yenI* still produces C4-HSL, C10-HSL, C12-HSL, 3-oxo-C4-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, 3-OH-C4-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C12-HSL and 3-OH-C14-HSL. For other AHL synthase mutants (Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenIyenRycoR*), the profiles were similar to that of the Δ*yenI* mutant (not shown). Interestingly, 3-OH-C4-HSL levels are similar in all strains tested.

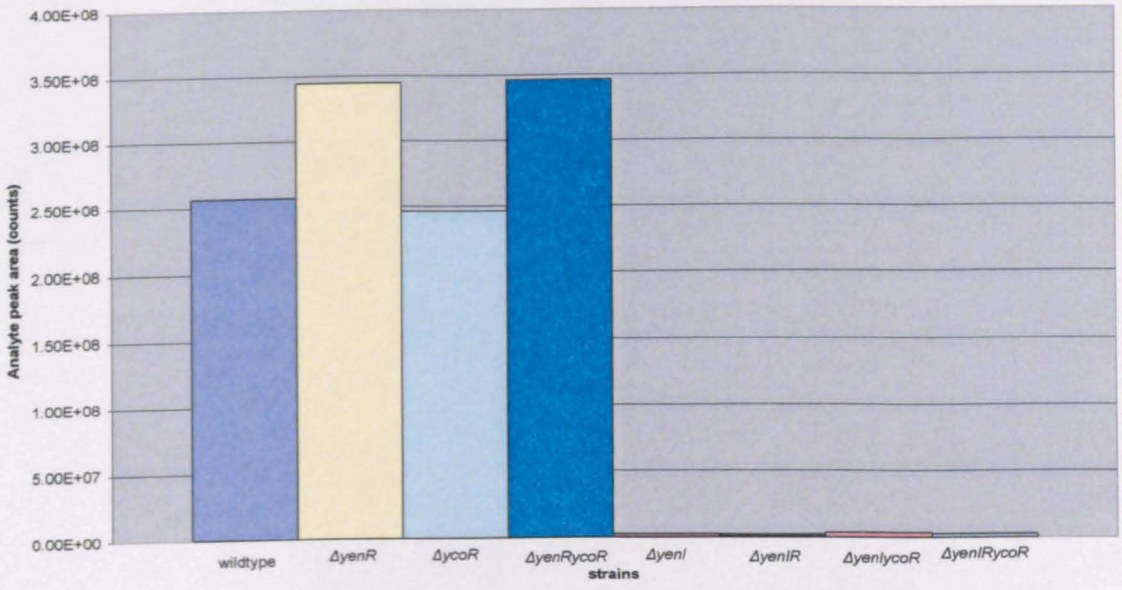


Figure 5.3. Chart comparing the total amount of AHLs produced by *Y. enterocolitica* 8081 wildtype and QS mutants. The R mutants (Δ*yenR*, Δ*ycoR* and Δ*yenRycoR*) showed similar total AHL levels to the wildtype while AHL synthase mutants (Δ*yenI*, Δ*yenIyenR*, Δ*yenIycoR* and Δ*yenIyenRycoR*) showed diminished amounts of AHLs produced but not completely so as there were still some low, detectable levels of AHLs.

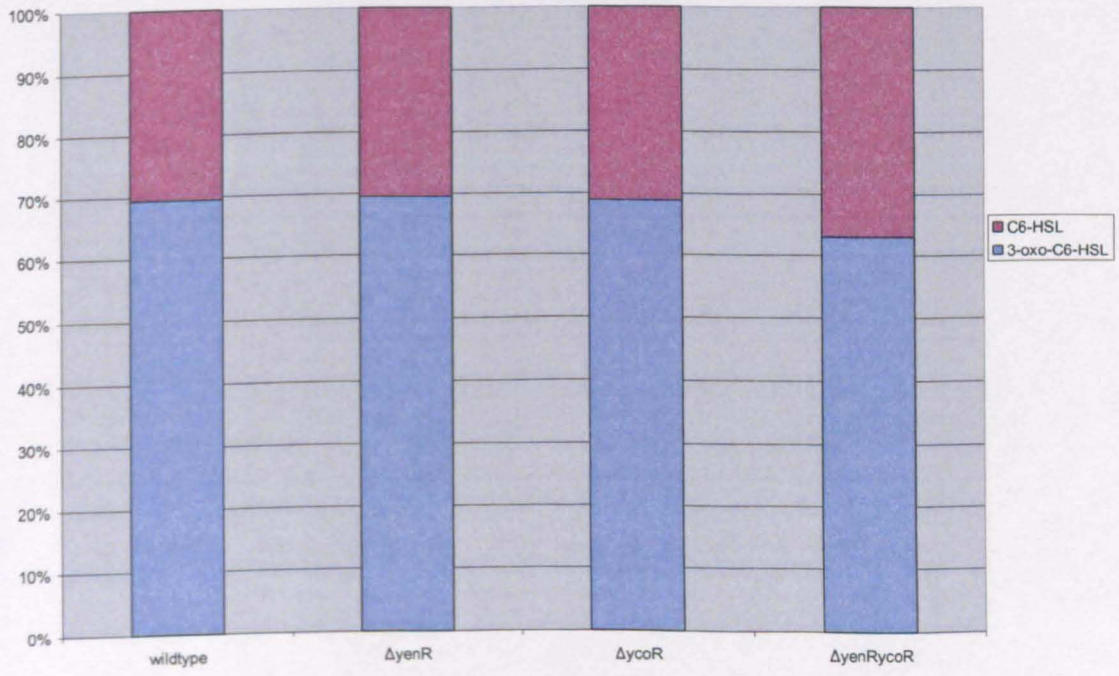


Figure 5.4. Chart comparing the C6-HSL and 3-oxo-C6-HSL production profiles of the *Y. enterocolitica* 8081 wildtype compared to the R mutants: Δ*yenR*, Δ*ycoR* and Δ*yenRycoR*.

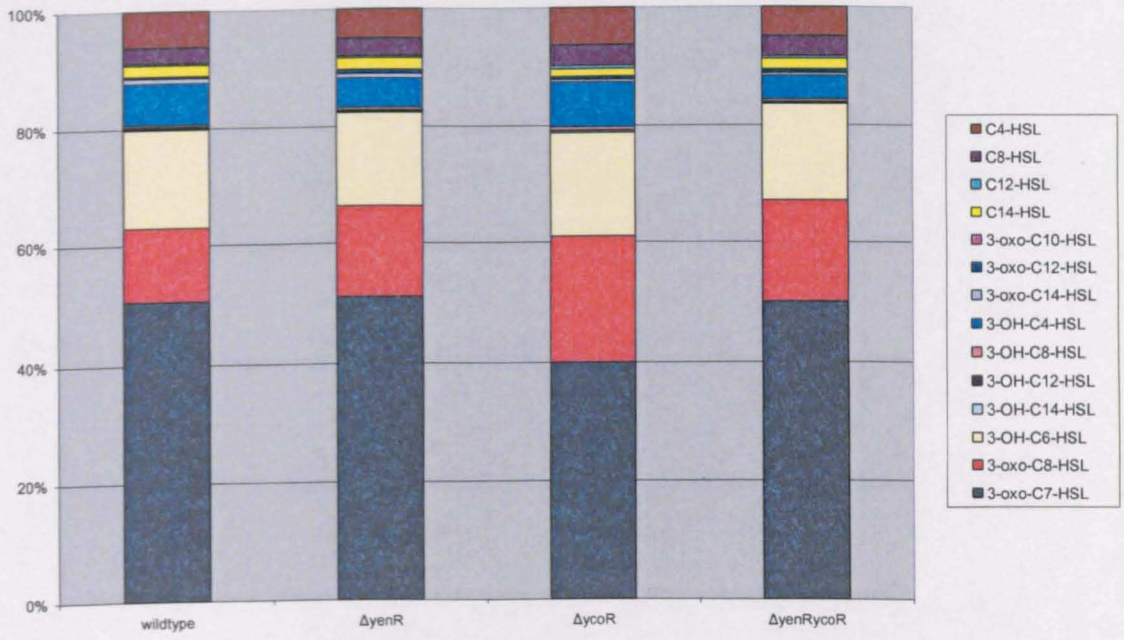


Figure 5.5. Chart comparing the AHL profiles (excluding C6-HSL and 3-oxo-C6-HSL) of the *Y. enterocolitica* 8081 wildtype compared with the R mutants: Δ*yenR*, Δ*ycoR* and Δ*yenRycoR*.

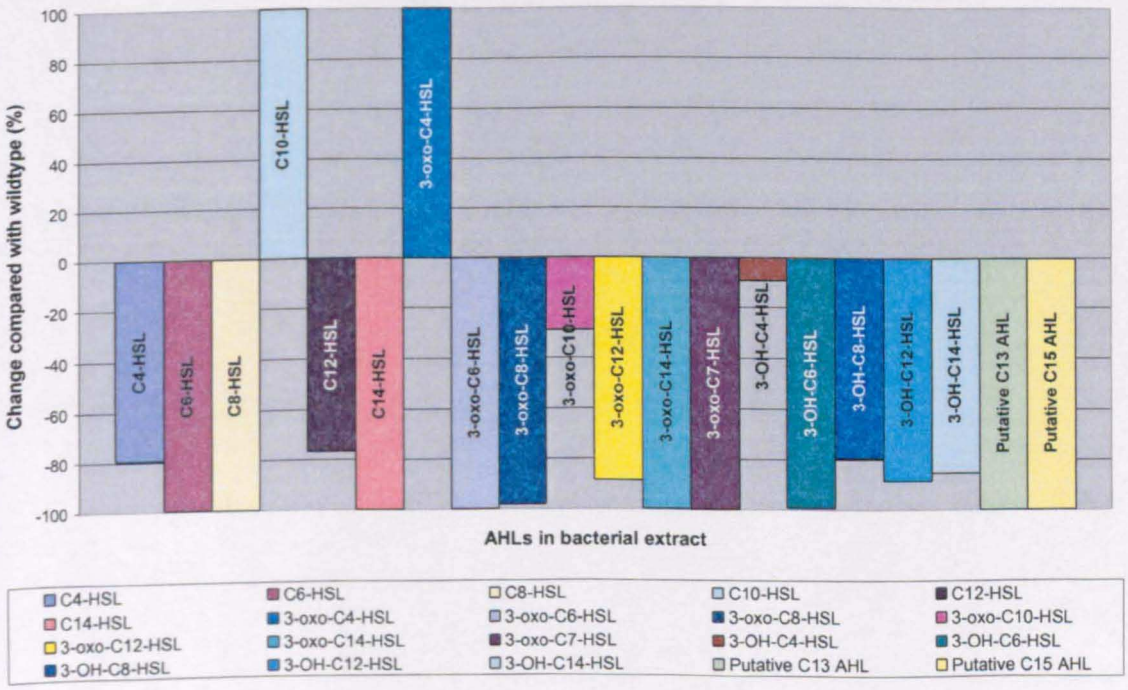


Figure 5.6. Percentage of change for AHLs produced by *Y. enterocolitica* 8081 Δ*yenI* mutant compared with wildtype.

5.2.3 Phenotypic studies on Y. enterocolitica 8081 *wildtype and QS mutants*

5.2.3.1 Swimming and swarming plate assay

Swimming and swarming motility of *Y. enterocolitica* 8081 wildtype and 5 QS mutants were tested (See Section 2.14.1) at 22°C and 37°C. The *Y. enterocolitica*, strain 90/54 (serotype O:9) wildtype and its Δ*yenI* mutant were included in this experiment as controls because 90/54 Δ*yenI* mutant was shown to be impaired in swimming motility and does not swarm (Atkinson *et al.*, 2006). All assays were done in triplicate. At 22°C, it was found that the swimming and swarming motility of the 8081 QS mutants were not affected which is in contrast to the impaired swimming motility and abolished swarming motility shown by the 90/54 (serotype O:9) Δ*yenI* mutant. There were no obvious differences in the distances of swarming/swimming from the central inoculation site for the 8081 QS mutants compared to 8081 wildtype. At 37°C, all strains neither swam nor swarmed. Table 5.2 shows the results of the swimming and swarming assay on the *Y. enterocolitica* wildtype and QS mutants 24 h after inoculation.

Table 5.2. Results of the swimming and swarming assay on the *Y. enterocolitica* wildtype and QS mutants 24 h after inoculation.

Strains	Swimming		Swarming	
	22 °C	37 °C	22 °C	37 °C
8081 wildtype	+++	-	+++	-
8081 Δ*yenI*	+++	-	+++	-
8081 Δ*yenR*	+++	-	+++	-
8081 Δ*ycoR*	+++	-	+++	-
8081 Δ*yenRycoR*	+++	-	+++	-
8081 Δ*yenIyenRycoR*	+++	-	+++	-
90/54 wildtype	+++	-	+++	-
90/54 Δ*yenI*	+	-	-	-

5.2.3.2 Agglutination assay

The agglutination phenotype is used as an indication of virulence in *Y. enterocolitica* attributed to YadA which is encoded on the pYVe plasmid. YadA is a homotrimer of approximately 45 kDa subunits that is 'lollipop'-shaped and is anchored to the outer membrane covering and conferring hydrophobic properties to the bacterial surface. *yadA* expression is induced at 37°C by the temperature-dependent transcriptional activator LcrF (El Tahir & Skurnik, 2001). The agglutination ability of *Y. enterocolitica* 8081 wildtype and its QS mutants were tested using a modified agglutination assay, performed according to Laird and Cavanaugh (1980) (See Section 2.14.3). All assays were done in triplicate. In this study, all *Y. enterocolitica* 8081 strains tested were agglutination-positive. Table 5.3 shows the results of the agglutination assay on the *Y. enterocolitica* 8081 wildtype and 5 QS mutants.

Table 5.3. Results of the agglutination assay on the *Y. enterocolitica* 8081 wildtype and 5 QS mutants

Strains	**Agglutination**
wildtype	+
Δ*yenI*	+
Δ*yenR*	+
Δ*ycoR*	+
Δ*yenRycoR*	+
Δ*yenIyenRycoR*	+

5.2.3.3 Haemagglutination assay

The haemagglutination phenotype is also used as an indication of virulence in *Y. enterocolitica* attributed to YadA. The ability of *Y. enterocolitica* 8081 wildtype and its QS mutants were tested (See Section 2.14.4). All assays were done in triplicate. In this study, all *Y. enterocolitica* strains tested were haemagglutination-negative at 22°C and haemagglutination-positive at 37°C. Table 5.4 shows the results of the haemagglutination assay on the *Y. enterocolitica* 8081 wildtype and 5 QS mutants.

Table 5.4. Results of the haemagglutination assay on the *Y. enterocolitica* 8081 wildtype and 5 QS mutants

Strains	**Haemagglutination**	
	22 °C	**37 °C**
wildtype	-	+
Δ*yenI*	-	+
Δ*yenR*	-	+
Δ*ycoR*	-	+
Δ*yenRycoR*	-	+
Δ*yenIyenRycoR*	-	+

5.2.3.4 Protein profile analysis

Four different protein profiles of *Y. enterocolitica* 8081 wildtype and five QS mutants (Δ*yenI*, Δ*yenR*, Δ*ycoR*, Δ*yenRycoR* and Δ*yenIyenRycoR*) were studied. The exoproteins from bacterial culture supernatant, cytoplasmic proteins, inner membrane proteins (IMP) and outer membrane proteins (OMP) from bacterial cultures grown at 22°C and 37°C were extracted and SDS-PAGE were carried out. All experiments were done in triplicate. There were no obvious differences between the QS mutants compared to wildtype for any of the protein extracts. However, temperature clearly has an effect because the protein profiles extracted at 22°C were different from those extracted at 37°C. Figures 5.7 to 5.10 show the four types of protein profiles of *Y. enterocolitica* 8081 wildtype and 5 QS mutants at 22°C and 37°C.

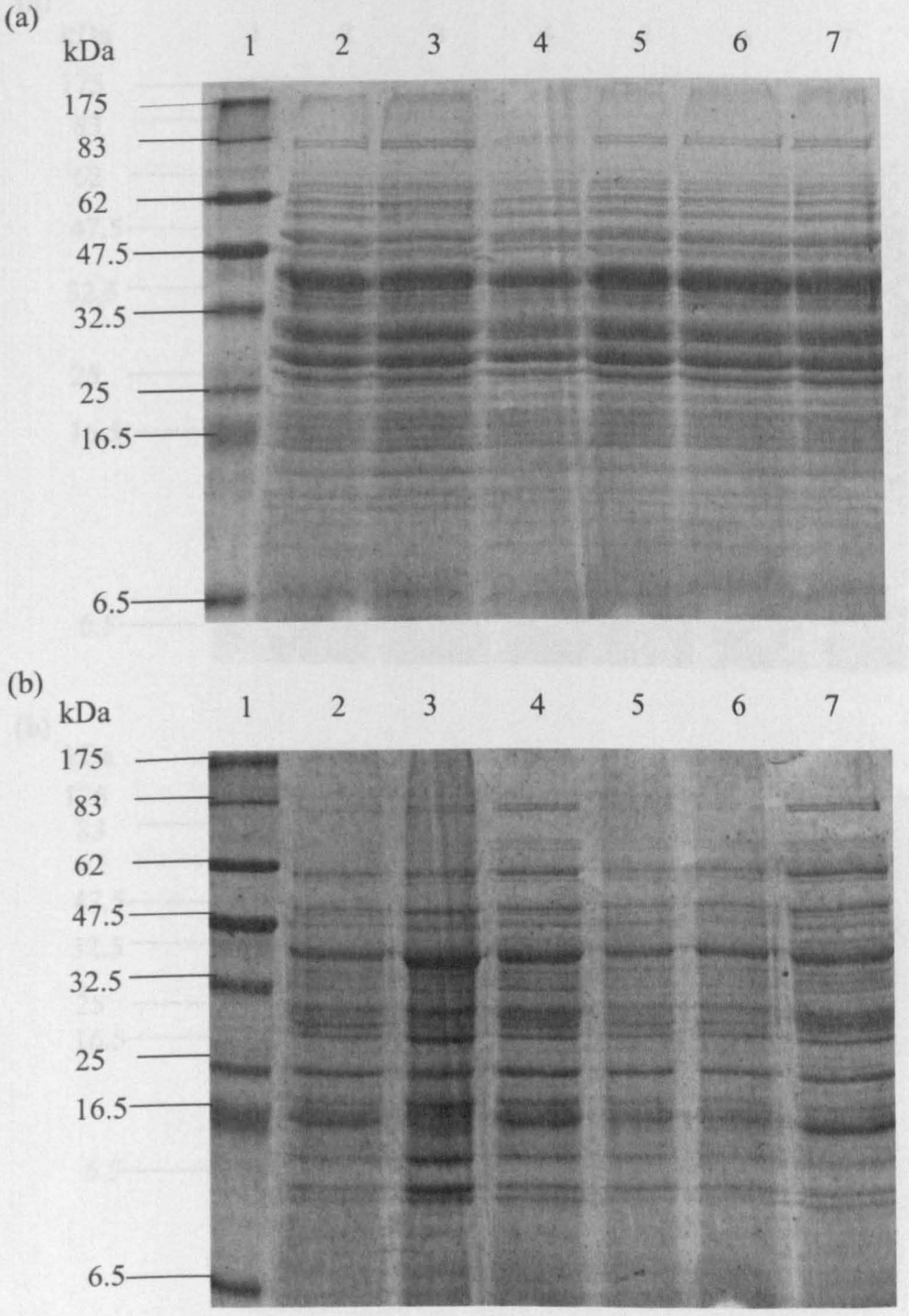


Figure 5.7. SDS-PAGE analysis of supernatant extracts of the *Y. enterocolitica* 8081 wildtype strain and QS mutants at (a) 22°C and (b) 37°C. Lane 1, Precision Plus Protein All Blue Standard (BioRad, UK); Lane 2, wildtype; Lane 3, Δ*yenI* mutant, Lane 4, Δ*yenR* mutant, Lane 5, Δ*ycoR* mutant, Lane 6, Δ*yenRycoR* mutant and Lane 7, Δ*yenIyenRycoR* mutant.

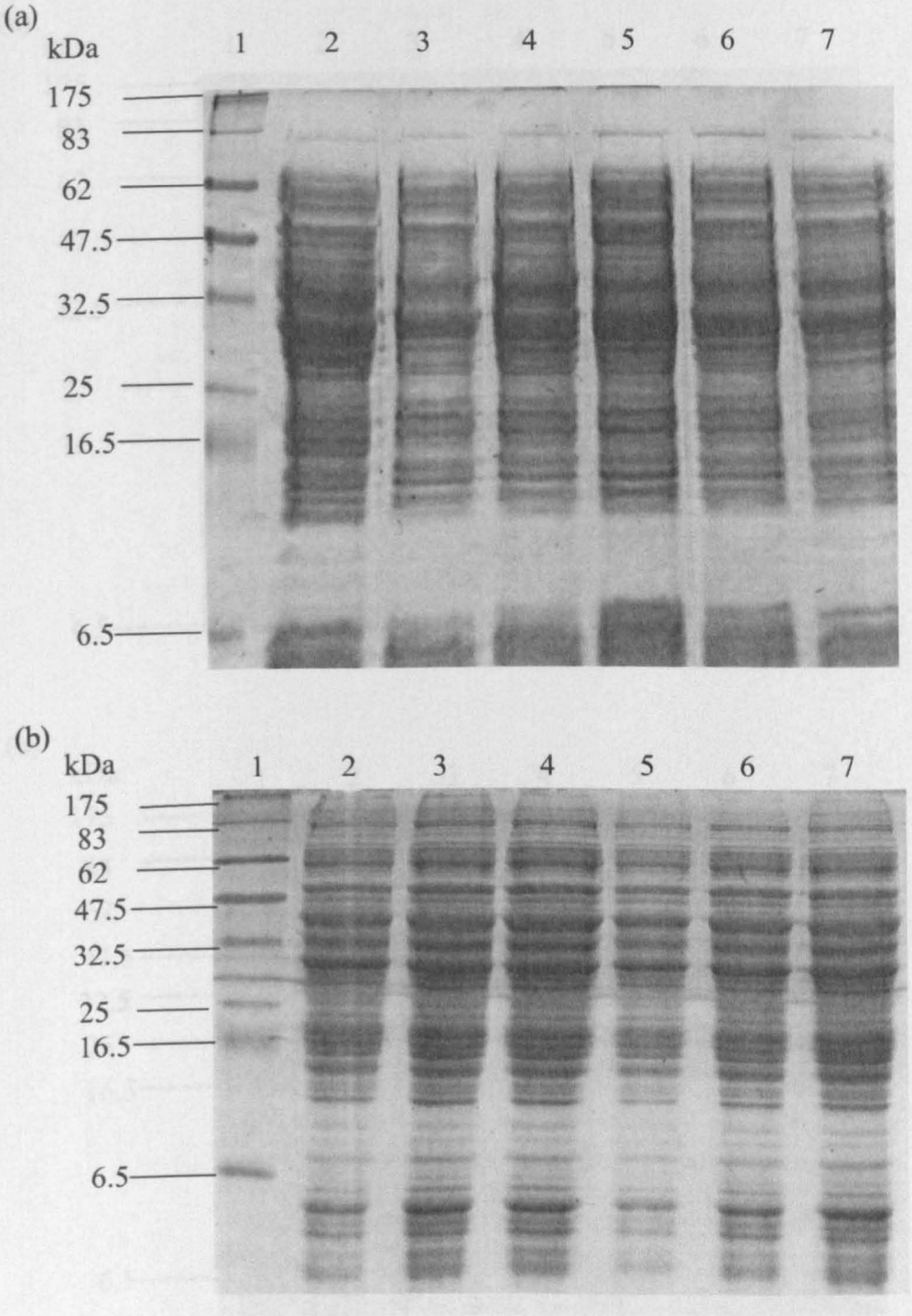


Figure 5.8. SDS-PAGE analysis of cytoplasmic extracts of the *Y. enterocolitica* 8081 wildtype strain and QS mutants at (a) 22°C and (b) 37°C. Lane 1, Precision Plus Protein All Blue Standard (BioRad, UK); Lane 2, wildtype; Lane 3, Δ*yenI* mutant, Lane 4, Δ*yenR* mutant, Lane 5, Δ*ycoR* mutant, Lane 6, Δ*yenRycoR* mutant and Lane 7, Δ*yenIyenRycoR* mutant.

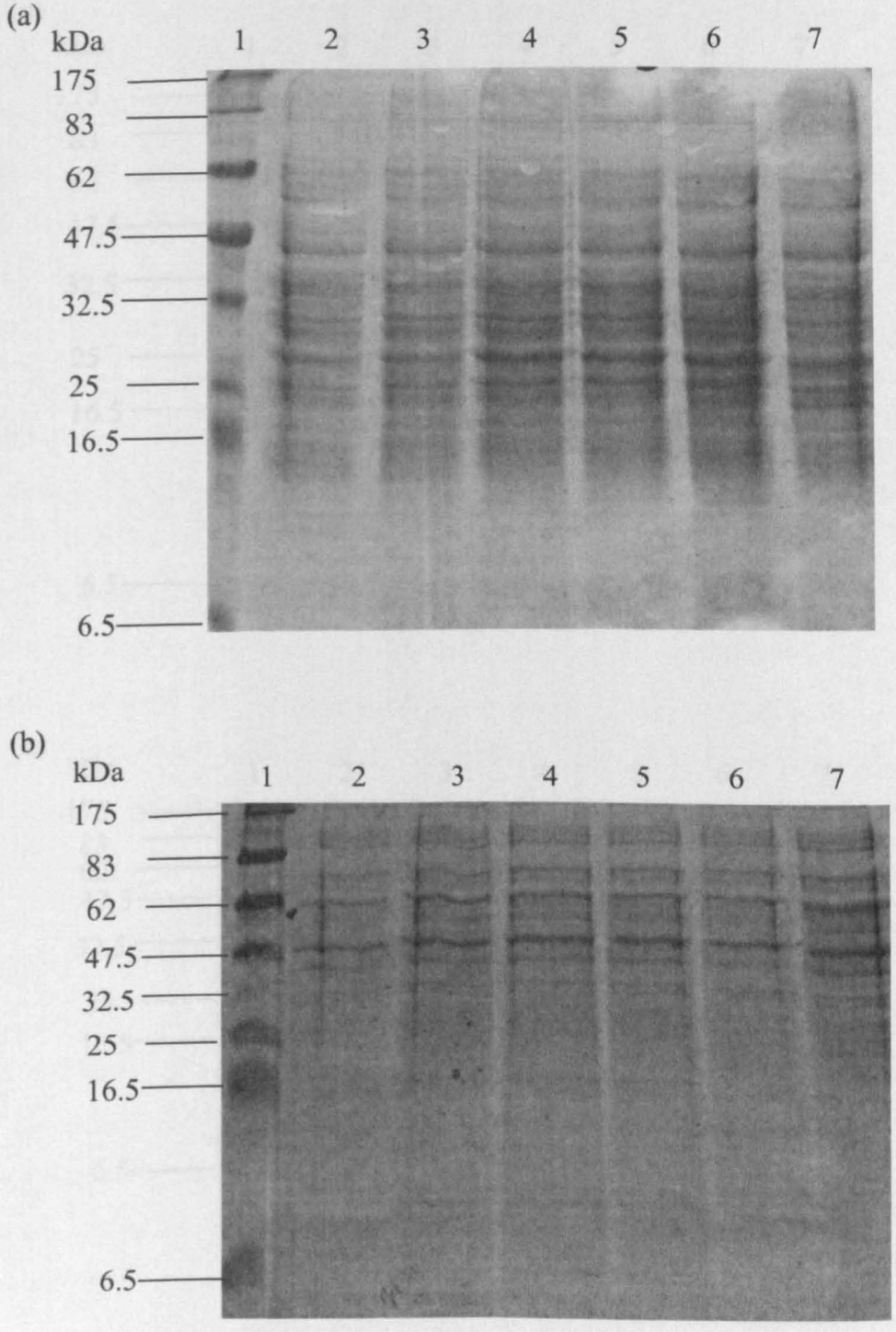


Figure 5.9. SDS-PAGE analysis of inner membrane proteins of the *Y. enterocolitica* 8081 wildtype strain and QS mutants at (a) 22°C and (b) 37°C. Lane 1, Precision Plus Protein All Blue Standard (BioRad, UK); Lane 2, wildtype; Lane 3, Δ*yenI* mutant, Lane 4, Δ*yenR* mutant, Lane 5, Δ*ycoR* mutant, Lane 6, Δ*yenRycoR* mutant and Lane 7, Δ*yenIyenRycoR* mutant.

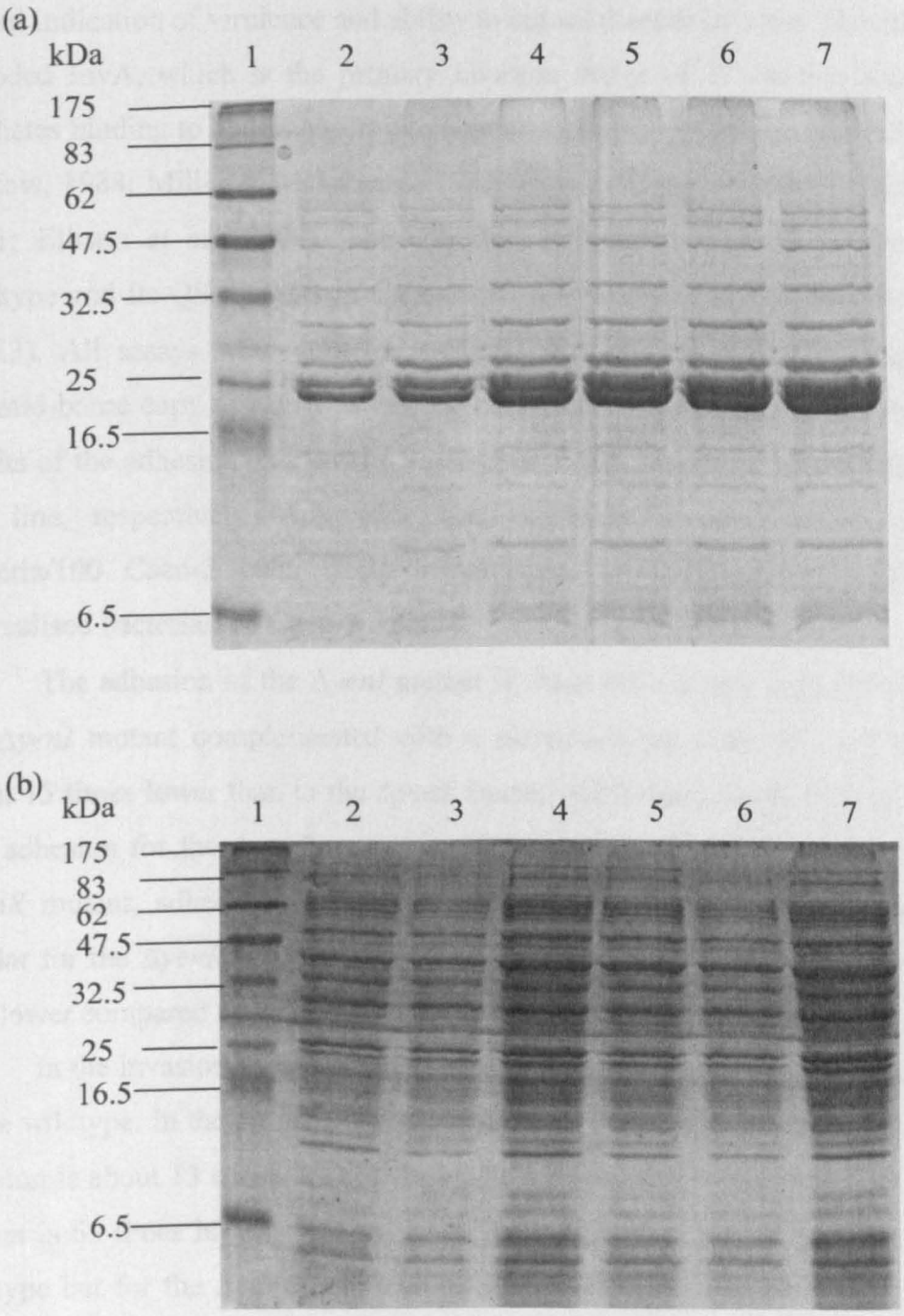


Figure 5.10. SDS-PAGE analysis of outer membrane proteins extracts of the *Y. enterocolitica* 8081 wildtype strain and QS mutants at (a) 22°C and (b) 37°C. Lane 1, Precision Plus Protein All Blue Standard (BioRad, UK); Lane 2, wildtype; Lane 3, Δ*yenI* mutant, Lane 4, Δ*yenR* mutant, Lane 5, Δ*ycoR* mutant, Lane 6, Δ*yenRycoR* mutant and Lane 7, Δ*yenIyenRycoR* mutant.

5.2.3.5 Adhesion and invasion assays in Caco-2 cell line

The ability of pathogenic *Yersinia* to adhere to and invade tissue culture cells is a vital indication of virulence and ability to induce disease in hosts. The chromosomally encoded InvA, which is the primary invasion factor of *Y. enterocolitica* and YadA mediates binding to and invasion into host epithelial cells (Isberg *et al.*, 1987; Miller & Falkow, 1988; Miller & Mekalanos, 1988; Pepe & Miller, 1990; El Tahir & Skurnik, 2001; Ellison *et al.*, 2004). The adhesion and invasion of *Y. enterocolitica* 8081 wildtype and its QS mutants in Caco-2 cell line were tested (See Sections 2.16.2 and 2.16.3). All assays were done in triplicate. A Δ*yenI* mutant complemented with a plasmid-borne copy of *yenI* was included in this study. Figures 5.11 and 5.12 show the results of the adhesion and invasion assays of *Y. enterocolitica* 8081 strains on Caco-2 cell line, respectively. Adherence was expressed as the number of associated bacteria/100 Caco-2 cells while invasiveness was expressed as the number of internalised bacteria/100 Caco-2 cells.

The adhesion of the Δ*yenI* mutant is about 80% higher than in the wildtype. In the Δ*yenI* mutant complemented with a plasmid-borne copy of *yenI*, adhesion was about 15 times lower than in the Δ*yenI* mutant and 8 times lower than in the wildtype. The adhesion for the Δ*yenR* mutant is 52% lower compared to wildtype while in the Δ*ycoR* mutant, adhesion is almost 2 fold higher compared to wildtype and this is similar for the Δ*yenRycoR* mutant. The Δ*yenIyenRycoR* mutant shows adhesion of ~7 fold lower compared to wildtype.

In the invasion assay, invasion of the Δ*yenI* mutant is 92 times higher compared to the wildtype. In the Δ*yenI* mutant complemented with a plasmid-borne copy of *yenI*, invasion is about 13 times lower than in the Δ*yenI* mutant. The invasion for the Δ*yenR* mutant is 65 times higher than wildtype. For the Δ*ycoR* mutant, invasion is similar to wildtype but for the Δ*yenRycoR* mutant, is 4 times higher than wildtype while in the Δ*yenIyenRycoR* mutant, the invasion is similar to wildtype.

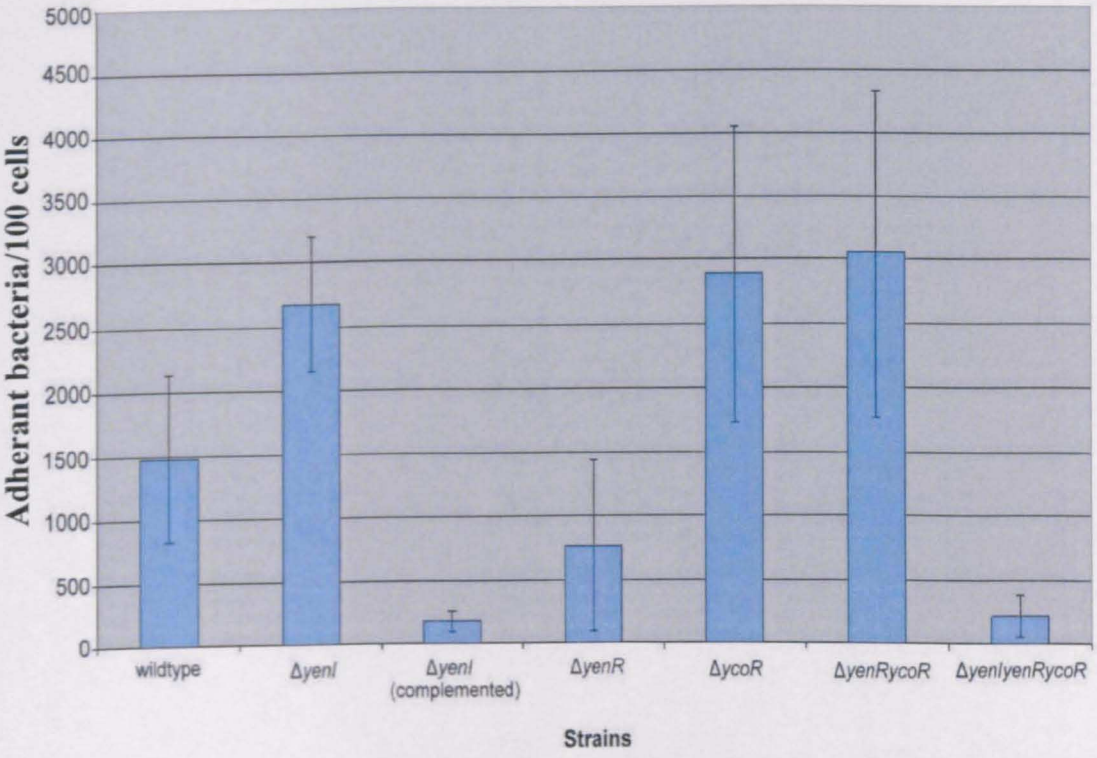


Figure 5.11. Adhesion of *Y. enterocolitica* 8081 strains to Caco-2 cell line. Unpaired T-test ($p \leq 0.05$) revealed that, when compared to wildtype or Δ*yenI* (complemented), only three pairings were significantly different: wildtype versus Δ*yenI* complemented mutant; wildtype versus Δ*yenIyenRycoR* mutant and Δ*yenI* versus Δ*yenI* (complemented)

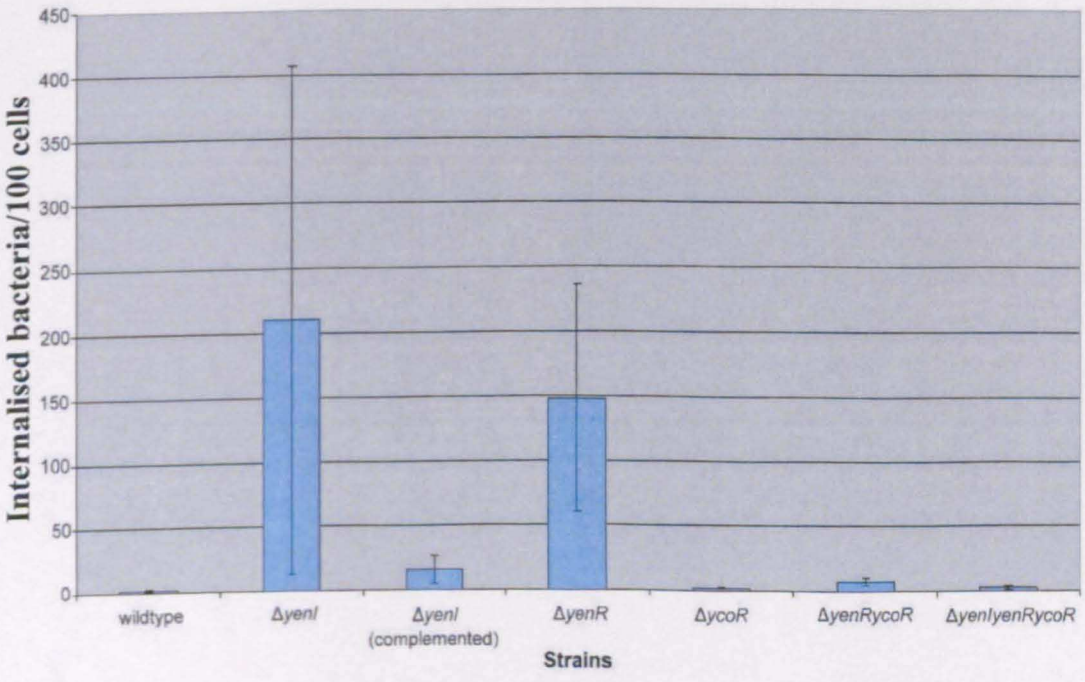


Figure 5.12. Invasion of Caco-2 cell line by *Y. enterocolitica* 8081 strains. Unpaired T-test ($p \leq 0.05$) revealed that, when compared to wildtype or Δ*yenI* (complemented), none of the pairings were significantly different.

5.2.3.6 Congo Red binding assay and plasmid loss study

The ability to bind Congo Red in *Y. enterocolitica* 8081 wildtype and its QS mutants were tested using a modified Congo Red agar (See Section 2.14.2) (Riley & Toma, 1989). Positive strains (CRMOX+) always produced a mixture of small red colonies (CRMOX+) and large colourless colonies (CRMOX-). Negative strains (CRMOX-) produced only large colourless colonies. The presence of CRMOX- colonies was due to a rapid loss of the pYVe plasmid *in vitro*. Consequently, subculture of CRMOX+ colonies onto CRMOX would consistently yield both CRMOX+ and CRMOX- colonies. Subculture of CRMOX- colonies from CRMOX+ strains would grow only CRMOX- colonies. All assays were done in triplicate.

Figure 5.13 shows CRMOX+ *Y. enterocolitica* 8081 wildtype (a), and a known CRMOX- *Y. enterocolitica* Δ*yenI* mutant which was cured of the pYVe plasmid (b). In this study, the *Y. enterocolitica* wildtype and QS mutants were able to bind Congo Red on CRMOX plates at 37°C and formed the characteristic small red colonies.

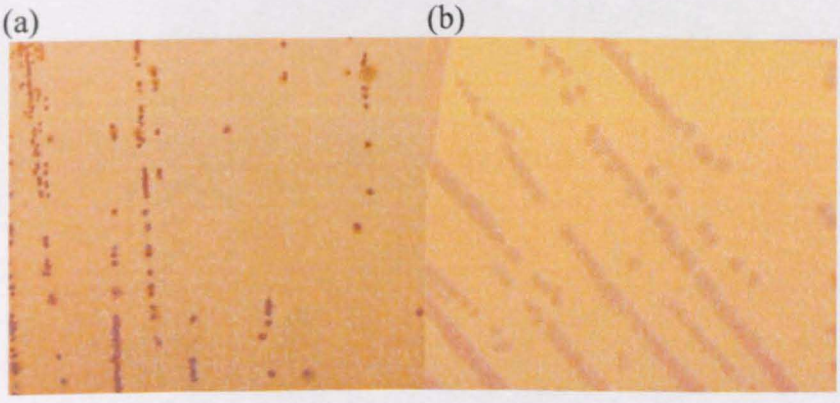


Figure 5.13. The appearances of a CRMOX+ strain, *Y. enterocolitica* 8081 wildtype (a), and a control CRMOX- strain *Y. enterocolitica* Δ*yenI* mutant which was cured of the pYVe plasmid (b). CRMOX+ strains always produced small red colonies and large colourless colonies. CRMOX- strains produced only large colourless colonies.

During sub-culture experiments which were intended to examine the growth of the QS mutants, each sample was checked on CRMOX plates to ensure that the strains maintained the pYVe plasmid. It was noted that the *yenI* mutants appeared to be rapidly losing the plasmid, as indicated by the increasing percentage of white colonies appearing on the CRMOX plates after only 4 subcultures when compared to the wildtype. These observations prompted a quantitative study of plasmid loss in the QS mutants and included a complemented Δ*yenI* strain. Rates of plasmid loss were observed by subculturing the strains in LB broth over 10 days, plating serially diluted cultures at 10^{-6} and the percentage of white colonies were determined for each day. All assays were done in triplicate. Figure 5.14(a) and (b) reveals that the pYVe plasmid is lost rapidly at 37°C in the QS mutants compared to wildtype, especially in the Δ*yenRycoR* mutant. Complementation *in trans* enabled the Δ*yenI* mutant to maintain the pYVe plasmid and was actually less likely to lose the plasmid when compared to wildtype (Figure 5.14a). At 22°C, only the Δ*yenRycoR* mutant lost the pYVe plasmid and this appeared to be constant (~20%) over 10 days (Figure 5.14b).

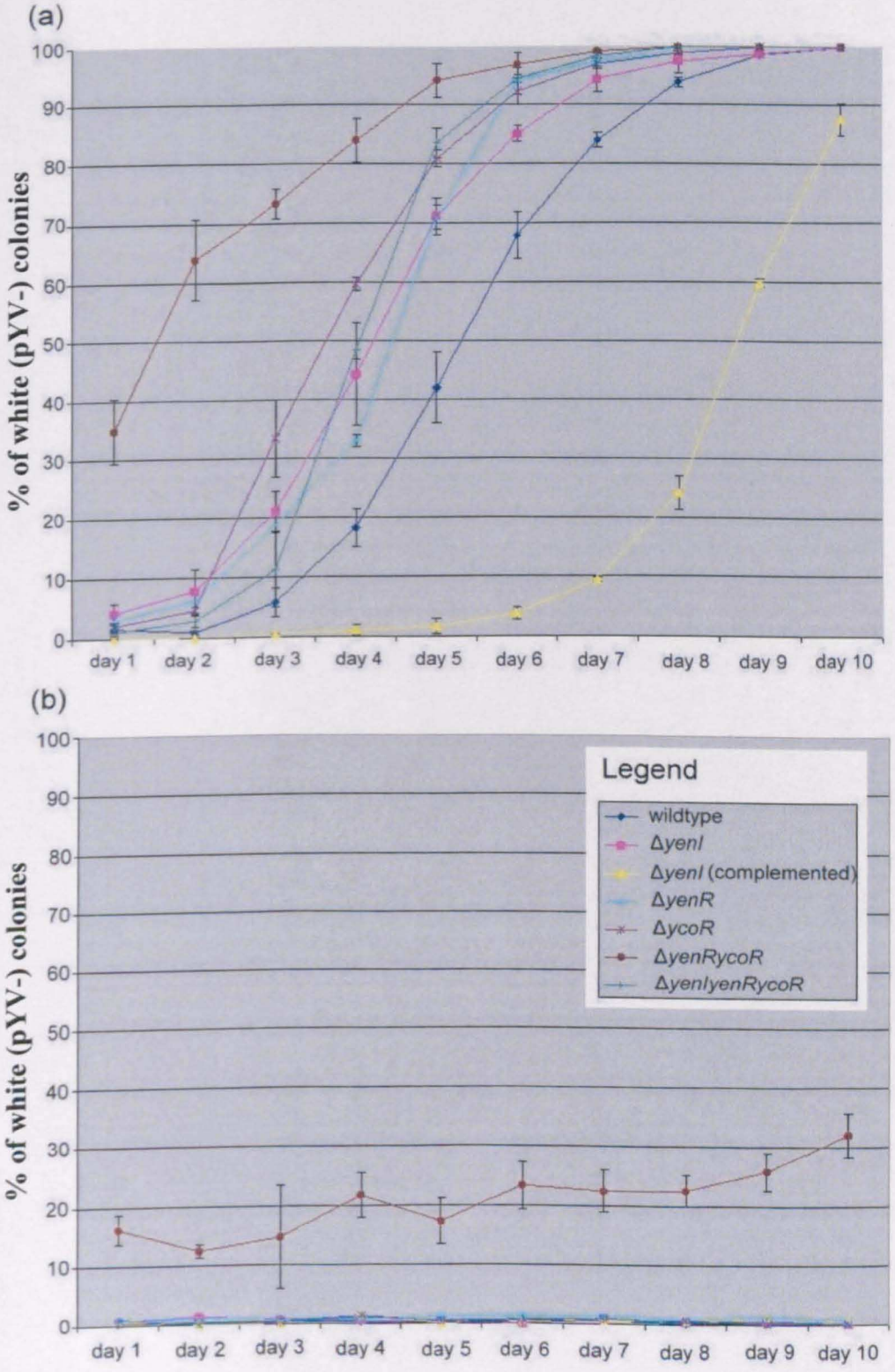


Figure 5.14. Rate of pYV plasmid loss in *Y. enterocolitica* 8081 wildtype and QS mutants at 37°C (a) and 22°C (b).

5.2.3.7 Bioluminescence-based gene expression studies

RQ-PCR confirmed that components of the QS system regulate the expression of *tyeA*, *virF*, *yscF*, *yadA*, *repA* and *spyA* (See Section 4.2.3). To examine in detail the expression of two of these genes, *virF* and *spyA*, *lux*-based promoter fusions were constructed and bioluminescence as a measure of expression was examined over an 18 h period. This method has been used previously to study gene expression in *Y. pseudotuberculosis* YPIII (Atkinson *et al.*, 2008). *virF* is of particular interest because of its role as a key regulator of many virulence plasmid-associated genes such as *yop* genes, *ylpA* (*Yersinia* lipoprotein A), *yadA* (adhesion factor) and *sycE* (chaperone protein for YopE) (See Section 4.2.2). *spyA* was targeted because pYVe plasmid loss studies (Section 5.2.3.6) suggest that QS regulates the maintenance of the pYVe plasmid in *Y. enterocolitica* 8081 strains. This was also supported by RQ-PCR results which indicate that *spyA* was downregulated in three QS mutants.

PCR primers were designed to amplify the promoter regions of *virF* and *spyA* incorporating *Apa*I and *Not*I ends. The resulting PCR products were cloned into similarly digested pBluescript to give pBlue::P_{virF} and pBlue::P_{spyA}. The *luxCDABE* cassette was excised from pBluelux (Atkinson *et al.*, 2008) and cloned into either pBlue::P_{virF} or pBlue::P_{spyA} as a *Sac*I fragment after which the P_{virF}::*luxCDABE* and P_{spyA}::*luxCDABE* cassettes were cloned as *Apa*I and *Not*I fragments into similarly digested pDM4 (Milton *et al.*, 1996) to give pYK800 and pYK801. Single crossover conjugation into the wildtype, Δ*yenI*, Δ*yenRycoR* and Δ*yenIyenRycoR* strains were performed as previously described (Atkinson *et al.*, 1999).

The expression of *virF* and *spyA* were determined as a function of temperature and growth phase by recording bioluminescence throughout growth in liquid culture (LB_{MOPS}) at 22°C or 37°C. The level of bioluminescence was expressed as light per unit optical density at 405nm (RLU/OD_{405}) (refer to Section 2.15). Examination of the growth curves of all the strains harbouring the 2 *lux*-fusion constructs revealed that the strains were not affected in growth due to the presence of the plasmid constructs.

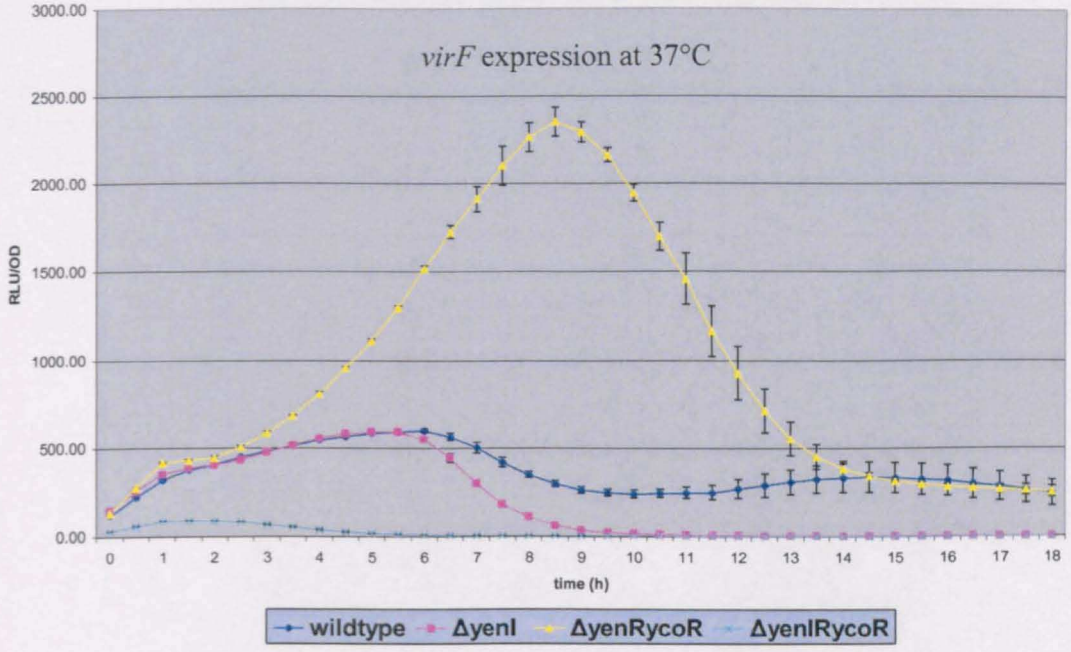
At 37°C, the expression of *virF* in the Δ*yenRycoR* mutant was ~10 fold higher compared with the wildtype, Δ*yenI* and Δ*yenIyenRycoR* strains (Figure 5.15). *virF* expression in the Δ*yenRycoR* mutant steadily rose to a maximum of ~2300 units at 8.5 h and then gradually fell to ~250 units at 18 h. Meanwhile the expression of *virF* in the wildtype remains low, peaking at ~600 units at 6 h, then falling to a constant 250 units. The expression of *virF* in the Δ*yenI* mutant is similar to the wildtype, peaking at ~600

units at 6 h and falling to near zero by 9 h. In the Δ*yenIyenRycoR* mutant, the expression was very low, remaining near zero for the duration of the experiment. At 22°C, the overall expression of *virF* is very low compared to 37°C (Figure 5.16). Expression of *virF* was ~6 fold lower in the Δ*yenIyenRycoR* mutant for ~9 h compared to the wildtype, Δ*yenI* and Δ*yenRycoR* strains. After 9 h, expression of *virF* starts to fall and by 18 h, the wildtype and all the mutants have similar expression levels.

At 37°C, the expression profiles of *spyA* in the wildtype and Δ*yenRycoR* mutant are almost identical (Figure 5.17). *spyA* expression steadily rose to a maximal level of ~2750 units at 5.5 h and then gradually fell to ~500 units by 18 h. Meanwhile the expression of *spyA* in the Δ*yenI* and Δ*yenIyenRycoR* mutants were relatively low, peaking at ~500 or ~750 units respectively. At 22°C, the expression of *spyA* is much lower compared to 37°C but the expression patterns were very similar at both temperatures for the wildtype and QS mutants (Figure 5.18).

Since the expression of *spyA* in the *yenI* mutants (Δ*yenI* and Δ*yenIyenRycoR*) were relatively low compared to wildtype at 37°C, we sought to investigate if addition of exogenous AHLs would restore expression of *spyA* in the *yenI* mutants to wildtype levels. Figure 5.19 shows that adding C6-HSL and/or 3-oxo-C6-HSL at 100 and 200 μM to the Δ*yenI* and Δ*yenIyenRycoR* mutants did not alter the expression of *spyA*.

(a)



(b)

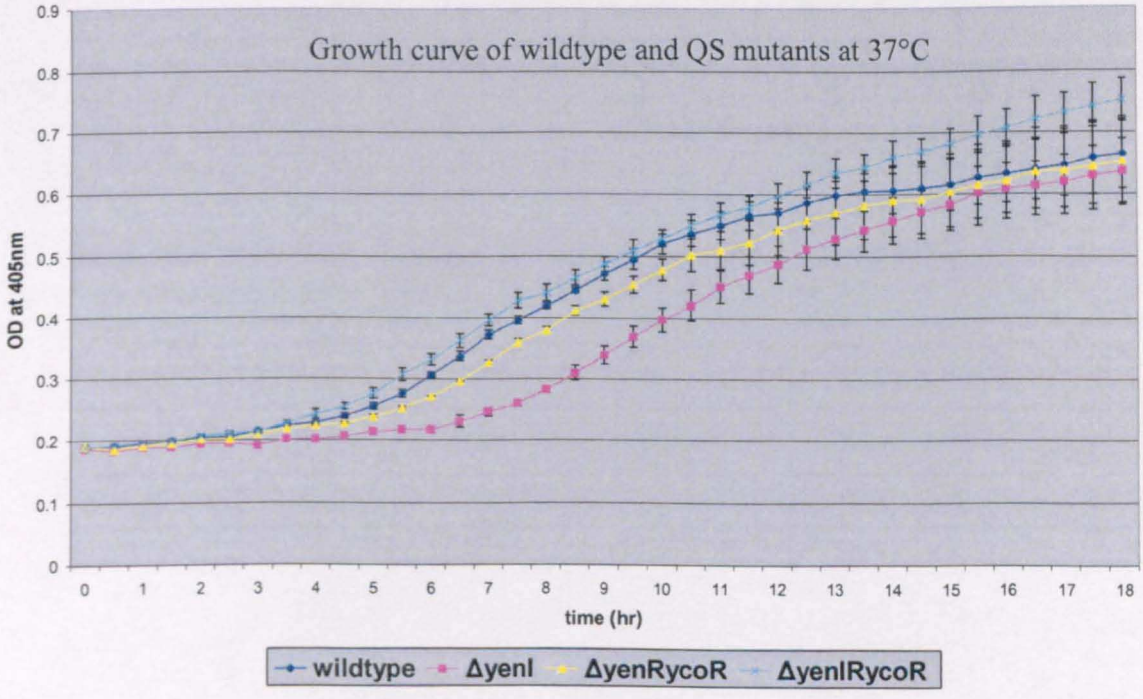
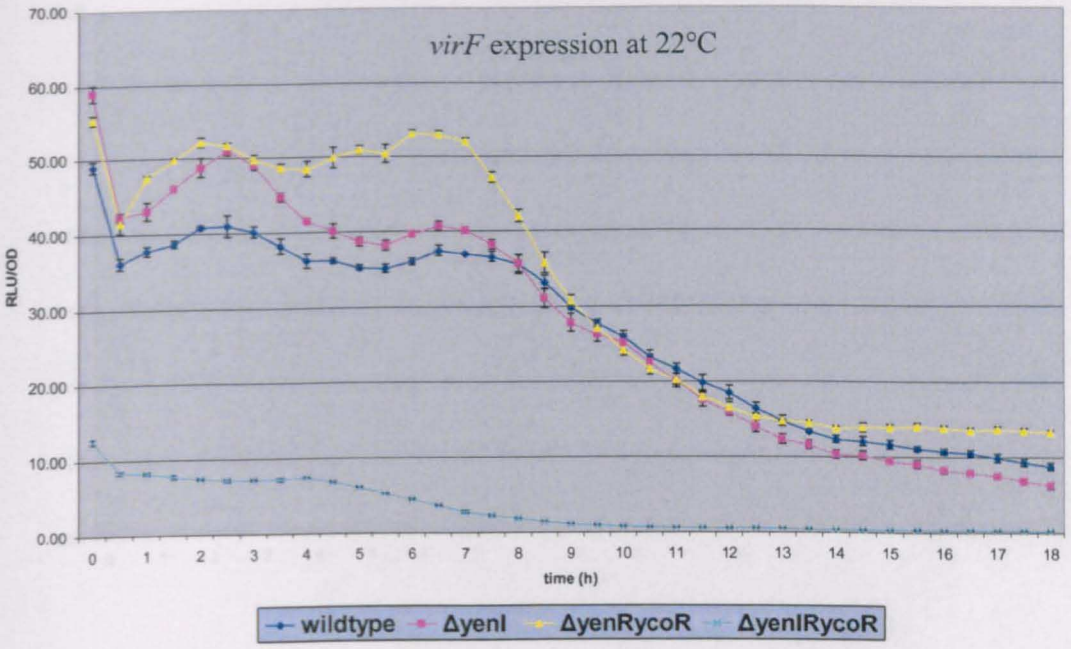


Figure 5.15. (a) Analysis of *virF* expression in *Y. enterocolitica* 8081 wildtype and QS mutants at 37 °C. (b) Growth curve of wildtype and QS mutants at 37°C.

(a)



(b)

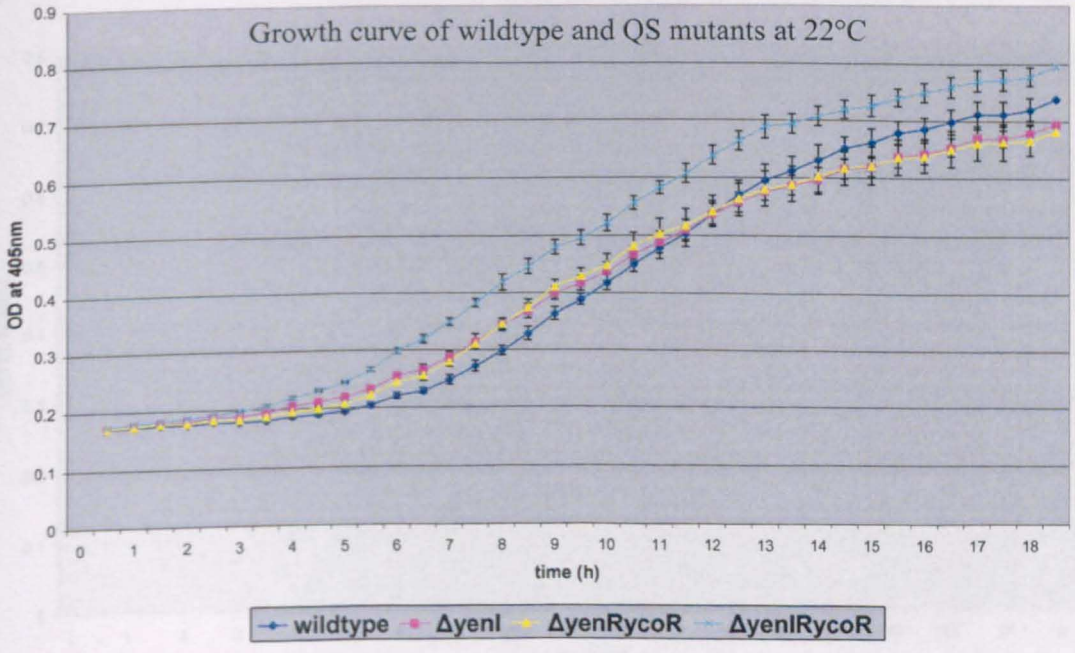
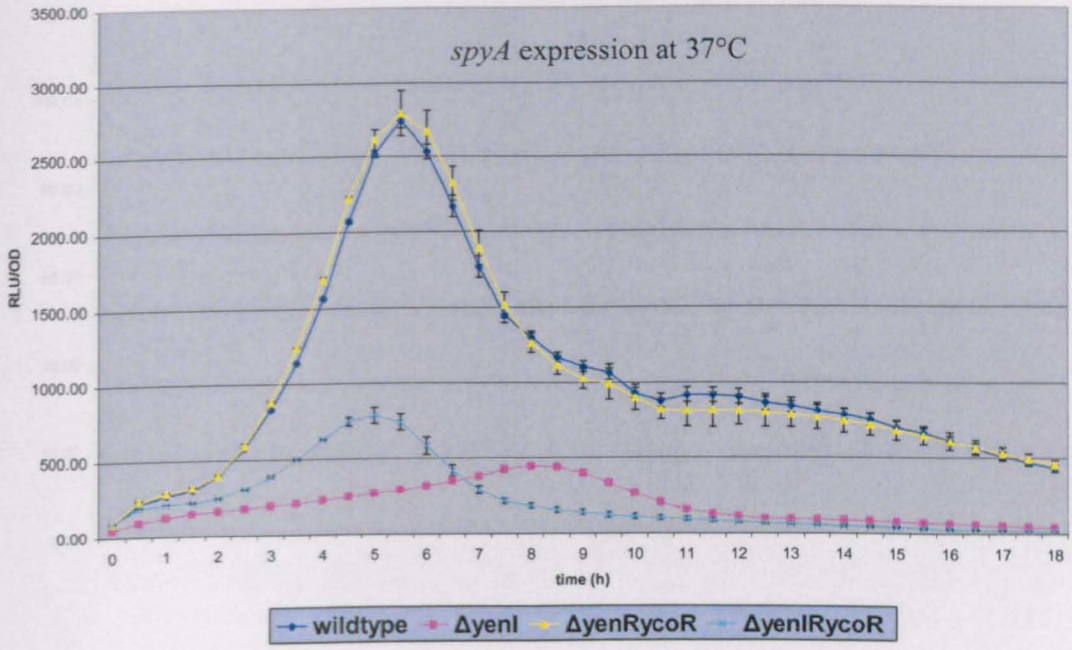


Figure 5.16. (a) Analysis of *virF* expression in *Y. enterocolitica* 8081 wildtype and QS mutants at 22 °C. (b) Growth curve of wildtype and QS mutants at 22°C.

(a)



(b)

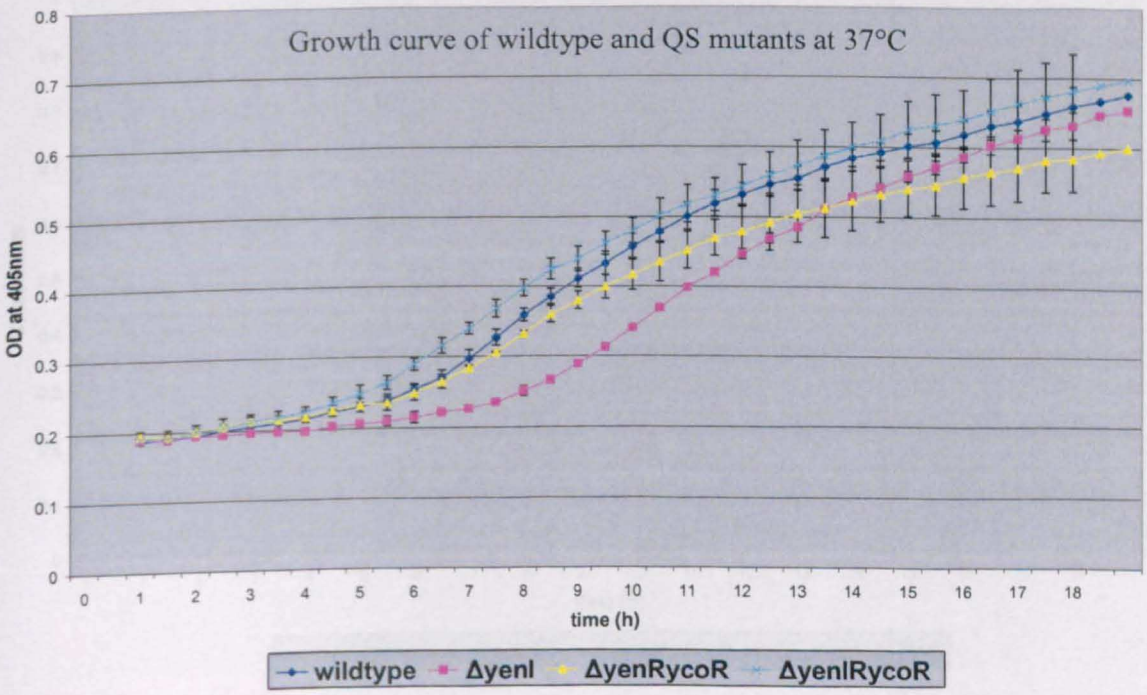
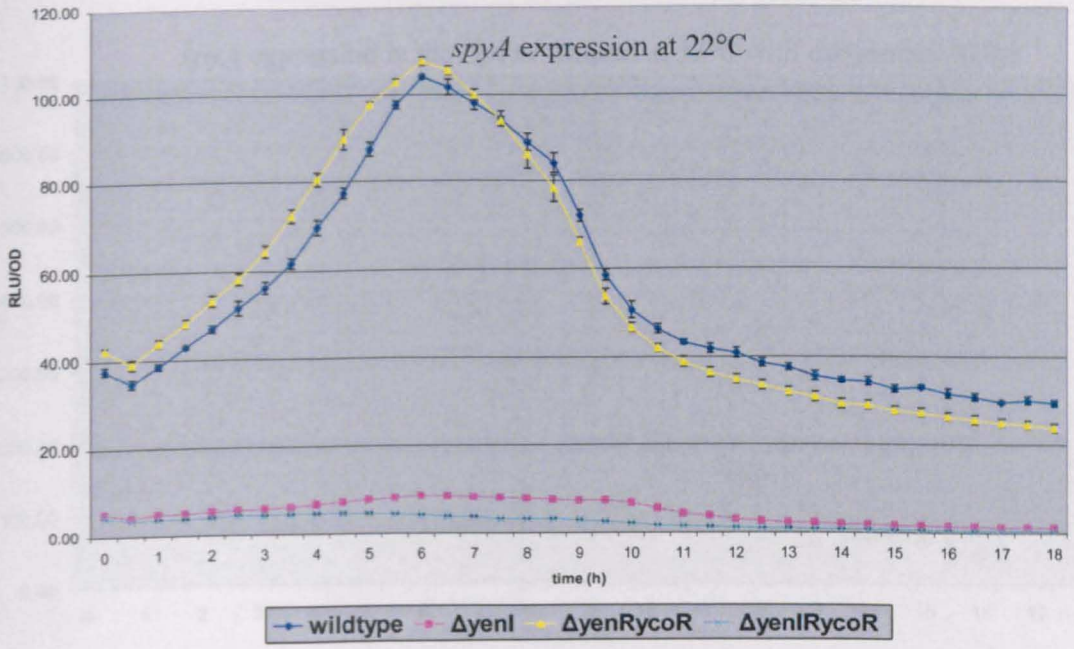


Figure 5.17. (a) Analysis of *spyA* expression in *Y. enterocolitica* 8081 wildtype and QS mutants at 37 °C. (b) Growth curve of wildtype and QS mutants at 37°C.

(a)



(b)

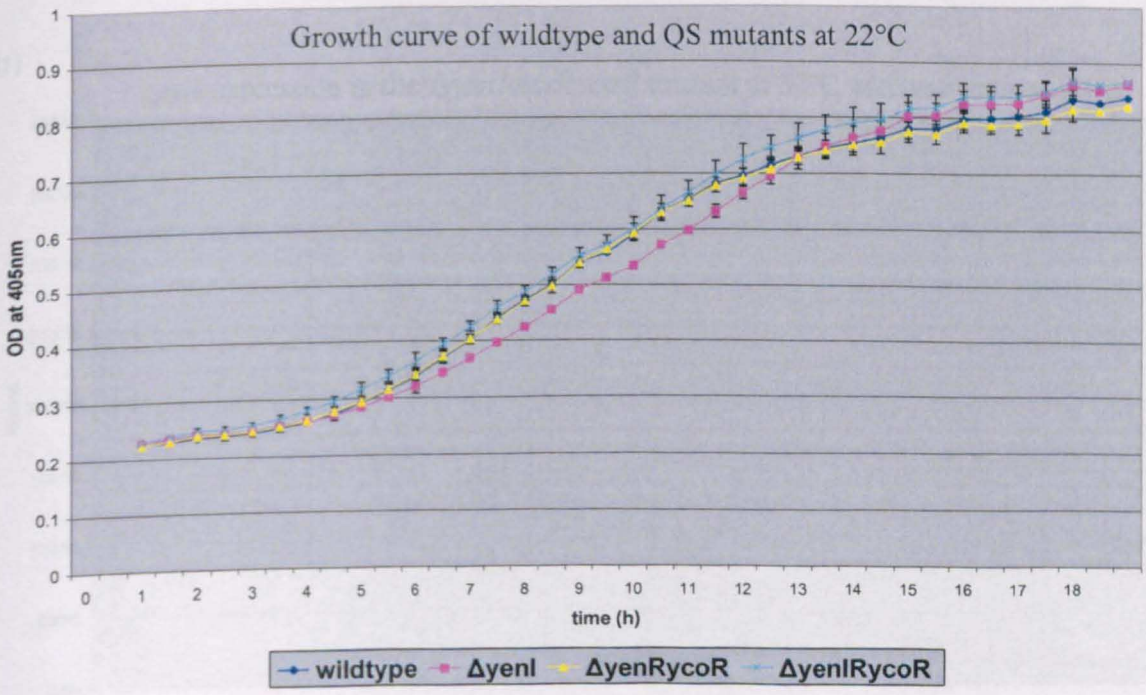
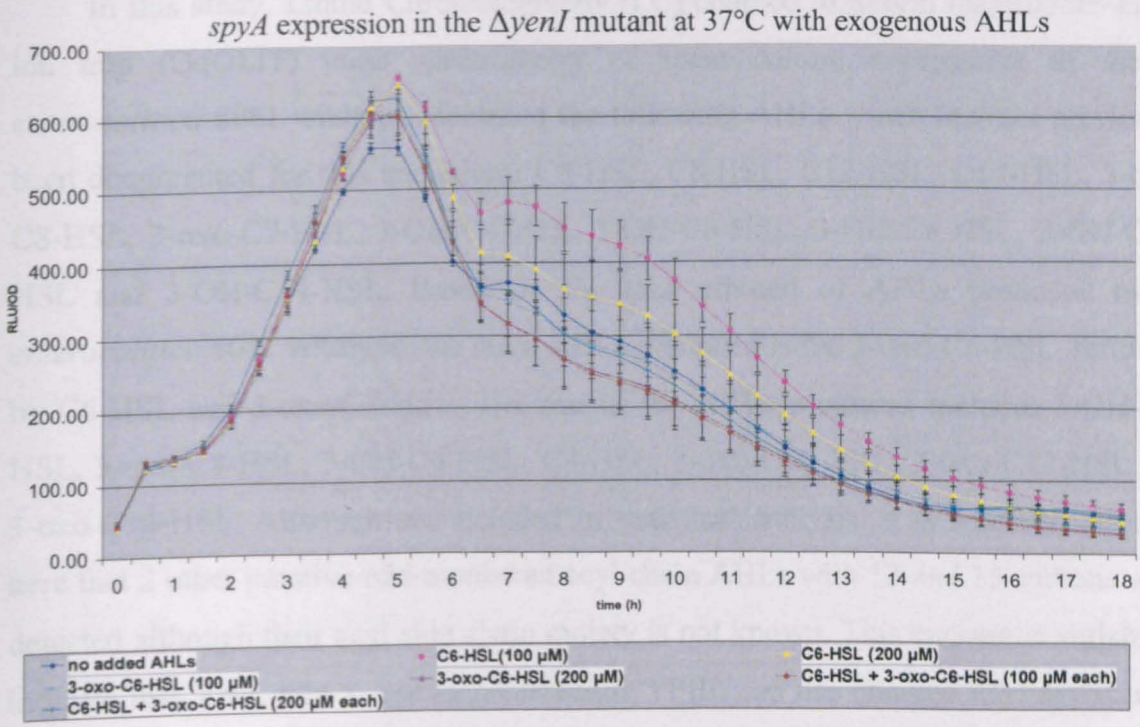


Figure 5.18. (a) Analysis of *spyA* expression in *Y. enterocolitica* 8081 wildtype and QS mutants at 22 °C. (b) Growth curve of wildtype and QS mutants at 22°C.

(a)



(b)

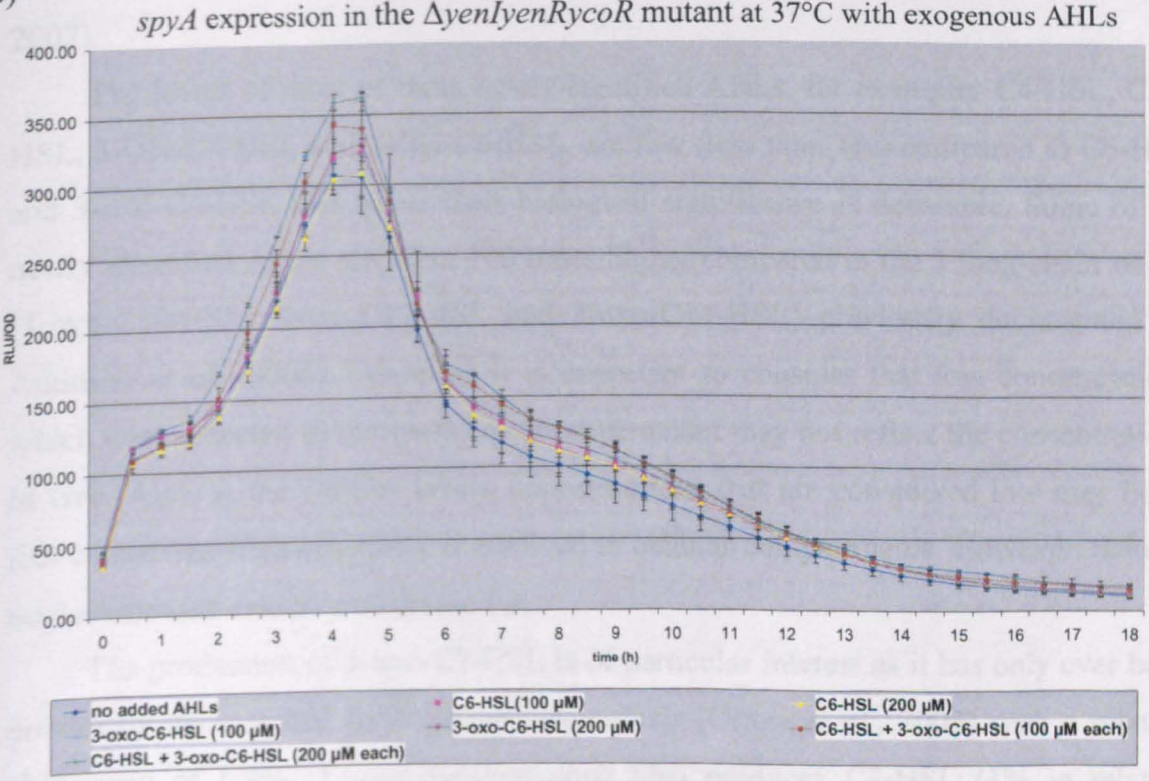


Figure 5.19. The addition of exogenous AHLs (C6-HSL and/or 3-oxo-C6-HSL at 100 and 200 µM) to the (a) Δ*yenI* and (b) Δ*yenIyenRycoR* mutants did not alter the expression of *spyA* at 37°C.

5.3 Discussion

In this study, Liquid Chromatography (LC) coupled to hybrid quadrupole–linear ion trap (QqQLIT) mass spectrometry of spent culture supernatant of the *Y. enterocolitica* 8081 wildtype identified the following AHLs which had not previously been documented for this bacterium: C4-HSL, C8-HSL, C12-HSL, C14-HSL, 3-oxo-C8-HSL, 3-oxo-C7-HSL, 3-OH-C4-HSL, 3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C12-HSL and 3-OH-C14-HSL. Based on the total amount of AHLs produced by *Y. enterocolitica* 8081 wildtype, the main AHLs produced is the 3-oxo-C6-HSL, followed by C6-HSL and 3-oxo-C7-HSL. The rest of the AHLs produced includes 3-OH-C6-HSL, 3-oxo-C8-HSL, 3-OH-C4-HSL, C4-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL. Although not included in statistical analysis, it is worth mentioning here that 2 other putative odd-numbered acyl chain AHLs with 13 and 15 carbons were detected although their acyl side chain moiety is not known. This enormous variability indicate that YenI, like *Y. pseudotuberculosis* YPIII, can use charged acyl-acyl-carrier protein (ACP) of almost any length as a substrate to synthesize AHLs although there are clear preferences for 3-oxo substituted C6, C7 and C8 acyl-ACPs (Ortori *et al.*, 2007).

The levels of most of these newly identified AHLs, for examples C4-HSL, C12-HSL, 3-OH-C4-HSL and 3-OH-C6-HSL, are low (less than 1%) compared to C6-HSL and 3-oxo-C6-HSL and hence their biological significance is debatable. Some of the newly identified AHLs are 10 to 700 times higher compared to the 3 long-chain AHLs (3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL) previously documented by Atkinson *et al.* (2006). However, it is important to consider that low concentrations which were detected in the spent culture supernatant may not reflect the concentrations *in vivo*. Also, at the cellular levels, concentrations that are considered low may be in fact considered high especially if confined in cellular compartments. However, there is no documented evidence of this so far.

The production of 3-oxo-C7-HSL is of particular interest as it has only ever been previously documented in *Y. pseudotuberculosis* (Ortori *et al.*, 2007) with a relative abundance of 6.9%. *Y. pseudotuberculosis* also produces C7-HSL (1% in relative abundance). Other than the two *Yersinia*, only two other species were documented to be producing AHLs with an odd number of carbons in their acyl chains; *Rhizobium*

leguminosarum (Lithgow *et al.*, 2000) and *Serratia marcescens* (Horng *et al.*, 2002) both of which produce C7-HSL. These odd numbered AHLs may arise through the use of propionyl-CoA and malonyl-CoA as acyl chain starter and extender units (Ingram *et al.*, 1977; O'Hagan, 1991). The synthesis of odd numbered AHLs is uncommon. However, fatty acids and polyketides with odd numbers of carbons in the chain have been described despite fatty acids containing an odd number of carbon atoms being less common than those with an even number of carbon atoms. These molecules typically originate from propionyl-CoA as a starter unit and malonyl-CoA as the extender unit or alternatively can arise by loss of one carbon from an even–numbered acid (Dewick, 2002).

If odd-numbered AHLs are produced *via* loss of one carbon from an even-numbered acid, the energy required would presumably be higher than producing even-numbered AHLs. Thus, the production of AHLs with odd number of carbons in their acyl chains by some bacteria is a peculiarity given that their 'expensive' production cost. Since the 'language' of AHLs is dominated by AHLs with even number of carbons in their acyl chains, it is possible that the bacteria that do invest their energy into producing odd-number acyl chain AHLs use them as a secret code to communicate amongst kin and preventing other bacteria in its niche from eavesdropping.

The reason why 3-oxo-C7-HSL (which makes up 5% of the total AHLs produced by *Y. enterocolitica* 8081 wildtype) has not been documented before is the limitations in the methodology used. In the TLC method, 3-oxo-C7-HSL probably co-migrated with the much more abundant 3-oxo-C6-HSL and C6-HSL and thus could not be distinguished separately by the biosensors. LC-MS methods rely on synthetic AHLs standards to identify AHLs and because synthetic 3-oxo-C7-HSL is not routinely used, the 3-oxo-C7-HSL was not detected. This demonstrated that the LC coupled to QqQLIT method is a superior method to comprehensively study the AHL profiles of bacteria as it can identify unknown AHLs in an unbiased manner although synthetic standards are still needed for structure confirmation at the endpoint. Furthermore, LC-QqQLIT also provides relative quantification of the amount of AHLs produced which is useful in data interpretation. Nevertheless, biosensors are still essential in preliminary screening for the presence of AHLs because the assays are relatively simple to perform and yield fast results.

Figures 5.4 and 5.5 show the AHL profiles of the *Y. enterocolitica* 8081 wildtype compared to the R mutants: Δ*yenR*, Δ*ycoR* and Δ*yenRycoR* which revealed

that the profiles for the wildtype and R mutants strains are very similar. This shows that the production of AHLs in *Y. enterocolitica* 8081 is independent of both transcriptional regulators. This is in contrast to *Y. pseudotuberculosis* YPIII where chromosomal *lux*-based promoter fusions to *ypsR*, *ypsI*, *ytbR* and *ytbI* revealed that the YpsRI system positively autoregulates itself and the expression of the *ytbRI* system. The *ytbRI* system is positively autoregulated but only at the level of *ytbI* expression. YtbRI does not control expression of *ypsR* or *ypsI* (Atkinson *et al.*, 2008; Patrick, H., unpublished data).

In previous studies, YenI was found to be responsible for the production of C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL and 3-oxo-C14-HSL (Throup *et al.*, 1995, Atkinson *et al.*, 2006). Evidence in this study confirmed that YenI is indeed responsible for the production of C6-HSL, 3-oxo-C6-HSL and 3-oxo-C14-HSL (see Figure 4.3). However, for 3-oxo-C10-HSL and 3-oxo-C12-HSL, the role of YenI is ambiguous because the Δ*yenI* strains still produce low levels of these 2 AHLs compared to the wildtype levels as detected by the highly sensitive LC-QqQLIT method. It is important to note that we have confirmed that the Δ*yenI* strains are true mutants by Southern hybridization and sequencing.

The C8-HSL, C14-HSL, 3-oxo-C7-HSL, 3-oxo-C8-HSL, 3-OH-C6-HSL; as well as the putative C13 and C15 AHLs are attributed to YenI as they were not detected in all Δ*yenI* strains. On the contrary, C4-HSL, C12-HSL, 3-OH-C4-HSL, 3-OH-C8-HSL, 3-OH-C12-HSL and 3-OH-C14-HSL are not entirely attributed to YenI because they are still detected in small amounts in Δ*yenI* strains. Interestingly, 3-OH-C4-HSL (1%) levels are similar in all the strains, suggesting the presence of at least one more additional AHL synthase in *Y. enterocolitica* 8081. Intriguingly, 2 types of AHLs, C10-HSL and 3-oxo-C4-HSL were detected in the Δ*yenI* mutants but not in wildtype which may also be attributed to an additional synthase which is recessive in the wildtype.

In other bacteria, the 3-OH-C4-HSL is produced by *Vibrio harveyi via* LuxM (Bassler, 1993). LuxM shows no homology to the LuxI family of AHL synthases (Bassler, 1993) but is similar to other AHL synthase; VanM of *V. anguillarum* which produces C6-HSL and 3-OH-C6-HSL (Milton *et al.*, 2001), and AinS of *V. fischeri* which produces C8-HSL (Gilson *et al.*, 1995). It is plausible that a homologue of LuxM, VanM and AinS is present in *Y. enterocolitica* but efforts to identify additional AHL synthase gene(s) in the genome sequence did not yield any significant matches

and so in conclusion, the C10-HSL and 3-oxo-C4-HSL produced only in the Δ*yenI* mutants must arise spontaneously or *via* a novel synthase.

Swarming motility is a flagellum-dependent behaviour that facilitates bacterial migration over solid surfaces and is distinct from swimming motility, which occurs in fluid environments. Such multicellular behaviour has been implicated in the formation of biofilms and in bacterial pathogenesis (Fraser & Hughes, 1999). Being motile and able to migrate to a favourable environment certainly contributes to the survival of enteropathogenic *Yersinia* during free-living stages of their life cycle but this attribute can also affect interactions with host organisms (Young, 2004). Motility is required to initiate invasion by *Y. enterocolitica* into human epithelial cells (Young *et al.*, 2000). Thus, motility is considered to contribute to virulence and it is possible that AHL-mediated QS may also play a role in this context. In this study, it was found that the swimming and swarming motility of QS mutants on plate assays were similar to that of the *Y. enterocolitica* 8081 wildtype. This is in contrast to a previous study on *Y. enterocolitica* 90/54 (serotype O:9), where the Δ*yenI* mutant showed impaired swimming motility and abolished swarming motility (Atkinson *et al.*, 2006) whereas the Δ*yenI* mutant of *Y. enterocolitica* 10460 was not affected in either swimming or swarming motility (Atkinson *et al.*, 2005). The 10460 strain (biotype 3, serotype O:1) is avirulent as it lacks the pYVe plasmid. At that time, it was implied that this disparity could be, at least in part, due to the presence/absence of the pYVe plasmid. However, the *Y. enterocolitica* 8081 QS mutants, which are pYVe+, behave in a similar manner to 10460. Thus, it is unlikely that the presence of the pYVe plasmid is a prerequisite for swarming and swimming motility but the mechanisms underlying the strain-dependent QS-associated motility phenotype remain to be elucidated.

We sought to investigate the adhesion and invasion ability of *Y. enterocolitica* 8081 wildtype and QS mutants in an *in vitro* model. The human colonic adenocarcinoma cell line, Caco-2, was chosen because it represents a model for enteropathogens for which the intestine is the usual site of entry or replication (Gaillard *et al.*, 1987) and it is highly susceptible to *Yersinia* infection (Curfs *et al.*, 1995). The adhesion of the Δ*yenI* mutant to Caco-2 cells was ~ 80% higher than the wildtype whereas adhesion of the Δ*yenI* mutant complemented with a plasmid-borne copy of *yenI*, the rate was ~15 times lower than in the Δ*yenI* mutant and 8 times lower than in the wildtype (Figure 5.11). These data suggest that the AHLs associated with YenI repress adhesion of *Y. enterocolitica* to Caco-2 cells. For the Δ*ycoR* and Δ*yenRycoR*

mutants, the adhesion is almost twice as high compared to wildtype. These data imply that YcoR also plays an important role in repression of adhesion and this may be in conjunction with the AHLs associated with *yenI*. The Δ*yenR* mutant is ~50% less adherent to the Caco-2 cells when compared to the wildtype and indicates that YenR is capable of activating adhesion although this is unlikely to be in conjunction with the AHLs exclusively synthesised by YenI. However, given that adhesion of the Δ*yenRycoR* mutant is comparable to that of the Δ*ycoR* mutant rather than that of the Δ*yenR* mutant, it is possible that the repressor effect exerted by YcoR may be dominant to the activator effect of YenR. If this was not the case and YenR was dominant to the activity of YcoR then we would expect adhesion of the Δ*yenRycoR* mutant to be comparable to that seen for the Δ*yenR* mutant alone. Complete deletion of the QS system (Δ*yenIyenRycoR*) lowers adhesion dramatically (about 7 times lower compared with wildtype) which suggests that under these conditions, an intact QS system may activate adhesion of *Y. enterocolitica* 8081 wildtype to Caco-2 cells.

In the invasion assay, the invasion by the Δ*yenI* mutant into Caco-2 cells was ~200 fold higher compared to the wildtype (Figure 5.12). However, the levels could be restored to those observed in the wildtype when a functional copy of *yenI* was introduced into the Δ*yenI* mutant *in trans*. The invasion by the Δ*yenR* mutant was 65 times higher than wildtype, suggesting that both the AHLs and YenR repress invasion. Invasion by the Δ*ycoR* mutant was similar to wildtype. Therefore, YcoR does not influence invasion, thus we would expect the Δ*yenRycoR* mutant to show the same invasion activity as the Δ*yenR* mutant. However, invasion by the Δ*yenRycoR* mutant was 19 fold lower than the Δ*yenR* mutant. The same result was observed in the Δ*yenIyenRycoR* mutant. Since the invasion by the Δ*yenI* and Δ*yenR* mutants are much higher than the wildtype and if YcoR plays no part, therefore we would expect the invasion by the Δ*yenIyenRycoR* mutant to be same as the Δ*yenI* and Δ*yenR* mutants. However, the Δ*yenIyenRycoR* mutants showed very low invasion activity, ~73 fold and ~51 fold lower than the Δ*yenI* and Δ*yenR* mutants respectively, indicating a role for YcoR. Both adhesion and invasion assays showed successful complementation of the Δ*yenI* with a plasmid-borne copy of *yenI*. These preliminary data clearly shows that QS does regulate adhesion of and invasion by *Y. enterocolitica* in Caco-2 cells in some way. Notably, the error bars for both assays are very high (See Figures 4.11 and 4.12) and statistical analyses (unpaired T-test, $p \leq 0.05$) to determine if the differences seen in the adhesion of /invasion by wildtype and QS mutants were significant revealed that

only three pairings in the adhesion assay: wildtype versus Δ*yenI* complemented mutant; wildtype versus Δ*yenIyenRycoR* mutant and Δ*yenI* versus Δ*yenI* (complemented) were indeed significant (data not shown). This is a problem which warrants further repeat assays and fine-tuning of the experimental techniques. Some elements of the adhesion and invasion assay contradict the RQ-PCR data (See Tables 4.3 and 4.4). In agreement, the adhesion assay showed that the Δ*yenR* mutant is less adherent to Caco-2 compared with wildtype. However, the Δ*yenRycoR* mutant appears to be more adherent compared with wildtype. In the invasion assay, the Δ*yenR* mutant is more invasive compared with wildtype which is in agreement with RQ-PCR results but unexpectedly the Δ*yenI* mutant is more invasive while the Δ*yenRycoR* mutant is not more invasive than wildtype. These discrepancies may be explained in that while *yadA* and *invA* expression in the RQ-PCR were measured at one time point, and thus are only a snapshot at that time point, this may not be predictive of the outcome of actual adhesion and invasion assays. Moreover, QS control of adhesion and invasion ability *via* YadA and InvA could possibly be at the posttranslational level, and not at the transcriptional level and thus is not reflected in the RQ-PCR data.

The fact that QS mutants lose the pYVe plasmid rapidly in the process of curing the strain of the temperature sensitive plasmid, pAJD434, is intriguing. Since the pYVe plasmid is a pre-requisite to *Yersinia* pathogenicity, we speculate that QS may control pathogenicity by regulating the replication and partition of the plasmid. We sought to quantify the rate of loss for the pYVe plasmid *in vivo* at 37°C. Studying the rate of plasmid loss quantitatively confirmed that QS mutants lose the pYVe plasmid at 37°C more rapidly compared to wildtype (See Figure 5.14) especially in the Δ*yenRycoR* mutant. The complementation of the Δ*yenI* mutant with a plasmid-borne copy of *yenI* resulted in a remarkable drop in the rate of plasmid loss, even much lower compared to wildtype, suggesting that AHLs associated with YenI plays an important role in activating the partition or replication of pYVe plasmid.

This is supported by data in the previous chapter where RQ-PCR (Section 4.2.3) showed that expression of *repA* (controls replication of the pYVe plasmid) at 37°C is upregulated in the Δ*yenR* mutant by 1.5 fold but is downregulated in the Δ*yenRycoR* mutant by 2.4 fold. The expression of *spyA* (controls the partition of the pYVe plasmid) is downregulated in the Δ*yenI* (1.8 fold), Δ*yenRycoR* (6.3 fold) and Δ*yenIyenRycoR* (3.3 fold) mutants. In this chapter, using the promoter fusion P_{spyA}::*luxCDABE*, it was shown that *spyA* expression at 37°C in wildtype is maximal (~2750 units) at the start of

exponential phase of growth (5.5 h) (Figure 5.18). This is expected because at the exponential phase, cells are dividing rapidly and therefore plasmids must be partitioning from mother cell to daughter cells. Subsequently at stationary phase, expression of *spyA* drops rapidly. In the Δ*yenI* and Δ*yenIyenRycoR* mutants, the differences compared to wildtype are dramatic. In the Δ*yenI* mutant, expression of *spyA* peaked at ~500 units at 8.5 h (mid-log phase of growth) while for the Δ*yenIyenRycoR* mutant, the expression peaked at ~750 units at 5 h. This suggests that AHLs associated with YenI may be activating or repressing the activity of YenR and YcoR. All these data show that partition of the pYVe plasmid is controlled by QS *via* AHLs associated with YenI. The fact that the expression of *spyA* in the wildtype mimics the growth curve of the bacteria shows that QS may (and logically should) influence the replication and partition of the pYVe plasmid. Consequently, because the pYVe plasmid is a pre-requisite of *Yersinia* pathogenicity, we speculate that QS controls pathogenicity by regulating the replication and partition of the pYVe plasmid. However, there is a conflicting element; the expression profile of *spyA* at 37°C in the Δ*yenRycoR* mutant is almost identical to the wildtype throughout the growth curve (Figure 5.18). This is unexpected as one would expect the expression of *spyA* to be lower comparatively to wildtype, resulting in the higher rate of pYVe loss as seen in the assay (Figure 5.14). We presume that plasmid replication and partition are linked and therefore a plausible explanation may be that the AHLs associated with YenI, together with an unknown regulatory protein, activates *spyA* while YenR and/or YcoR activate *repA*. It is also possible that the 2 sets of genes may act in a hierarchical manner where the effect of AHLs associated with YenI overrides the effect of YenR/YcoR given that *spyA* expression in the Δ*yenIyenRycoR* mutant remained low throughout the growth curve.

The addition of exogenous AHLs (C6-HSL and 3-oxo-C6-HSL at 100 and 200μM) to the Δ*yenI* and Δ*yenIyenRycoR* mutants did not alter the expression of *spyA* at 37°C (See Figure 5.19). These observations echo two previous studies in *Y. enterocolitica* 90/54, where provision of exogenous AHLs did not restore swarming or swimming in the *yenI* mutant and in *Y. pseudotuberculosis* where exogenously supplied AHLs could not restore the expression of *flhDC* and *fliA* in *ypsR/ytbI*, *ypsI/ytbR* and *ypsI/ytbI* mutants despite uptake of the AHLs into the cell (Atkinson *et al.*, 2006; 2008).

It was shown in the microarray analysis in the previous chapter that T3SS-related are regulated by QS including genes encoding the components of the injectisome, the Yops proteins, Yops chaperones and regulatory proteins (VirF and

TyeA). However, no observation indicating differences in Yops expression or secretion were made in the SDS-PAGE analysis of protein extracts of *Y. enterocolitica* 8081 wildtype and five QS mutants (Figures 5.10 to 5.14). This could be due to the limitation of the SDS-PAGE method used to visualise the proteins, which separates the protein by size alone. More sophisticated methods like 2-dimensional electrophoresis, which separates the proteins by a second property (e.g. isoelectric point), would separate the proteins more effectively. This would enhance visualisation of any differences, if any, on the protein profiles and make comparisons of the protein profiles of different strains more comprehensive.

In promoter fusions studies using the P_{virF}::*luxCDABE* construct, it was shown that at 37°C, the expression of *virF* in the Δ*yenRycoR* mutant was much higher compared to the wildtype, Δ*yenI* and Δ*yenIyenRycoR* strains (Figure 5.15). This suggests that *yenR* and *ycoR* work in tandem to repress the expression of *virF* in the wildtype. The expression of *virF* in the wildtype peaked at ~600 units at 6 h and maintained at a constant ~250 units until 18 h. In the Δ*yenI* mutant, the expression of *virF* is similar to the wildtype until 6 h, at the start of the log phase, but the expression level is not maintained and dropped to zero by 9 h. This shows that the AHLs associated with *yenI* are essential for sustained expression of *virF*. In the Δ*yenIyenRycoR* mutant, the effect is even more dramatic, the expression of *virF* is very low throughout the growth phase. This further supports that an intact QS system contributing to regulating the virulence of *Y. enterocolitica* 8081. At 22°C, the expression of *virF* is at much lower levels compared to 37°C (Figure 5.16). The RQ-PCR results confirmed that the expression of *virF* in the Δ*yenIyenRycoR* mutant is upregulated by 2.0 fold (Table 4.3). Taken together, these data is in agreement with the fact that *virF* is transcribed strongly only at 37°C (de Rouvroit *et al.*, 1992).

Falker *et al.* (2007) showed that strains of *Y. enterocolitica* overproducing Dam are hypermotile and the expression of the master regulator of flagellum biosynthesis, *flhDC*, is upregulated. Although *dam* and the putative *dam* were up and down-regulated in the different QS mutants (See Table 4.2), swimming motility plate assays showed that the QS mutants were unaffected in its motility compared to wildtype. This is probably due to insufficient *dam* expression in the upregulated strains to induce hypermotility as in Dam-overproducing strains and *dam* expression is still sufficient in the downregulated strains to maintain normal motility. However, the Δ*yenI* and Δ*yenR* mutants, like Dam-overproducing strains (Falker *et al.*, 2007) showed increased

invasion in eukaryotic cells (See Figure 4.14). Falker *et al.* (2007) suggested that the hyperinvasive phenotype in Dam-overproducing strains maybe due to altered lipopolysaccharide (LPS) O-antigen composition on the bacterial surface. It is therefore plausible that QS mutants have altered LPS O-antigen composition which may account for the increased invasion and adhesion of QS mutants into Caco-2 cells.

In conclusion, LC-QqQLIT analysis of spent culture supernatant of the *Y. enterocolitica* 8081 wildtype identified a remarkable profile of 16 AHLs, 11 of which had not previously been documented for this bacterium. The production of 3-oxo-C7-HSL is of particular interest and we suggest that this AHL is used as a unique signal amongst kin to prevent 'eavesdropping' by other bacteria in a given niche although further work is needed to determine if 3-oxo-C7-HSL is unique to *Yersinia* spp. We have also described some phenotypic changes that occurs which can be attributed to mutations made to the QS system of *Y. enterocolitica* 8081. We have shown that QS plays an important role in the adhesion and invasion ability of *Y. enterocolitica* 8081 in the Caco-2 cell line virulence. QS also appears to regulate virulence *via* maintenance of the pYVe plasmid and expression of the transcriptional activator, *virF*.

CHAPTER 6

GENERAL DISCUSSION

Species-wide analysis of a *Y. enterocolitica* genome database revealed a conserved QS system consisting of *yenIR* in all strains studied and the presence of an 'orphan' *luxR* homologue, *ycoR*, which is only conserved in the hypervirulent USA strains. Other key findings are that mutations to the QS system resulted in the alteration of expression in a number of genes which revealed correlations between QS and virulence gene regulation (expression of *virF* and *tyeA*), virulence factors (expression of *yadA* and *invA*) and maintenance of the pYVe plasmid (expression of *repA* and *spyA*) in *Y. enterocolitica* 8081.

Protein motif analysis revealed that YcoR has highly conserved regions in the AHL binding domain (N terminal) and DNA binding domain (C terminal) when compared with known LuxR homologues, which strongly suggests that YcoR is indeed a LuxR homologue. The transcriptional profiles of *Y. enterocolitica* 8081 QS mutants (Δ*yenI*; Δ*yenR*; Δ*ycoR*; Δ*yenRycoR* and Δ*yenIyenRycoR*) revealed some clues as to the role of YcoR within the QS system of *Y. enterocolitica* 8081. The data suggest that YenR represses *ycoR* at 22°C while YcoR represses *yenI* at 22°C but activates *yenI* at 37°C (See Table 4.2, no. 209-211 and 458-460). However, the total amount of AHLs extracted from the R mutants: Δ*yenR*, Δ*ycoR* and Δ*yenRycoR* were similar to wildtype (Figure 5.3) although these extractions were done at 30°C. In future work, it would be crucial to examine whether AHL production in the R mutants (Δ*yenR*, Δ*ycoR* and Δ*yenRycoR*) is different at 22°C and 37°C. Also, to prove experimentally that YcoR is truly a LuxR homologue, the interrelationship between YenI, YenR and YcoR could be examined using chromosomal *lux*–based promoter fusions to *yenR*, *yenI* and *ycoR*. This strategy was successfully used to determine the interrelationship between the YpsRI and YtbRI loci of *Y. pseudotuberculosis* using chromosomal *lux*–based promoter fusions to *ypsR*, *ypsI*, *ytbR* and *ytbI* (Atkinson *et al.*, 2008; Patrick, H., personal communication).

Orphan LuxR homologues are QS transcriptional regulators which are not associated with an adjacent *N*-acyl-homoserine lactone synthase gene (Fuqua, 2006). Some studies suggest that an extra receptor expands the QS regulatory network to sense non-cognate AHLs. For example, QscR (orphan transcriptional regulator of *P. aeruginosa*) has a broader signal specificity than LasR (main AHL regulator of *P. aeruginosa*) and it has been suggested that the bacterium uses this ability to identify neighbouring bacteria and thus integrating monospecies and multispecies signalling (Lazdunski *et al.*, 2004; Waters & Bassler, 2005; Fuqua, 2006). This type of

relationship may exist between *Y. enterocolitica* and other bacteria and even with eukaryotic host cell in the gut in which YcoR may play a part in recognising non-cognate AHLs. It has been long known that enteropathogenic *Yersinia* have some form of communication with the human gut which results in their tropism for lymphoid tissues and their capacity to resist the non-specific immune response and even to modulate host physiology and immunity (Koutsoudis *et al.*, 2006; Xavier & Bassler, 2005). Therefore it is possible that these interactions occur *via* QS and YcoR may play a part in the process.

The presence/absence of *ycoR* in the USA and UK strains indicate that there are genetic differences in the QS systems of 'New World' (North America) and 'Old World' (Europe and Japan) strains of *Y. enterocolitica*. It is possible that the UK strains lost *ycoR* based on the presence of remnant intergenic DNA still remaining near the missing *ycoR* locus. By extension, we speculate that other non-UK 'Old World' strains also do not have *ycoR*. In the future, it would be crucial to include non-UK 'Old World' strains to study the heterogeneity of the *Y. enterocolitica* QS system and whether this heterogeneity has any effect on disease epidemiology.

Using mass spectrometry, it has been determined that the *Y. enterocolitica* 8081 wildtype produces 16 AHLs, 11 of which have not been previously documented. Like *Y. pseudotuberculosis* YPIII YpsI and YtbI (Ortori *et al.*, 2007), YenI is able to use charged acyl-acyl-carrier protein (ACP) of almost any length to synthesize AHLs but with preferences for 3-oxo substituted or unsubstituted C6, C7 and C8. The levels of most of these newly identified AHLs are less than 1% of the total amount of AHLs produced and therefore their biological significance is debatable. However, these low concentrations were detected in the spent culture supernatant and hence may not reflect the concentrations *in vivo*.

Motility is considered a virulence factor in *Y. enterocolitica* (Fraser & Hughes, 1999; Young *et al.*, 2000; Young, 2004) and AHL-mediated QS plays a role in this context. In this study, plate assays showed some inconsistencies in the swimming and swarming motility in the QS mutants of different *Y. enterocolitica* strains. Similar to the Δ*yenI* mutant of strain 10460 (pYVe$^-$) (Atkinson *et al.*, 2005), the motility in the 8081 QS mutants (pYVe$^+$) were not affected when compared to the wildtype. This is in contrast to strain 90/54 (pYVe$^+$), where its Δ*yenI* mutant showed impaired swimming motility and abolished swarming motility (Atkinson *et al.*, 2006). Thus, we have concluded that this disparity is not due to the presence/absence of the pYVe plasmid. In

future work, the disparities mentioned above could be better explained if, like mentioned earlier, the interrelationship between YenI, YenR and YcoR were studied using chromosomal *lux*–based promoter fusions to *yenR*, *yenI* and *ycoR*.

Preliminary data showed that when compared with the wildtype, the QS mutants showed altered levels of adhesion to and invasion of a Caco-2 cell line. AHLs associated with YenI and YcoR repress adhesion of *Y. enterocolitica* to Caco-2 cells while YenR activates adhesion. However, the repressor effect exerted by YcoR may be dominant to the activator effect of YenR. It was also shown that a complete deletion of the QS system (Δ*yenIyenRycoR*) lowers adhesion dramatically which suggests that an intact QS system may activate adhesion of *Y. enterocolitica* 8081 wildtype.

The invasion assay suggests that both the AHLs associated with YenI and in conjunction with YenR repress invasion whereas YcoR has no effect. Notably, there are some inconsistencies in both the adhesion and invasion assays which complicated data interpretation. Furthermore, the error bars in both assays were very high which resulted in the assays lacking statistical significance (data not shown). Thus, further repeat assays are necessary to fine-tune the experimental techniques. Despite these problems, we conclude that QS does regulate the adhesion of and invasion by *Y. enterocolitica* as complementation with a plasmid-borne copy of *yenI* to the Δ*yenI* mutant appear to reverse the upregulation of both adhesion and invasion seen in the Δ*yenI* mutant. In future work, it would be useful to include the double mutants, Δ*yenIyenR* and Δ*yenIycoR* in the adhesion and invasion assays to better explain how the two R proteins work with the AHLs. Also essential would be to include *in trans* complementation of *yenR* and *ycoR* as well as adding exogenous AHLs to the Δ*yenI* and Δ*yenIyenRycoR* mutants to examine if this would alter their adhesion and invasion ability back to wildtype levels.

According to the microarray data, the T3SS-related genes appeared to be regulated by the QS system including genes encoding the components of the injectisome, the Yops proteins, Yops chaperones and regulatory proteins (VirF and TyeA). However, no observation indicating differences in Yops expression or secretion were made in the SDS-PAGE analysis of protein extracts of *Y. enterocolitica* 8081 wildtype and five QS mutants (Figures 5.7 to 5.10). In future work, it would be beneficial to include the double Δ*yenIyenR* and Δ*yenIycoR* mutants for a more comprehensive analysis. Also, it would be more informative if additional SDS-PAGE

analyses are done using proteins extracted in conditions permissive for Yops expression i.e. in rich medium depleted of Ca^{2+} at 37°C.

One of the most intriguing observations made in this study was that QS mutants lose the pYVe plasmid at 37°C more rapidly compared to wildtype. The role of QS was further confirmed by the complementation of the Δ*yenI* mutants with a plasmid-borne copy of *yenI* which resulted in a remarkable drop in the rate of plasmid loss, to a level even lower than the level in wildtype. As mentioned earlier, as the pYVe plasmid is a pre-requisite of *Yersinia* pathogenicity, we speculate that QS may control pathogenicity by regulating the replication and partition of the plasmid. The effect of QS on pYVe replication and partition is supported by RQ-PCR and *spyA* promoter-*lux* fusion studies (P_{spyA}::*luxCDABE*). In future work, a more comprehensive study of plasmid loss in *Y. enterocolitica* 8081 should include the double Δ*yenIyenR* and Δ*yenIycoR* mutants, as well as *in trans* complementation of *yenR* and *ycoR* in these mutants. Addition of exogenous AHLs to the Δ*yenI* mutants would also be informative. These additional work may reconcile some of the disagreements seen between the *spyA* promoter-*lux* fusion studies and the plasmid loss assay where the Δ*yenRycoR* mutant which readily loses pYVe despite *spyA* expression remaining similar to the wildtype. There could be a yet unknown regulator which could respond to AHLs affecting *spyA* expression. It would also be interesting to include the 90/54 wildtype strain to see if the absence of *ycoR* would mean that the rate of plasmid loss this strain will be similar to the 8081 Δ*ycoR*.

On the other hand, the complete regulatory mechanism involving the QS system, *spyA* and *repA*, could not be studied as attempts to construct the promoter fusion P_{repA}::*luxCDABE* were unsuccessful. Nevertheless, these findings in *Y. enterocolitica* 8081 on the QS control of virulence plasmid replication and partition implicate that *Y. pseudotuberculosis* and *Y. pestis* may have a similar mechanism, and this could extend to other QS+ bacteria which possesses a virulence plasmid, for examples, *A. tumefaciens* (Watson *et al.*, 1975; Zhang *et al.*, 1993; Piper *et al.*, 1993) and *V. anguillarum* (Crosa *et al.*, 1980; Milton *et al.*, 1997).

It is a recurring theme in this study that QS in *Y. enterocolitica* regulates expression of virulence-related genes. QS control of virulence-related genes is clearly in conjunction with temperature in a hierarchical manner. Since QS cannot override the temperature control of motility, temperature control is dominant over QS in this context. The correlation of QS and virulence has been documented previously in

Yersinia. Chen *et al.* (2006) compared the antibody profiles elicited by *Y. pestis* wildtype and a QS mutant strain (*ΔypeRIyepRI*). It was shown that QS affects the expression of many virulence associated proteins of *Y. pestis*, including F1 (a protective antigen), LcrV (putative V antigen and antihost protein/regulator), KatY (a catalase-peroxidase) and pH6 (antigen precursor and adhesin), which were not expressed or expressed at relatively lower levels in the QS mutant (Chen *et al.*, 2006). More recently, using ELISA (Enzyme-linked immunosorbent assay), Western blot, DNA microarray analysis, and real time PCR, Gelhaus *et al.* (2009) demonstrated that the addition of C8-HSL or oxo-C8-HSL, but not C6-HSL or oxo-C6-HSL, to *Y. pestis* cultures significantly downregulates LcrV protein expression. However, it is important to note that in this study; 1 mM of each AHL was used, which is considered to be a very high and non-physiological concentration. LcrV is important as a key *Y. pestis* antigen, immune regulator, and component of the T3SS. It was also shown that other T3SS genes are down-regulated by C8-HSL or oxo-C8-HSL, including *yopQ*, *yopM*, *yopJ* and *yscE* (Gelhaus *et al.*, 2009). Since both *Y. enterocolitica* (this study) and *Y. pestis* (Chen *et al.*, 2006; Gelhaus *et al.*, 2009) showed correlation of QS to virulence, it is of no surprise that a transcriptomic study on QS mutants of *Y. pseudotuberculosis* YPIII also suggests that QS is involved in the TTSS regulon (Atkinson *et al.*, unpublished data). These findings pave the way for a new area of study in *Yersinia* research as a whole.

QS regulation of virulence is already well-studied in *P. aeruginosa*. The synthesis and secretion of a number of virulence factors in *P. aeruginosa* are controlled by QS. QS is crucial in the pathogenesis of *P. aeruginosa* infections, as demonstrated using mouse models (Rumbaugh *et al.*, 1999; Pearson *et al.*, 2000) and virulence factor gene expression in the lungs of cystic fibrosis patients (Erickson *et al.*, 2002). A large number of QS-regulated genes that encode known or probable virulence genes were also identified in a microarray-based study, further emphasizing the role of QS in *P. aeruginosa* pathogenesis (Wagner *et al.*, 2003).

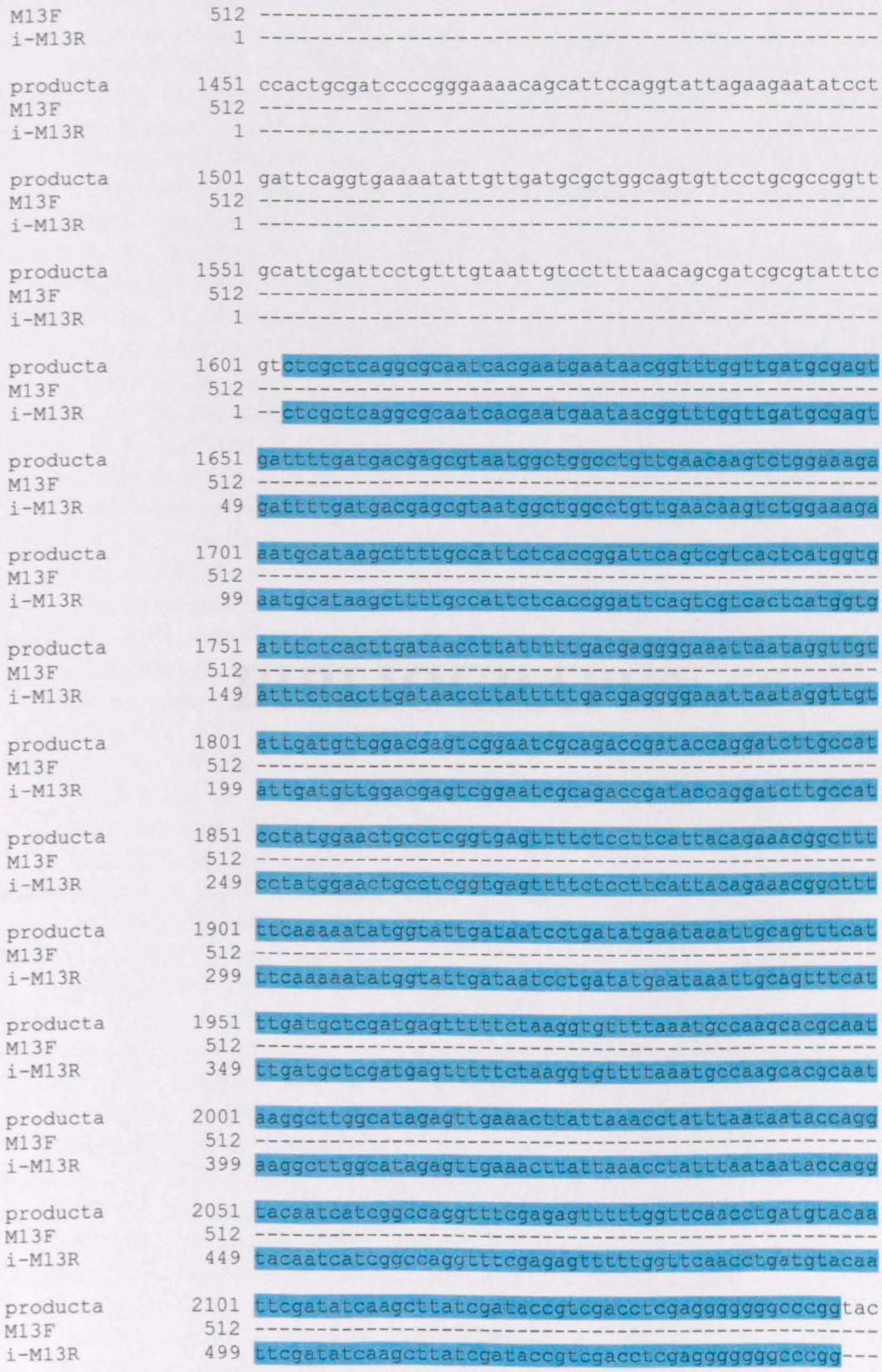
In conclusion, this study showed the correlation of QS to virulence gene regulation; virulence factors and maintenance of the pYVe plasmid in *Y. enterocolitica* 8081. To our knowledge, these findings, particularly the novel QS-linked genetic regulation of the replication and partition of a plasmid, have not been previously documented. Because of the overwhelming number and types of genes that have appeared in the microarray data, only a limited selection were chosen for further work

and discussion here. Many other groups of genes which appeared to be QS regulated warrants further studies and analyses, for examples, genes on the High Pathogenicity Island (HPI), DNA methylation genes (*dam*), genes involved in tryptophan, histidine, glutamate/aspartate and SAM (precursor of AHLs) metabolism.

APPENDICES

Appendix 1 - Sequencing results and alignment for the Δ*yenR* mutant

```
producta        651 tggagctccaccgcggtggcggccgctctagaactagtggatccccgggg
M13F              1 ----------------------------------------tccccgggg
i-M13R            1 ----------------------------------------------------

producta        701 ctgcaggaattgtacttggtgaatctgaagtgccctggtgttggcgaaaa
M13F             10 ctgcaggaattgtacttggtgaatctgaagtgccctggtgttggcgaaaa
i-M13R            1 ----------------------------------------------------

producta        751 tatattcttttcagactaactttcttaacggcagtgcgctaagatactag
M13F             60 tatattcttttcagactaactttcttaacggcagtgcgctaagatactag
i-M13R            1 ----------------------------------------------------

producta        801 ttctattatattaaaatctaattctgcaaagcaaaaacagtttaattaac
M13F            110 ttctattatattaaaatctaattctgcaaagcaaaaacagtttaattaac
i-M13R            1 ----------------------------------------------------

producta        851 ataggggtataataatagctagccttaggtctagttatctaaactatact
M13F            160 ataggggtataataatagctagccttaggtctagttatctaaactatact
i-M13R            1 ----------------------------------------------------

producta        901 ttggtctaggttttctcttatcgggcgtcaattttatataagaaatgttt
M13F            210 ttggtctaggttttctcttatcgggcgtcaattttatataagaaatgttt
i-M13R            1 ----------------------------------------------------

producta        951 gtgaggatatgttataccgcattttctattgaaaacaaggcgataaaatt
M13F            260 gtgaggatatgttataccgcattttctattgaaaacaaggcgataaaatt
i-M13R            1 ----------------------------------------------------

producta       1001 gtattgttacattatacacagagtagaattggcctattatgataattgaa
M13F            310 gtattgttacattatacacagagtagaattggcctattatgataattgaa
i-M13R            1 ----------------------------------------------------

producta       1051 agccacgttgtgtctcaaaatctctgatgttacattgcacaagataaaaa
M13F            360 agccacgttgtgtctcaaaatctctgatgttacattgcacaagataaaaa
i-M13R            1 ----------------------------------------------------

producta       1101 tatatcatcatgaacaataaaactgtctgcttacataaacagtaatacaa
M13F            410 tatatcatcatgaacaataaaactgtctgcttacataaacagtaatacaa
i-M13R            1 ----------------------------------------------------

producta       1151 ggggtgttatgagccatattcaacgggaaacgtcttgctcgaggccgcga
M13F            460 ggggtgttatgagccatattcaacgggaaacgtcttgctcgaggccgcga
i-M13R            1 ----------------------------------------------------

producta       1201 ttaaattccaacatggatgctgatttatatgggtataaatgggctcgcga
M13F            510 tt--------------------------------------------------
i-M13R            1 ----------------------------------------------------

producta       1251 taatgtcgggcaatcaggtgcgacaatctatcgattgtatgggaagcccg
M13F            512 ----------------------------------------------------
i-M13R            1 ----------------------------------------------------

producta       1301 atgcgccagagttgtttctgaaacatggcaaaggtagcgttgccaatgat
M13F            512 ----------------------------------------------------
i-M13R            1 ----------------------------------------------------

producta       1351 gttacagatgagatggtcagactaaactggctgacggaatttatgcctct
M13F            512 ----------------------------------------------------
i-M13R            1 ----------------------------------------------------

producta       1401 tccgaccatcaagcattttatccgtactcctgatgatgcatggttactca
```



Legend: product a, the predicted sequence of the cloned Δ*yenR* mutant in pBluescript vector, M13F and i-M13R are the sequencing results of the forward and reverse strand using the m13F and m13R primers, respectively.

BIBLIOGRAPHY

Achtman, M., K. Zurth, G. Morelli, G. Torrea, A. Guiyoule & E. Carniel (1999) *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA* **96**: 14043-14048.

Achtman, M., G. Morelli, P. Zhu, T. Wirth, I. Diehl, B. Kusecek, A. J. Vogler, D. M. Wagner, C. J. Allender, W. R. Easterday, V. Chenal-Francisque, P. Worsham, N. R. Thomson, J. Parkhill, L. E. Lindler, E. Carniel & P. Keim (2004) Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc. Natl. Acad. Sci. USA* **101**: 17837-17842.

Ahmer, B. M. M. (2004) Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Mol. Microbiol.* **52**: 933-945.

Ahmer, B. M. M., J. van Reeuwijk, C. D. Timmers, P. J. Valentine & F. Heffron (1998) *Salmonella typhimurium* encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *J. Bacteriol.* **180**: 1185-1193.

Aili, M., E. L. Isaksson, S. E. Carlsson, H. Wolf-Watz, R. Rosqvist & M. S. Francis (2008) Regulation of *Yersinia* Yop-effector delivery by translocated YopE. *Int. J. Med. Microbiol.* **298**: 183-192.

Allaoui, A., R. Schulte & G. R. Cornelis (1995) Mutational analysis of the *Yersinia enterocolitica virC* operon: characterization of *yscE, R, G, I, J,* K required for Yop secretion and *yscH* encoding YopR. *Mol. Microbiol.* **18**: 343-355.

Andersen, J. B., A. Heydorn, M. Hentzer, L. Eberl, O. Geisenberger, B. B. Christensen, S. Molin & M. Givskov (2001) gfp-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl. Environ. Microbiol.* **67**: 575-585.

Andersson, K., N. Carballeira, K. E. Magnusson, C. Persson, O. Stendahl, H. Wolf-Watz & M. Fallman (1996) YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signalling associated with phagocytosis. *Mol. Microbiol.* **20**: 1057-1069.

Athanasopoulos, V., J. Praszkier & A. J. Pittard (1995) The replication of an IncL/M plasmid is subject to antisense control. *J. Bacteriol.* **177**: 4730-4741.

Atkinson, S. (1999) Quorum Sensing in *Yersinia pseudotuberculosis*. PhD thesis. Nottingham: University of Nottingham.

Atkinson, S., C.-Y. Chang, H. L. Patrick, C. M. F. Buckley, Y. Wang, R. E. Sockett, M. Cámara & P. Williams (2008) Functional interplay between the *Yersinia pseudotuberculosis* YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of *flhDC* and *fliA*. *Mol. Microbiol.* **69**: 137-151.

Atkinson, S., C.-Y. Chang, E. R. Sockett, M. Camara & P. Williams (2006) Quorum sensing in *Yersinia enterocolitica* controls swimming and swarming motility. *J. Bacteriol.* **188**: 1451-1461.

Atkinson, S., R. E. Sockett, M. Cámara & P. Williams (2004) *N*-acylhomoserine lactone-mediated quorum sensing in *Yersinia*. In: *Yersinia*: Molecular and cellular biology. E. Carniel & B. J. Hinnesbusch (eds). Norfolk, UK: Horizon Bioscience. pp. 75-90.

Atkinson, S., R. E. Sockett, M. Cámara & P. Williams (2005) Quorum Sensing and the Lifestyle of *Yersinia*. *Curr. Issues Mol. Biol.* **8**: 1-10.

Atkinson, S., J. P. Throup, G. S. A. B. Stewart & P. Williams (1999) A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping. *Mol. Microbiol.* **33**: 1267-1277.

Badie, G., D. M. Heithoff, R. L. Sinsheimer & M. J. Mahan (2007) Altered levels of *Salmonella* DNA adenine methylase are associated with defects in gene expression, motility, flagellar synthesis, and bile resistance in the pathogenic strain 14028 but not in the laboratory strain LT2. *J. Bacteriol.* **189**: 1556-1564.

Balbontin, R., G. Rowley, M. G. Pucciarelli, J. Lopez-Garrido, Y. Wormstone, S. Lucchini, F. Garcia-Del Portillo, J. C. Hinton & J. Casadesus (2006) DNA adenine methylation regulates virulence gene expression in *Salmonella enterica* serovar *Typhimurium*. *J. Bacteriol.* **188**: 8160-8168.

Bassler, B. L. (2002) Small Talk: Cell-to-cell communication in bacteria. *Cell* **109**: 421-424.

Bassler, B. L., E. P. Greenberg & A. M. Stevens (1997) Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* **179**: 4043-4045.

Bassler, B. L., M. Wright, R. E. Showalter & M. R. Silverman (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9**: 773-786.

BBC News (2009) Plague death toll rises in China. http://news.bbc.co.uk/go/pr/fr/-/1/hi/world/asia-pacific/8182734.stm.

Beer, K. B. & V. L. Miller (1992) Amino acid substitutions in naturally occurring variants of ail result in altered invasion activity. *J. Bacteriol.* **174**: 1360-1369.

Bell, K. S., M. Sebaihia, L. Pritchard, M. T. G. Holden, L. J. Hyman, M. C. Holeva, N. R. Thomson, S. D. Bentley, L. J. C. Churcher, K. Mungall, R. Atkin, N. Bason, K. Brooks, T. Chillingworth, K. Clark, J. Doggett, A. Fraser, Z. Hance, H. Hauser, K. Jagels, S. Moule, H. Norbertczak, D. Ormond, C. Price, M. A. Quail, M. Sanders, D. Walker, S. Whitehead, G. P. C. Salmond, P. R. J. Birch, J. Parkhill & I. K. Toth (2004) Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proc. Natl. Acad. Sci. USA* **101**: 11105-11110.

Birtalan, S. C., R. M. Phillips & P. Ghosh (2002) Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. *Mol. Cell.* **9**: 971-980.

Black, D. S. & J. B. Bliska (2000) The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. *Mol. Microbiol.* **37**: 515-527.

Blocker, A., N. Jouihri, E. Larquet, P. Gounon, F. Ebel, C. Parsot, P. Sansonetti & A. Allaoui (2001) Structure and composition of the *Shigella flexneri* 'needle complex', a part of its type III secreton. *Mol. Microbiol.* **39**: 652-663.

Boland, A. & G. R. Cornelis (1998) Role of YopP in suppression of tumor necrosis factor α release by macrophages during *Yersinia* infection. *Infect. Immun.* **66**: 1878-1884.

Boland, A., M. P. Sory, M. Iriarte, C. Kerbourch, P. Wattiau & G. R. Cornelis (1996) Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. *EMBO J.* **15**: 5191-5201.

Bölin, I. & H. Wolf-Watz (1984) Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. *Infect. Immun.* **43**: 72-78.

Bottone, E. J. (1997) *Yersinia enterocolitica*: The charisma continues. *Clin. Microbiol. Rev.* **10**: 257-276.

Bottone, E. J. (1999) *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microb. Infect.* **1**: 323-333.

Bourgois, J. J., F. E. Sluse, F. Baguet & J. Mallefet (2001) Kinetics of light emission and oxygen consumption by bioluminescent bacteria. *J. Bioenerg. Biomembr.* **33**: 353-363.

Boyd, A. P., I. Lambermont & G. R. Cornelis (2000) Competition between the Yops of *Yersinia enterocolitica* for delivery into eukaryotic cells: role of the SycE chaperone binding domain of YopE. *J. Bacteriol.* **182**: 4811-4821.

Buckley, C. (2002) Quorum sensing in *Yersinia pseudotuberculosis*. PhD thesis. Nottingham: University of Nottingham.

Burr, T., A. M. L. Barnard, M. J Corbett, C. L. Pemberton, N. J. L Simpson & G. P. C. Salmond (2006) Identification of the central quorum sensing regulator of virulence in the enteric phytopathogen, *Erwinia carotovora*: the VirR repressor. *Mol. Microbiol.* **59**: 113-125.

Burrows, T. W. & S. Jackson (1956) The virulence-enhancing effect of iron on nonpigmented mutants of virulent strains of *Pasteurella pestis*. *Br. J. Exp. Pathol.* **37**: 577-583.

Butler, T. (1994) *Yersinia* infections: centennial of the discovery of the plague bacillus. *Clin. Infect. Dis.* **19**: 655-663.

Cámara, M., P. Williams & A. Hardman. (2002) Controlling infection by tuning in and turning down the volume of bacterial small-talk. *Lancet Infect. Dis.* **2**: 667-676.

Cambronne, E. D., L. W. Cheng & O. Schneewind (2000) LcrQ/YscM1, regulators of the *Yersinia* yop virulon, are injected into host cells by a chaperone-dependent mechanism. *Mol. Microbiol.* **37**: 263-273.

Carlone, G. M., M. L. Thomas, H. S. Rumschlag & F. O. Sottnek (1986) Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *J. Clin. Microbiol.* **24**: 330-332.

Carniel, E. (2001) The *Yersinia* high-pathogenicity island: an iron-uptake island. *Microb. Infect.* **3**: 561-569.

Carniel, E., I. Guilvout & M. Prentice (1996) Characterization of a large chromosomal "high-pathogenicity island" in biotype 1B *Yersinia enterocolitica*. *J. Bacteriol.* **178**: 6743-6751.

Casadesus, J. & D. Low (2006) Epigenetic gene regulation in the bacterial world. *Microbiol. Mol. Biol. Rev.* **70**: 830-856.

Chai, Y., J. Zhu & S. C. Winans (2001) TrlR, a defective TraR-like protein of *Agrobacterium tumefaciens*, blocks TraR function *in vitro* by forming inactive TrlR:TraR dimers. *Mol. Microbiol.* **40**: 414-421.

Chain, P. S., E. Carniel, F. W. Larimer, J. Lamerdin, P. O. Stoutland, W. M. Regala, A. M. Georgescu, L. M. Vergez, M. L. Land, V. L. Motin, R. R. Brubaker, J. Fowler, J. Hinnebusch, M. Marceau, C. Medigue, M. Simonet, V. Chenal-Francisque, B. Souza, D. Dacheux, J. M. Elliott, A. Derbise, L. J. Hauser & E. Garcia (2004) Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA.* **101**: 13826-13831.

Champion, O. L., M. W. Gaunt, O. Gundogdu, A. Elmi, A. A. Witney, J. Hinds, N. Dorrell & B. W. Wren (2005) Comparative phylogenomics of the food-borne pathogen *Campylobacter jejuni* reveals genetic markers predictive of infection source. *Proc. Natl. Acad. Sci. USA* **102**: 16043-16048.

Chang, A. C. & S. N. Cohen (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**: 1141-1156.

Chang, C.-Y. (2006) Quorum sensing, motility and biofilm formation in *Yersinia pseudotuberculosis*. PhD thesis. Nottingham: University of Nottingham.

Chen, D. S. & M. M. Davis (2006) Molecular and functional analysis using live cell microarrays. *Curr. Opin. Chem. Biol.* **10**: 28-34.

Chen, X., S. Schauder, I. Pelczer, B. L. Bassler, F. M. Hughson, N. Potier & A. van Dorsselaer (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549.

Chen, Z., B. Li, J. Zhang, L. Qin, D. Zhou, Y. Han, Z. Du, Z. Guo, Y. Song & R. Yang (2006) Quorum sensing affects virulence-associated proteins F1, LcrV, KatY and pH6 etc. of *Yersinia pestis* as revealed by protein microarray-based antibody profiling. *Microb. Infect.* **8**: 2501-2508.

Cheng, L. W., O. Kay & O. Schneewind (2001) Regulated secretion of YopN by the type III machinery of *Yersinia enterocolitica*. *J. Bacteriol.* **183**: 5293-5301.

Cheng, L. W. & O. Schneewind (2000) *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J. Bacteriol.* **182**: 3183-3190.

Chhabra, S. R., C. Harty, D. S. W. Hooi, M. Daykin, P. Williams, G. Telford, D. I. Pritchard & B. W. Bycroft (2003) Synthetic analogues of the bacterial signal (quorum sensing) molecule *N*-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. *J. Med. Chem.* **46**: 97-104.

China, B., T. Michiels & G. R. Cornelis (1990) The pYV plasmid of *Yersinia* encodes a lipoprotein, YlpA, related to TraT. *Mol. Microbiol.* **4**: 1585-1593.

Chugani, S. A., M. Whiteley, K. Lee, M. D. D'Argenio, C. Manoil & E. P. Greenberg (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **98**: 2752-2757.

Clarke, M. B., D. T. Hughes, C. Zhu, E. C. Boedeker & V. Sperandio (2006) The QseC sensor kinase: A bacterial adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **103**: 10420-10425.

Clarke, M. B. & V. Sperandio (2005) Transcriptional regulation of *flhDC* by QseBC and σ^{28} (FliA) in enterohaemorrhagic *Escherichia coli*. *Mol. Microbiol.* **57**: 1734-1749.

Cornelis, G., J. C. Vanootegem & C. Sluiters (1987) Transcription of the yop regulon from *Y. enterocolitica* requires *trans* acting pYV and chromosomal genes. *Microb. Pathog.* **2**: 367-379.

Cornelis, G. R. (2002a) *Yersinia* type III secretion: send in the effectors. *J. Cell. Biol.* **158**: 401-408.

Cornelis, G. R. (2002b) The Yersinia Ysc-Yop 'Type III' Weaponry. *Nature* **3**: 742-752.

Cornelis, G. R. & H. Wolf-Watz (1997) The *Yersinia* Yop virulon: A bacterial system for subverting eukaryotic cells. *Mol. Microbiol.* **23**: 861-867.

Cornelis, G. R., C. Sluiters, I. Delor, D. Geib, K. Kaniga, C. Lambert de Rouvroit, M. P. Sory, J. C. Vanooteghem & T. Michiels (1991) *ymoA*, a *Yersinia enlerocolitica* chromosomal gene modulating the expression of virulence functions. *Mol. Microbiol.* **5**: 1023-1034.

Cornelis, G. R., C. Sluiters, D. Lambert de Rouvroit & T. Michiels (1989) Homology between VirF, the transcriptional activator of the *Yersinia* virulence regulon, and AraC, the *Escherichia coli* arabinose operon regulator. *J. Bacteriol.* **171**: 254-262.

Couturier, M., F. Bex, P. L. Bergquist & W. K. Maas (1988) Identification and classification of bacterial plasmids. *Microbiol. Rev.* **52**: 375-395.

Crosa, J. H., L. L. Hodges & M. H. Schiewe (1980) Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum*. *Infect. Immun.* **27**: 897-902.

Curfs, J. H. A. J., J. F. G. M. Meis, J. A. M. Fransen, H. A. L. van der Lee & J. A. A. Hoogkamp-Korstanje (1995) Interactions of *Yersinia enterocolitica* with polarized human intestinal Caco-2 cells. *Med. Microbiol. Immunol.* **184**: 123-127.

Darby, C., J. W. Hsu, N. Ghori & S. Falkow (2002) *Caenorhabditis elegans*; plague bacteria biofilm blocks food intake. *Nature* **417** 243-244.

Datsenko, K. A. & B. L. Wanner (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.USA* **97**: 6640-6645.

Davey, R. B., P. I. Bird, S. M. Nikoletti, J. Praszkier & J. Pittard (1984) The use of mini-Gal plasmids for rapid incompatibility grouping of conjugative R plasmids. *Plasmid* **11**: 234-242.

Davies, J. M. (1994) *Basic Cell Culture: A Practical Approach*. Oxford University Press.

Day, J. B., F. Ferracci & G. V. Plano (2003) Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN*, *tyeA*, *sycN*, *yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. *Mol. Microbiol.* **47**: 807-823.

Day, J. B. & G. V. Plano (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol. Microbiol.* **30**: 777-788.

Day, J. B. & G. V. Plano (2000) The *Yersinia pestis* YscY protein directly binds YscX, a secreted component of the type III secretion machinery. *J. Bacteriol.* **182**: 1834-1543.

de Almeida, A. M. P., A. Guiyoule, I. Guilvout, I. Iteman, G. Baranton & E. Carniel (1993) Chromosomal *irp2* gene in *Yersinia*: distribution, expression, deletion and impact on virulence. *Microb. Pathog.* **14**: 9-21.

de Kievit, T. R. & B. H. Iglewski (2000) Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **68**: 4839-4849.

de Rouvroit, C. L., C. Sluiters & G. R. Cornelis (1992) Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. *Mol. Microbiol.* **6**: 395-409.

del Solar, G. & M. Espinosa (2000) Plasmid copy number control: an ever-growing story. *Mol. Microbiol.* **37**: 492-500.

del Solar, G., R. Giraldo, M. J. Ruiz-Echevarria, M. Espinosa & R. Diaz-Orejas (1998) Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **62**: 434-464.

Denecker, G., W. Declercq, C. A. W. Geuijen, A. Boland, R. Benabdillah, M. van Gurp, M.-P. Sory, P. Vandenabeele & G. R. Cornelis (2001) *Yersinia enterocolitica* Yop-P-induced apoptosis of macrophages involves the apoptotic signaling cascade upstream of bid. *J. Biol. Chem.* **276**: 19706-19714.

Denecker, G., S. Tötemeyer, L. J. Mota, P. Troisfontaines, I. Lambermont, C. Youta, I. Stainier, M. Ackermann & G. R. Cornelis (2002) Effect of low- and high-virulence *Yersinia enterocolitica* strains on the inflammatory response of human umbilical vein endothelial cells. *Infect. Immun.* **70**: 3510-3520.

Deng, W., V. Burland, G. Plunkett III, A. Boutin, G. F. Mayhew, P. Liss, N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz, J. D. Fetherston, L. E. Lindler, R. R. Brubaker, G. V. Plano, S. C. Straley, K. A. McDonough, M. L. Nilles, J. S. Matson, F. R. Blattner & R. D. Perry (2002) Genome sequence of *Yersinia pestis* KIM. *J. Bacteriol.* **184**: 4601-4611.

Devine, J. H., C. Countryman & T.O Baldwin (1988) Nucleotide sequence of the *luxR* and *luxI* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. *Biochemistry* **27**: 873-842.

Dewick, P. M. (2002) *Medicinal natural products. A biosynthetic approach.* John Wiley & Sons, Chichester.

Dhingra, V., M. Gupta, T. Andacht & Z. F. Fu (2005) New frontiers in proteomics research: A perspective. *Int. J. Pharm.* **299**: 1-18.

Di Biase, A. M., G. Petrone, M. P. Conte, L. Seganti, M. G. Ammendolia, A. Tinari, F. Iosi, M. Marchetti & F. Superti (2000) Infection of human enterocyte-like cells with rotavirus enhances invasiveness of *Yersinia enterocolitica* and *Y. pseudotuberculosis*. *J. Med. Microbiol.* **49**: 897-904.

Donabedian, H. (2003) Quorum sensing and its relevance to infectious disease. *J. Infect.* **46**: 207-214.

Dong, Y.-H. & L.-H. Zhang (2005) Quorum sensing and quorum-quenching enzymes *J. Microbiol.* **43**: 101-109.

Dorman, C. J., J. C. Hinton & A. Free (1999) Domain organization and oligomerization among H-NS-like nucleoid-associated proteins in bacteria. *Trends Microbiol.* **7**: 124-128.

Doyle, M. P. (1985) Food-borne pathogens of recent concern. *Annu. Rev. Nutr.* **5**: 25-41.

Dufour, P., S. Jarraud, F. Vandenesch, T. Greenland, R. P. Novick, M. Bes, J. Etienne & G. Lina (2002) High genetic variability of the *agr* locus in *Staphylococcus* species. *J. Bacteriol.* **184**: 1180-1186.

Dukuzumuremyi, J. M., R. Rosqvist, B. Hallberg, B. Akerstrom, H. Wolf-Watz & K. Schesser (2000) The *Yersinia* protein kinase A is a host factor inducible RhoA/Rac binding virulence factor. *J. Biol. Chem.* **275**: 35281-35290.

Dunny, G. M. & S. C. Winans (1999) Bacterial life: Neither lonely nor boring. In: Cell-cell signalling in bacteria. G. M. Dunny & S. C. Winans (eds). Washington D.C.: ASM Press, pp. 1-5.

Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson & N. J. Oppenheimer (1981) Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**: 2444-2449.

Edqvist, P. J., J. Olsson, M. Lavander, L. Sundberg, A. Forsberg, H. Wolf-Watz & S. A. Lloyd (2003) YscP and YscU regulate substrate specificity of the *Yersinia* type III secretion system. *J. Bacteriol.* **185**: 2259-2266.

Egland, K. A. & E. P. Greenberg (1999) Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter. *Mol. Microbiol.* **31**: 1197-1204.

El Tahir, Y. & M. Skurnik (2001) YadA, the multifaceted *Yersinia* adhesin. *Int. J. Med. Microbiol.* **291**: 209-218.

Ellison, D. W., M. B. Lawrenz & V. L. Miller (2004) Invasin and beyond: regulation of *Yersinia* virulence by RovA. *Trends Microbiol.* **12**: 296-300.

Engebrecht, J. & M. Silverman (1984) Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**: 4154-4158.

Erickson, D. L., R. Endersby, A. Kirkham, K. Stuber, D. D. Vollman, H. R. Rabin, I. Mitchell & D. G. Storey (2002) *Pseudomonas aeruginosa* quorum sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infect. Immun.* **70**: 1783-1790.

Erova, T. E., L. Pillai, Fadl, A. A. , J. Sha, S. Wang, C. L. Galindo & A. K. Chopra (2006) DNA adenine methyltransferase influences the virulence of *Aeromonas hydrophila*. *Infect. Immun.* **74**: 410-424.

Evdokimov, A. G., D. E. Anderson, K. M. Routzahn & D. S. Waugh (2001) Unusual molecular architecture of the *Yersinia pestis* cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. *J. Mol. Biol.* **312**: 807-821.

Evdokimov, A. G., J. E. Tropea, K. M. Routzahn & D. S. Waugh (2002) Crystal structure of the *Yersinia pestis* GTPase activator YopE. *Protein Sci.* **11**: 401-408.

Falker, S., J. Schilling, M. A. Schmidt & G. Heusipp (2007) Overproduction of DNA adenine methyltransferase alters motility, invasion, and the lipopolysaccharide O-antigen composition of *Yersinia enterocolitica*. *Infect. Immun.* **75**: 4990-4997.

Feldman, M. F., S. Muller, E. Wuest & G. R. Cornelis (2002) SycE allows secretion of YopE-DHFR hybrids by the *Yersinia enterocolitica* type III Ysc system. *Mol. Microbiol.* **46**: 1183-1197.

Fields, K. A., M. L. Nilles, C. Cowan & S. C. Straley (1999) Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. *Infect. Immun.* **67**: 5395-5408.

Fogh, J., J. M. Fogh & T. Orfeo (1977) One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *JNCI* **59**: 221-226.

Forsberg, A., I. Bolin, L. Norlander & H. Wolf-Watz (1987) Molecular cloning and expression of calcium regulated, plasmid-coded proteins of *Y. pseudotuberculosis*. *Microb. Pathog.* **2**: 123-137.

Forsberg, A., A. M. Viitanen, M. Skurnik & H. Wolf-Watz (1991) The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. *Mol. Microbiol.* **5**: 977-986.

Forsberg, A. & H. Wolf-Watz (1988) The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-plB 1-encoded trans-acting elements controlled by temperature and calcium. *Mol. Microbiol.* **2**: 121-133.

Fraser, G. M. & C. Hughes (1999) Swarming motility. *Curr. Opin. Microbiol.* **2**: 630-635.

Frithz-Lindsen, E., R. Rosqvist, L. Johansson & A. Forsberg (1995) The chaperone-like protein YerA of *Yersinia pseudotuberculosis* stabilizes YopE in the cytoplasm but is dispensable for targeting to the secretion loci. *Mol. Microbiol.* **16**: 635-647.

Fuqua, C. (2006) The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. *J. Bacteriol.* **188**: 3169-3171.

Fuqua, C. & E. P. Greenberg (2002) Listening in on bacteria: Acyl-homoserine lactone signalling. *Nature Rev. Mol. Cell. Biol.* **3**: 685-695.

Fuqua, C., S. C. Winans & E. P. Greenberg (1996) Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. *Ann. Rev. Microbiol.* **50**: 727-751.

Gaillard, J. L., P. Berche, J. Mounier, S. Richard & P. Sansonetti (1987) *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun.* **55**: 2822-2829.

Gallant, C. V., C. Daniels, J. M. Leung, A. S. Ghosh, K. D. Young, L. P. Kotra & L. L. Burrows (2005) Common β-lactamases inhibit bacterial biofilm formation. *Mol. Microbiol.* **58**: 1012-1024.

Galyov, E. E., S. Hakansson, A. Forsberg & H. Wolf-Watz (1993) A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. *Nature* **361**: 730-732.

Gao, M., H. Chen, A. Eberhard, M. R. Gronquist, J. B. Robinson, Barry G. Rolfe & W. D. Bauer (2005) *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. *J. Bacteriol.* **187**: 7931-7944.

Garcia-Del Portillo, F., M. G. Pucciarelli & J. Casadesus (1999) DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* **96**: 11578-11583.

Gelhaus, H. C., D. A. Rozak, W. C. Nierman, D. Chen, M. Z. J. J. Varga, R. L. Ulrich & J. J. Adamovicz (2009) Exogenous *Yersinia pestis* quorum sensing molecules *N*-octanoyl-homoserine lactone and *N*-(3-oxooctanoyl)-homoserine lactone regulate the LcrV virulence factor. *Microb. Pathog.* **46**: 283-287.

Gerdes, K., J. Moller-Jensen & R. Bugge Jensen (2000) Plasmid and chromosome partitioning: surprises from phylogeny. *Mol. Microbiol.* **37**: 455-466.

Gilson, L., A. Kuo & P. V. Dunlap (1995) AinS and a new family of autoinducer synthesis proteins. *J. Bacteriol.* **177**: 6946-6951.

Goguen, J. D., J. Yother & S. C. Straley (1984) Genetic analysis of the low calcium response in *Yersinia pestis* mu dl(Ap *lac*) insertion mutants. *J. Bacteriol.* **160**: 842-848.

Greenberg, E. P. (1997) Quorum sensing in gram-negative bacteria: acylhomoserine lactone signalling and cell-cell communication. *ASM News* **63**: 371-377.

Grosdent, N., I. Maridonneau-Parini, M. P. Sory & G. R. Cornelis (2002) Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. *Infect. Immun.* **70**: 4165-4176.

Haine, V., A. Sinon, F. Van Steen, S. Rousseau, M. Dozot, P. Lestrate, C. Lambert, J.-J. Letesson & X. De Bolle (2005) Systematic targeted mutagenesis of *Brucella melitensis* 16M reveals a major role for GntR regulators in the control of virulence. *Infect. Immun.* **73**: 5578-5586.

Haller, J. C., S. Carlson, K. J. Pederson & D. E. Pierson (2000) A chromosomally encoded type III secretion pathway *in Yersinia enterocolitica* is important in virulence. *Mol. Microbiol.* **36**: 1436-1446.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**: 557-580.

Hastings, J. W. (1996) Chemistries and colors of bioluminescent reactions: a review. *Gene* **173**: 5-11.

Heesemann, J. & L. Grüter (1987) Genetic evidence that the outer membrane protein YOP1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. *FEMS Microbiol. Lett.* **40**: 37-41.

Hinnebusch, B. J., E. R. Fischer & T. G. Schwan (1998) Evaluation of the role of the *Yersinia pestis* plasminogen activator and other plasmid-encoded factors in temperature-dependent blockage of the flea. *J. Infect. Dis.* **178**: 1406-1415.

Hinnebusch, B. J., R. D. Perry & T. G. Schwan (1996) Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. *Science* **273**: 367-370.

Hinnebusch, B. J., M.-L. Rosso, T. G. Schwan & E. Carniel (2002) High-frequency conjugative transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. *Mol. Microbiol.* **46**: 349-354.

Hoffmann, R., K. van Erp, K. Trulzsch & J. Heesemann (2004) Transcriptional responses of murine macrophages to infection with *Yersinia enterocolitica*. *Cell. Microbiol.* **6**: 377-390.

Hoheisel, J. D. (2006) Microarray technology: beyond transcript profiling and genotype analysis. *Nat. Rev. Genet.* **7**: 200-210.

Hoiczyk, E. & G. Blobel (2001) Polymerization of a single protein of the pathogen *Yersinia enterocolitica* into needles punctures eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **98**: 4669-4674.

Holmström, A., J. Olsson, P. Cherepanov, E. Maier, R. Nordfelth, J. Pettersson, R. Benz, H. Wolf-Watz & A.Forsberg (2001) LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. *Mol. Microbiol.* **39**: 620-632.

Holmström, A., J. Petterson, R. Rosqvist, S. Hakansson, F. Tafazoli, M. Fallman, K. E. Magnusson, H. Wolf-Watz & A. Forsberg (1997) YopK of *Yersinia pseudotuberculosis* controls translocation of Yop effectors across the eukaryotic cell membrane. *Mol. Microbiol.* **24**: 73-91.

Hooper, L. V. & J. I. Gordon (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115-1118.

Horng, Y.-T., S.-C. Deng, M. Daykin, P.-C. Soo, J.-R. Wei, K.-T. Luh, S.-W. Ho, S. Swift, H.-C. Lai & P. Williams (2002) The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. *Mol. Microbiol.* **45**: 1655-1671.

Howard, S. L., M. W. Gaunt, J. Hinds, A. A. Witney, R. Stabler & B. W. Wren (2006) Application of comparative phylogenomics to study the evolution of *Yersinia enterocolitica* and to identify genetic differences relating to pathogenicity. *J. Bacteriol.* **188**: 3645-3653.

Hughes, D. T. & V. Sperandio (2008) Inter-kingdom signalling: communication between bacteria and their hosts. *Nat. Rev. Microbiol.* **6**: 111-120.

Ingram, L. O., L. S. Chevalier, E. J. Gabbay, K. D. Ley & K. Winters (1977) Propionate induced synthesis of odd chain length fatty acids by *Escherichia coli*. *J. Bacteriol.* **131**: 1023-1025.

Iriarte, M. & G. R. Cornelis (1999) The 70-kilobase virulence plasmid of *Yersiniae*. In: Pathogenicity islands and other mobile elements. J. B. Kaper and B. Hacker (eds). Washington D. C.: American Society for Microbiology. pp. 91-127.

Iriarte, M., I. Stainier, V. Mikulskis & G. R. Cornelis (1995) The *fliA* gene encoding σ28 in *Yersinia enterocolitica*. *J. Bacteriol.* **177**: 2299-2304.

Isberg, R. R. (1989) Determinants for the thermoinducible cell binding and plasmid-encoded cellular penetration detected in the absence of the *Yersinia pseudotuberculosis* invasin protein. *Infect. Immun.* **57**: 1998-2005.

Isberg, R. R., D. L. Voorhis & S. Falkow (1987) Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* **50**: 769-778.

Isherwood, K. E. (2001) Quorum sensing in *Yersinia pestis*. PhD thesis. Nottingham: University of Nottingham.

Jarrett, C. O., E. Deak, K. E. Isherwood, P. C. Oyston, E. R. Fischer, A. R. Whitney, S. D. Kobayashi, F. R. DeLeo & B. J. Hinnebusch (2004) Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. *J. Infect. Dis.* **190**: 783-792.

Jeffrey, S. K., T. Matthew & R. H. Alexander (2007) A role for type I signal peptidase in *Staphylococcus aureus* quorum sensing. *Mol. Microbiol.* **65**: 780-798.

Ji, G., R. Beavis & R. P. Novick (1997) Bacterial interference caused by autoinducing peptide variants. *Science* **276**: 2027-2030.

Ji, G., R. C. Beavis & R. P. Novick (1995) Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. USA* **92**: 12055-12059.

Jiang, Y., M. Camara, S. R. Chhabra, K. R. Hardie, B. W. Bycroft, A. Lazdunski, G. P. C. Salmond, G. S. A. B. Stewart & P. Williams (1998) *In vitro* biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-L-homoserine lactone. *Mol. Microbiol.* **28**: 193-203.

Joshua, G. W. P., A. V. Karlyshev, M. P. Smith, K. E. Isherwood, R. W. Titball, & B. W. Wren (2003) A *Caenorhabditis elegans* model of *Yersinia* infection: biofilm formation on a biotic surface. *Microbiol.* **149**: 3221-3229.

Journet, L., C. Agrain, P. Broz & G. R. Cornelis (2003) The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* **1757-1760.**

Juhas, M., L. Wiehlmann, B. Huber, D. Jordan, J. Lauber, P. Salunkhe, A. S. Limpert, F. von Gotz, I. Steinmetz, L. Eberl & B. Tummler (2004) Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiol.* **150**: 831-841.

Julio, S. M., D. M. Heithoff, D. Provenzano, K. E. Klose, R. L. Sinsheimer, D. A. Low & M. J. Mahan (2001) DNA adenine methylase is essential for viability and plays a role in the pathogenesis of *Yersinia pseudotuberculosis* and *Vibrio cholerae*. *Infect. Immun.* **69**: 7610-7615.

Julio, S. M., D. M. Heithoff, R. L. Sinsheimer, D. A. Low & M. J. Mahan (2002) DNA adenine methylase overproduction in *Yersinia pseudotuberculosis* alters YopE expression and secretion and host immune responses to infection. *Infect. Immun.* **70**: 1006-1009.

Kimbrough, T. G. & S. I. Miller (2000) Contribution of *Salmonella typhimurium* type III secretion components to needle complex formation. *Proc. Natl. Acad. Sci. USA* **97**: 11008-11013.

Kinder, S. A., J. L. Badger, G. O. Bryant, J. C. Pepe & V. L. Miller (1993) Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O8 and construction of a transformable R-M+ mutant. *Genes Dev.* **136**: 271-275.

Kirwan, J. P., T. A. Gould, H. P. Schweizer, S. W. Bearden, R. C. Murphy & M. E. A. Churchill (2006) Quorum-sensing signal synthesis by the *Yersinia pestis* acyl-homoserine lactone synthase YspI. *J. Bacteriol.* **188**: 784-788.

Kolibachuk, D. & E. P. Greenberg (1993) The *Vibrio fischeri* luminescence gene activator LuxR is a membrane-associated protein. *J. Bacteriol.* **175**: 7307-7312.

Koster, M., W. Bitter, H. de Cock, A. Allaoui, G. R. Cornelis & J. Tommassen (1997) The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Mol. Microbiol.* **26**: 789-797.

Koutsoudis, M. D., D. Tsaltas, T. D. Minogue & S. B. von Bodman (2006) Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. USA* **103**: 5983-5988.

Kubori, T., Y. Matsushima & D. Nakamura (1998) Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* **280**: 602-605.

Kubori, T., A. Sukhan, S.-I. Aizawa & J. E. Galan (2000) Molecular characterization and assembly of the needle complex of the *Salmonella typhimurium* type III protein secretion system. *Proc. Natl. Acad. Sci. USA* **97**: 10225-10230.

Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Laird, W. J. & D. C. Cavanaugh (1980) Correlation of autoagglutination and virulence of *Yersiniae*. *J. Clin. Microbiol.* **11**: 430-432.

Latifi, A., M. Foglino, K. Tanaka, P. Williams & A. Lazdunski (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol. Microbiol.* **21**: 1137-1146.

Lawton, D. G., C. Longstaff, B. A. Wallace, J. Hill, S. E. Leary, R. W. Titball & K. A. Brown (2002) Interactions of the type III secretion pathway proteins LcrV and LcrG from Yersinia pestis are mediated by coiled-coil domains. *J. Biol. Chem.* **277**: 38714-38722.

Lazdunski, A. M., I. Ventre & J. N. Sturgis (2004) Regulatory circuits and communication in gram negative bacteria. *Nat. Rev. Microbiol.* **2**: 581-592.

Ledgham, F., I. Ventre, C. Soscia, M. Foglino, J. N. Sturgis & A. Lazdunski (2003) Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. *Mol. Microbiol.* **48**: 199-210.

Lequette, Y., J-H. Lee, F. Ledgham, A. Lazdunski & E. P. Greenberg (2006) A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. *J. Bacteriol.* **188**: 3365-3370.

Lesic, B. & E. Carniel (2004) The High-Pathogenicity Island: A broad-host-range pathogenicity island. In: Yersinia: Molecular and cellular biology. E. Carniel & B. J. Hinnesbusch (eds). Norfolk, UK: Horizon Bioscience. pp. 285-317.

Li, L.-L., J. E. Malone & B. H. Iglewski (2007) Regulation of the *Pseudomonas aeruginosa* quorum-sensing regulator VqsR. *J. Bacteriol.* **189**: 4367-4374.

Lindberg, M., K. Jonsson, H. Muller, H. Jonsson, C. Signas, M. Hook, R. Raja, G. Raucci & G. M. Anantharamaiah (1990) Fibronectin-binding proteins in *Staphylococcus aureus*. In: Molecular biology of the staphylococci. R. P. Novick (ed.). New York, N.Y.: VCH. pp. 343-356.

Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dye, P. Williams & J. A. Downie (2000) The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. *Mol. Microbiol.* **37**: 81-97.

Lovallo, W. R. & T. L. Thomas (2007) Stress hormones in psychophysiological research: Emotional, behavioral and cognitive implication. In: Handbook of psychophysiology. J. T. Cacioppo, L. G. Tassinary and G. G. Berntson (eds). Cambridge: Cambridge University Press. pp. 342-367.

Lyon, G. J., P. Mayville, T. W. Muir & R. P. Novick (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in

part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. *Proc. Natl. Acad. Sci. USA* **97**: 13330-13335.

Malott, R. J., E. P. O'Grady, J. Toller, S. Inhulsen, L. Eberl & P. A. Sokol (2009) A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. *J. Bacteriol.* **191**: 2447-2460.

Matson, J. S. & M. L. Nilles (2002) Interaction of the *Yersinia pestis* type III regulatory proteins LcrG and LcrV occur at a hydrophobic interface. *BMC Microbiol.* **2**: 16-25.

Marceau, M. (2005) Transcriptional regulation in *Yersinia*: an update. *Curr. Issues Mol. Biol.* **7**: 151-178.

Marenne, M.-N., L. Journet, L. J. Mota & G. R. Cornelis (2003) Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: role of LcrV, YscF and YopN. *Microb. Pathog.* **35**: 243-258.

Marenne, M.-N., L. J. Mota & G. R. Cornelis (2004) The pYV plasmid and the Ysc-Yop type III secretion system. In: *Yersinia*: Molecular and cellular biology. E. Carniel & B. J. Hinnesbusch (eds). Norfolk, UK: Horizon Bioscience. pp. 319-348.

Mario, J., W. Lutz, S. Prabhakar, L. Jörg, B. Jan, & T. Burkhard (2005) GeneChip expression analysis of the VqsR regulon of *Pseudomonas aeruginosa* TB. *FEMS Microbiol. Lett.* **242**: 287-295.

Martinez, E., B. Bartolome & F. de la Cruz (1988) pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* alpha reporter gene of pUC8/9 and pUC18/19 plasmids. *Genes Dev.* **68**.

Maxson, M. E. & A. J. Darwin (2004) Identification of inducers of the *Yersinia enterocolitica* phage shock protein system and comparison to the regulation of the RpoE and Cpx extracytoplasmic stress responses. *J. Bacteriol.* **186**: 4199-4208.

McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart & P. Williams (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiol.* **143**: 3703-3711.

McGowan, S., M. Sebaihia, S. Jones, B. Yu, N. Bainton, P. F. Chan, B. Bycroft, G. S. A. B. Stewart, P. Williams & G. P. C. Salmond (1995) Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator. *Microbiol.* **141**: 541-550.

McGowan, S. J., M. Sebaihia, L. E. Porter, G. S. A. B. Stewart, P. Williams, B. W. Bycroft & G. P. C. Salmond (1996) Analysis of bacterial carbapenem antibiotic production genes reveals a novel β-lactam biosynthesis pathway. *Mol. Microbiol.* **22**: 415-426.

McNally, A., T. Cheasty, C. Fearnley, R. W. Dalziel, G. A. Paiba, G. Manning & D. G Newell (2004) Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999-2000. *Lett. Appl. Microbiol.* **39**: 103-108.

Michael, B., J. N. Smith, S. Swift, F. Heffron & B. M. M. Ahmer (2001) SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. *J. Bacteriol.* **183**: 5733-5742.

Michiels, T. & G. R. Cornelis (1991) Secretion of hybrid proteins by the *Yersinia* Yop export system. *J. Bacteriol.* **173**: 1677-1685.

Michiels, T., P. Wattiau, R. Brasseur, J. M. Ruysschaert & G. R. Cornelis (1990) Secretion of Yop proteins by *Yersiniae*. *Infect. Immun.* **58**: 2840-2849.

Miller, S., K. Xavier, S. Campagna, M. Taga, M. Semmelhack, B. Bassler & F. Hughson (2004) *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol. Cell* **15**: 677-687.

Miller, V. L. (1992) *Yersinia* invasion genes and their products. *ASM News* **58**: 26-33.

Miller, V. L. & J. J. Mekalanos (1988) A novel suicide vector and its use in construction of insertion mutation: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **177**: 2575-2583.

Miller, V. L. & S. Falkow (1988) Evidence for two genetic loci from *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect. Immun.* **56**: 1242-1248.

Milton, D. L., A. Hardman, M. Camara, S. R. Chhabra, B. W. Bycroft, G. S. A. B. Stewart & P. Williams (1997) Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identifacation of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. *J. Bacteriol.* **179**: 3004-3012.

Milton, D. L., V. J. Chalker, D. Kirke, A. Hardman, M. Camara & P. Williams (2001) The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and N-hexanoylhomoserine lactone. *J. Bacteriol.* **183**: 3537-3547.

Milton, D. L., R. O'Toole, P. Horstedt & H. Wolf-Watz (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J. Bacteriol.* **178**: 1310-1319.

Miyahara, M., T. Maruyama, A. Wake & K. Mise (1988) Widespread occurrence of the restriction endonuclease *Yen*I, an isoschizomer of *Pst*I, in *Yersinia enterocolitica* serotype O8. . *Appl. Environ. Microbiol.* **54**: 577-580.

Moré, M. I., L. D. Finger, J. L. Stryker, C. Fuqua, A. Eberhard & S. C. Winans (1996) Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science* **272**: 1655-1658.

Mulder, B., T. Michiels, M. Simonet, M. P. Sory & G. R. Cornelis (1989) Identification of additional virulence determinants on the pYV plasmid of *Yersinia enterocolitica* W227. *Infect. Immun.* **57**: 2534-2541.

Murphy, K. C. (1998) Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *J. Bacteriol.* **180**: 2063-2071.

Nasser, W., M. L. Bouillant, G. Salmond & S. Reverchon (1998) Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol. Microbiol.* **29**: 1391-1405.

Navarro, L., N. M. Alto & J. E. Dixon (2005) Functions of the *Yersinia* effector proteins in inhibiting host immune responses. *Curr. Opin. Microbiol.* **8**: 21-27.

Navarro, L., A. Koller, R. Nordfelth, H. Wolf-Watz, S. Taylor & J. E. Dixon (2007) Identification of a molecular target for the *Yersinia* protein kinase A. *Mol. Cell.* **26**: 465-477.

Nealson, K. H., T. Platt & J. W. Hastings (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **104**: 313-322.

Nijvipakul, S., J. Wongratana, C. Suadee, B. Entsch, D. P. Ballou & P. Chaiyen (2008) LuxG is a functioning flavin reductase for bacterial luminescence. *J. Bacteriol.* **190**: 1531-1538.

Nilles, M. L., A. W. Williams, E. Skrzypek & S. C. Straley (1997) *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low-Ca^{2+} response. *J. Bacteriol.* **179**: 1307-1316.

Nilles, M. L., K. A. Fields & S. C. Straley (1998) The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. *J. Bacteriol.* **180**: 3410-3420.

Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* **48**: 1429-1449.

Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch & S. Moghazeh. (1995) The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol. Gen. Genet.* **248**: 446-458.

Novick, R. P., R. C. Clowes, S. N. Cohen, R. D. Curtiss, N. Datta & S. Falkow (1976) Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriol. Rev.* **40**: 168-189.

O'Hagan, D. (1991) *The Polyketide Metabolites*. Ellis Harwood, London.

Offermanns, S., C. F. Toombs, Y. H. Hu & M. I. Simon (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature* **389**: 183-186.

Oger, P., K. S. Kim, R. L. Sackett, K. R. Piper & S. K. Farrand (1998) Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. *Mol. Microbiol.* **27**: 277-288.

Ortori, C. A., S. Atkinson, S. R. Chhabra, M. Cámara, P. Williams & D. A. Barrett (2007) Comprehensive profiling of *N*-acylhomoserine lactones produced by *Yersinia pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry. *Anal. Bioanal. Chem.* **387**: 497-511.

Oshima, T., C. Wada, Y. Kawagoe, T. Ara, M. Maeda, Y. Masuda, S. Hiraga & H. Mori (2002) Genome-wide analysis of deoxyadenosine methyltransferase-mediated control of gene expression in *Escherichia coli*. *Mol. Microbiol.* **45**: 673-695.

Pappas, K. M., C. L. Weingart & S. C. Winans (2004) Chemical communication in proteobacteria: biochemical and structural studies of signal synthases and receptors required for intercellular signalling. *Mol. Microbiol.* **53**: 755-769.

Parker, C. T. & V. Sperandio (2009) Cell-to-cell signalling during pathogenesis. *Cell. Microbiol.* **11**: 363-369.

Parkhill, J., B.W. Wren, N. R. Thomson, R.W. Titball, M. T. G. Holden, M. B. Prentice, M. Sebaihia, K. D. James, C. Churcher, K. L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeño-Tárraga, T. Chillingworth, A. Cronin, R. M. Davies, P. Davis, G. Dougank, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. F. Oyston, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, & B. G. Barrell (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**: 523-527.

Pearson, J. P., M. Feldman, B. H. Iglewski & A. Prince (2000) *Pseudomonas aeruginosa* cell-cell signalling is required for virulence in a model of acute pulmonary infection. *Infect. Immun.* **68**: 4331-4334.

Pellock, B. J., M. Teplitski, R. P. Boinay, W. D. Bauer & G. C. Walker (2002) A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J. Bacteriol.* **184**: 5067-5076.

Pelludat, C., M. Hogardt & J. Heeseman (2002) Transfer of the core region genes of the *Yersinia enterocolitica* WAC Serotype O:8 High-Pathogenicity Island to *Y. enterocolitica* MRS40, a strain with low levels of pathogenicity, confers a

yersiniabactin biosynthesis phenotype and enhanced mouse virulence *Infect. Immun.* **70**: 1832-1841.

Peng, H. L., R. P. Novick, B. Kreiswirth, J. Kornblum & P. Schlievert (1988) Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J. Bacteriol.* **170**: 4365-4372.

Pepe, J. C., J. L. Badger & V. L. Miller (1994) Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica inv* gene. *Mol. Microbiol.* **11**: 123-135.

Pepe, J. C. & V. L. Miller (1990) The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. *J. Bacteriol.* **172**: 3780-3789.

Pepe, J. C. & V. L. Miller (1993) *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. *Proc. Natl. Acad. Sci. USA* **90**: 6473-6477.

Pepe, J. C., M. R. Wachtel, E. Wagar & V. L. Miller (1995) Pathogenesis of defined invasion mutants of *Yersinia enterocolitica* in a BALB/c mouse model of infection. *Infect. Immun.* **63**: 4837-4848.

Perez-Gutierrez, C., C. M. Llompart, M. Skurnik & J. A. Bengoechea (2007) Expression of the *Yersinia enterocolitica* pYV-Encoded type III secretion system is modulated by lipopolysaccharide O-antigen status. *Infect. Immun.* **75**: 1512-1516.

Perry, R. D. (2004) *Yersinia*. In: Iron transport in bacteria: Molecular genetics, biochemistry, and role in pathogenicity and ecology. J. H. Crosa, A. R. Mey & S. M. Payne (eds). Washington, D.C.: ASM Press. pp. 219-248.

Perry, R. D. & J. D. Fetherston (1997) *Yersinia pestis* - Etiologic agent of plague. *Clin. Microbiol. Rev.* **10**: 35-66.

Perry, R. D. & J. D. Fetherston (2004) Iron and Heme Uptake Systems. In: *Yersinia*: Molecular and cellular biology. E. Carniel & B. J. Hinnesbusch (eds). Norfolk, UK: Horizon Bioscience. pp. 257-283.

Perry, R. D., S. C. Straley, J. D. Fetherston, D. J. Rose, J. Gregor & F. R. Blattner (1998) DNA sequencing and analysis of the low-Ca^{2+}-response plasmid pCD1 of *Yersinia pestis* KIM5. *Infect. Immun.* **66**: 4611-4623.

Persson, C., R. Nordfelth, A. Holmstrom, S. Hakansson, R. Rosqvist & H. Wolf-Watz (1995) Cell-surface bound *Yersinia* translocate the protein tyrosine phosphatase YopH by a polarized mechanism into the target cell. *Mol. Microbiol.* **18**: 135-150.

Pettersson, J., A. Holmström, J. Hill, S. Leary, E. Frithz-Lindsten, A. von Euler-Matell, E. Carlsson, R. Titball, A. Forsberg & H. Wolf-Watz (1999) The V-antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. *Mol. Microbiol.* **32**: 961-976.

Pettersson, J., R. Nordfelth, E. Dubinina, T. Bergman, M. Gustafsson, K. E. Magnusson & H. Wolf-Watz (1996) Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**: 1231-1233.

Pirhonen, M., D. Felgo, R. Heikinheimo, & E. T. Palva (1993) A small diffusible molecule is responsible for the global control of virulence and exoenzyme production in *Erwinia carotovora*. *EMBO J.* **12**: 2467-2476.

Portnoy, D. A., S. L. Moseley & S. Falkow (1981) Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. *Infect. Immun.* **31**: 775-782.

Praszkier, J., T. Wei, K. Siemering & J. Pittard (1991) Comparative analysis of the replication regions of IncB, IncK, and IncZ plasmids. *J. Bacteriol. Rev.* **173**: 2393-2397.

Prentki, P. & H. M. Krisch (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**: 303-313.

Qin, Y., Z.-Q. Luo, A. J. Smyth, P. Gao, S. B. von Bodman & S. K. Farrand (2000) Quorum-sensing signal binding results in dimerization of TraR and its release from membranes into the cytoplasm. *EMBO J.* **19**: 5212-5221.

Ramamurthi, K. S. & O. Schneewind (2002) Type III protein secretion in *Yersinia* species. *Annu. Rev. Cell Dev. Biol.* **18**: 107-133.

Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss & R. P. Novick (1985) Regulation of exoprotein gene expression by *agr*. *Mol. Gen. Genet.* **202**: 58-61.

Reverchon, S., M. L. Bouillant, G. Salmond & W. Nasser (1998) Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. *Mol. Microbiol.* **29**: 1407-1418.

Riley, G. & S. Toma (1989) Detection of pathogenic *Yersinia enterocolitica* by using congo red-magnesium oxalate agar medium. *J. Clin. Microbiol.* **27**: 213-214.

Rimpilainen, M., A. Forsberg & H. Wolf-Watz (1992) A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. *J. Bacteriol.* **174**: 3355-3363.

Robins-Browne, R. M. & J. K. Prpic (1985) Effects of iron and desferrioxamine on infections with *Yersinia enterocolitica*. *Infect. Immun.* **47**: 774-779.

Rogers, S., M. Girolami, W. Kolch, K. M. Waters, T. Liu, B. Thrall & H. S. Wiley (2008) Investigating the correspondence between transcriptomic and proteomic expression profiles using coupled cluster models. *Bioinformatics* **24**: 2894-2900.

Rohde, J. R., X. S. Luan, H. Rohde, J. M. Fox & S. A. Minnich (1999) The *Yersinia enterocolitica* pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37°C. *J. Bacteriol.* **181**: 4198-4204.

Ruckdeschel, K., A. Roggenkamp, V. LaFont, P. Mangeat, J. Heesemann & B. Rouot (1997) Interaction of *Yersinia enterocolitica* with macrophages leads to macrophage cell death through apoptosis. *Infect. Immun.* **65**: 4813-4821.

Rumbaugh, K. P., J. A. Griswold, B. H. Iglewski & A. N. Hamood (1999) Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect. Immun.* **67**: 5854-5862.

Sambrook, J., E. F. Fritsch & T. Maniatis (1989) *Molecular cloning-A laboratory manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, USA.

Sarker, M. R., M. P. Sory, B. A. P., M. Iriarte & G. R. Cornelis (1998) LcrG is required for efficient translocation of *Yersinia* Yop effector proteins into eukaryotic cells. *Infect. Immun.* **66**: 2976-2979.

Sauvonnet, N., B. Pradet-Balade, J. A. Garcia-Sanz, and & G. R. Cornelis (2002) Regulation of mRNA expression in macrophages after *Yersinia enterocolitica* infection: Role of different Yop effectors. *J. Biol. Chem.* **277**: 25133-25142.

Schauder, S. & B. L. Bassler (2001) The languages of bacteria. *Genes Dev.* **15**: 1468-1480.

Schauder, S., K. Shokat, M. G. Surette & B. L. Bassler (2001) The LuxS family of bacterial autoinducers: Biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41**: 463-476.

Schena, M., D. Shalon, R. W. Davis & P. O. Brown (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**: 467-470.

Schesser, K., A. K. Spiik, J. M. Dukuzumuremyi, M. F. Neurath, S. Pettersson & H. Wolf-Watz (1998) The *yopJ* locus is required for *Yersinia*-mediated inhibition of NF-kappaB activation and cytokine expression. YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol. Microbiol.* **28**: 1067-1079.

Schierbeek, A. (1959) *Measuring the Invisible World. The Life and Works of Antoni van Leeuwenhoek FRS.* Abelard-Schuman Ltd., London and New York.

Sesma, A., G. W. Sundin & J. Murillo (2000) Phylogeny of the replication regions of pPT23A-like plasmids from *Pseudomonas syringae*. *Microbiol.* **146**: 2375-2384.

Shao, F., P. O. Vacratsis, Z. Bao, K. E. Bowers, C. A. Fierke & J. E. Dixon (2003) Biochemical characterization of the *Yersinia* YopT protease: cleavage site and recognition elements in Rho GTPases. *Proc. Natl. Acad. Sci. USA* **100**: 904-909.

Sjoblom, S., G. Brader, G. Koch & P. E. Tapio (2006) Cooperation of two distinct ExpR regulators controls quorum sensing specificity and virulence in the plant pathogen *Erwinia carotovora*. *Mol. Microbiol.* **60**: 1474-1489.

Skryzpek, E. & S. C. Straley (1993) LcrG, a secreted protein involved in negative regulation of the low-calcium response in *Yersinia pestis*. *J. Bacteriol.* **175**: 3520-3528.

Skurnik, M. (1995) Role of YadA in *Yersinia enterocolitica*-induced reactive arthritis: a hypothesis. *Trends Microbiol.* **3**: 318-319

Skurnik, M., I. Bölin, H. Heikkinen, S. Piha & H. Wolf-Watz (1984) Virulence plasmid-associated autoagglutination in *Yersinia* spp. *J. Bacteriol.* **158**: 1033-1036.

Skurnik, M., A. Peippo & E. Ervela (2000) Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b. *Mol. Microbiol.* **37**: 316-330.

Skurnik, M. & P. Toivanen (1992) LcrF is the temperature-regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *J. Bacteriol.* **174**: 2047-2051.

Smith, D., J.-H. Wang, J. E. Swatton, P. Davenport, B. Price, H. Mikkelsen, H. Stickland, K. Nishikawa, N. Gardiol, D. R. Spring & M. Welch (2006) Variations on a theme: diverse *N*-acyl homoserine lactone-mediated quorum sensing mechanisms in Gram-negative bacteria. *Science Progress* **89**: 167-211.

Snellings, N. J., M. Popek & L. E. Lindler (2001) Complete DNA sequence of *Yersinia enterocolitica* serotype 0:8 low-calcium-response plasmid reveals a new virulence plasmid-associated replicon. *Infect. Immun.* **69**: 4627-4638.

Sobek, J., K. Bartscherer, A. Jacob, J. D. Hoheisel & P. Angenendt (2006) Microarray technology as a universal tool for high-throughput analysis of biological systems. *Comb. Chem. High. Throughput Screen.* **9**: 365-380.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98:** 503-517.

Sperandio, V., C. C. Li & J. B. Kaper (2002) Quorum sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the

locus of enterocyte effacement pathogenicityisland in enterohemorrhagic *E. coli*. *Infect. Immun.* **70**: 3085-3093.

Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin & J. B. Kaper (1999) Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**: 15196-15201.

Sperandio, V., A. G. Torres, J. A. Girón & J. B. Kaper (2001) Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol.* **183**: 5187-5197.

Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro & J. B. Kaper (2003) Bacteria-host communication: The language of hormones. *Proc. Natl. Acad. Sci. USA* **100**: 8951-8956.

Stevens, A. M. & E. P. Greenberg (1997) Quorum sensing in *Vibrio fischeri*: Essential elements for activation of the luminescence genes. *J. Bacteriol.* **179**: 557-562.

Stevens, A.M. & E. P. Greenberg (1999) Transcriptional activation by LuxR. In: Cell-cell signaling in bacteria. G. M. Dunny and S. C. Winans (eds). Washington D.C.: ASM Press. pp. 231-242.

Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix & K. A. Fields (1993) Regulation by Ca^{2+} in the *Yersinia* low-Ca^{2+} response. *Mol. Microbiol.* **8**: 1005-1010.

Surette, M. G., M. B. Miller & B. L. Bassler (1999) Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc. Natl. Acad. Sci. USA* **96**: 1639-1644.

Swift, S., K. E. Isherwood, S. Atkinson, P. Oyston & G. S. A. B. Stewart (1999a) Quorum sensing in *Aeromonas* and *Yersinia*. In: Cell-cell signaling in bacteria. G. H. R. England, N. J. Bainton, and D. M. Roberts (eds). Washington D.C.: ASM Press. pp. 85-104.

Swift, S., P. Williams & G. S. A. B. Stewart (1999b) *N*-acylhomoserine lactones and quorum sensing in Proteobacteria. In: Cell-cell signaling in bacteria. G. M. Dunny and S. C. Winans (eds). Washington D.C.: ASM Press. pp. 291-313.

Swift, S., M. K. Winson, P. F. Chan, N. J. Bainton, M. Birdsall, P. J. Reeves, E. D. Rees, S. R. Chhabra, P. J. Hill, J. P. Throup, B. W. Bycroft, G. P. C. Salmond, P. Williams & G. S. A. B. Stewart (1993) A novel strategy for the isolation of *luxI* homologues: evidence for the widespreas distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol. Microbiol.* **10**: 511-520.

Taga, M. E. & B. L. Bassler (2003) Chemical communication among bacteria. *Proc. Natl. Acad. Sci. USA* **100**: 14549-14554.

Taga, M. E., J. L. Semmelhack & B. L. Bassler (2001) The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol. Microbiol.* **42**: 777-793.

Thomson, N. R., S. Howard, B. W. Wren, M. T. G. Holden, L. Crossman, G. L. Challis, C. Churcher, K. Mungall, K. Brooks, T. Chillingworth, T. Feltwell, Z. Abdellah, H. Hauser, K. Jagels, M. Maddison, S. Moule, M. Sanders, S. Whitehead, M. A. Quail, G. Dougan, J. Parkhill & M. B. Prentice (2006) The complete genome sequence and comparative genome analysis of the high pathogenicity *Yersinia enterocolitica* strain 8081. *PLoS Genet.* **2**: e206. doi:210.1371/journal.pgen.0020206.

Throup, J. P., M. Cámara, N. J. Bainton, G. S. Briggs, S. R. Chhabra, B. W. Bycroft, P. Williams & G. S. A. B. Stewart (1995) Characterisation of the *yenI/yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acyl homoserine lactone signal molecules. *Mol. Microbiol.* **17**: 345-356.

Townsend, M. K., N. J. Carr, J. G. Iyer, S. M. Horne, P. S. Gibbs & B. M. Prüß (2008) Pleiotropic phenotypes of a *Yersinia enterocolitica flhD* mutant include reduced lethality in a chicken embryo model. *BMC Microbiology* **8**: 1471-2180.

Trasak, C., G. Zenner, A. Vogel, G. Yuksekdag, R. Rost, I. Haase, M. Fischer, L. Israel, A. Imhof, S. Linder, M. Schleicher & M. Aepfelbacher (2007) *Yersinia* protein kinase YopO is activated by a novel G-actin binding process. *J. Biol. Chem.* **282**: 2268-2277.

Trosky, J. E., A. D. B. Liverman & K. Orth (2008) *Yersinia* outer proteins: Yops. *Cell. Microbiol.* **10**: 557-565.

Viboud, G. I. & J. B. Bliska (2005) *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu. Rev. Microbiol.* **59**: 69-89.

von Bodman, S. B., J. M. Willey & S. P. Diggle (2008) Cell-cell communication in bacteria: United we stand. *J. Bacteriol.* **190**: 4377-4391.

Wagner, V.E., D. Bushnell, L. Passador, A. I. Brooks & B. H. Iglewski (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J. Bacteriol.* **185**: 2080-2095.

Walters, M. & V. Sperandio (2006) Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **74**: 5445-5455.

Wang, X.D., P. A. J. de Boer, & L. I. Rothfield (1991) A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*. *EMBO J.* **10**: 3363-3372.

Waters, C. M. & B. L. Bassler (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell. Dev. Biol.* **21**: 319-346.

Watson, M. E., Jr., J. Jarisch & A. L. Smith (2004) Inactivation of deoxyadenosine methyltransferase (dam) attenuates *Haemophilus influenzae* virulence. *Mol. Microbiol.* **53**.

Watson, B., T. C. Currier, M. P. Gordon, M. D. Chilton & E. W. Nester (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **123**: 255-264.

Wattiau, P. & G. R. Cornelis (1993) SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. *Mol. Microbiol.* **8**: 123-131.

Wheeler, D. B., A. E. Carpenter & D. M. Sabatini (2005) Cell microarrays and RNA interference chip away at gene function. *Nat. Genet.* **37**: S25-S30

Whitehead, N. J., A. M. L. Barnard, H. Slater, N. J. L. Simpson & G. P. C. Salmond (2001) Quorum-sensing in gram-negative bacteria. *FEMS Microbiol. Rev.* **25**: 365-404.

Whiteley, M. & E. P. Greenberg (2001) Promoter specificity elements in *Pseudomonas aeruginosa* quorum sensing-controlled genes. *J. Bacteriol.* **183**: 5529-5534.

Wiley, D. J., R. Nordfeldth, J. Rosenzweig, C. J. DaFonseca, R. Gustin, H. Wolf-Watz & K. Schesser (2006) The Ser/Thr kinase activity of the *Yersinia* protein kinase A (YpkA) is necessary for full virulence in the mouse, mollifying phagocytes, and disrupting the eukaryotic cytoskeleton. *Microb. Pathog.* **40**: 234-243.

Wilkins, M. R., C. Pasquali, R. D. Appel, K. Ou, O. Golaz, J.-C. Sanchez, J. X. Yan, A. A. Gooley, G. Hughes, I. Humphery-Smith, K. L. Williams & D. F. Hochstrasser (1996) From proteins to proteomes: Large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Nature Biotechnol.* **14**: 61-65.

Williams, A. W. & S. C. Straley (1998) YopD of *Yersinia pestis* plays a role in negative regulation of the low calcium response in addition to its role in translocation of Yops. *J. Bacteriol.* **180**: 350-358.

Williams, P. (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiol.* **153**: 3923-3938.

Winson, M. K., S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams & G. S. A. B. Stewart (1998a) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol. Lett.* **163**: 185-192.

Winson, M. K., S. Swift, P. J. Hill, C. M. Sims, G. Griesmayr, B. W. Bycroft, P. Williams & G. S. A. B. Stewart (1998b) Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn*5* constructs. *FEMS Microbiol. Lett.* **163**: 193-202.

Winzer, K., K. R. Hardie, N. Burgess, N. Doherty, D. Kirke, M. T. G. Holden, R. Linforth, K. A. Cornell, A. J. Taylor, P. J. Hill & P. Williams (2002a) LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone. *Microbiology-SGM* **148**: 909-922.

Winzer, K., K. R. Hardie & P. Williams (2002b) Bacterial cell-to-cell communication: Sorry, can't talk now - gone to lunch! *Curr. Opin. Microbiol.* **5**: 216-222.

Withers, H., S. Swift & P. Williams (2001) Quorum sensing as an integral component of gene regulatory networks in gram-negative bacteria. *Curr. Opin. Microbiol.* **4**: 186-193.

Woestyn, S., M. P. Sory, A. Boland, O. Lequenne & G. R. Cornelis (1996) The cytosolic SycE and SycH chaperones of *Yersinia* protect the region of YopE and YopH involved in translocation across eukaryotic cell membranes. *Mol. Microbiol.* **20**: 1261-1271.

Wren, B. W. (2003) The *Yersiniae* - A model genus to study the rapid evolution of bacterial pathogens. *Nat. Rev. Microbiol.* **1**: 55-64.

Xavier, K. B. & B. L. Bassler (2005) Interference with AI-2-mediated bacterial cell-cell communication. *Nature* **437**: 750-753.

Yanisch-Perron, C., J. Vieira & J. Messing (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Genes Dev.* **33**: 103-119.

Yates, E. A., B. Philipp, C. Buckley, S. Atkinson, S. R. Chhabra, E. Sockett, M. Goldner, Y. Dessaux, M. Cámara, H. Smith & P. Williams (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect. Immun.* **70**: 5635-5646.

Yother, J. & J. D. Goguen (1985) Isolation and characterization of Ca^{2+}-blind mutants of *Yersinia pestis*. *J. Bacteriol.* **164**: 704-711.

Young, G. M. (2004) Flagella: Organelles for motility and protein secretion. In: *Yersinia*: Molecular and cellular biology. E. Carniel & B. J. Hinnesbusch (eds). Norfolk, UK: Horizon Bioscience. pp. 243-256.

Young, G. M., J. L. Badger & V. L. Miller (2000) Motility is required to initiate host cell invasion by *Yersinia enterocolitica*. *Infect. Immun.* **68**: 4323-4326.

Zhang, R.-G., T. Pappas, J. L. Brace, P. C. Miller, T. Oulmassov, J. M. Molyneaux, J. C. Anderson, J. K. Bashkin, S. C. Winans & A. Joachimiak (2002) Structure of a bacterial quorum sensing transcription factor complexed with pheromone and DNA. *Nature* **417**: 971-974.

Zhu, J. & S. C. Winans (1998) Activity of the quorum-sensing regulator TraR of *Agrobacterium tumefaciens* is inhibited by a truncated, dominant defective TraR-like protein. *Mol. Microbiol.* **27**: 289-297.

Zhu, J. & S. C. Winans (1999) Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. *Proc. Natl. Acad. Sci. USA* **96**: 4832-4837.

Zumbihl, R., M. Aepfelbacher, A. Andor, C. A. Jacobi, K. Ruckdeschel, B. Rouot & J. Heesemann (1999) The cytotoxin YopT of *Yersinia enterocolitica* induces modification and cellular redistribution of the small GTP-binding protein RhoA. *J. Biol. Chem.* **274**: 29289-29293.