Quorum Sensing and the Regulation of Multicellular Behaviour in *Yersinia pseudotuberculosis*

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Declaration

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

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Abstract

Yersinia pseudotuberculosis is a human enteropathogen and is the direct ancestor of *Yersinia pestis*, the causative agent of plague. For its pathogenicity *Y. pseudotuberculosis* harbours a virulence plasmid (pYV) encoding genes for a type-III-secretion system (T3SS) which inhibits host phagocytosis and inflammatory responses.

Y. pseudotuberculosis regulates key phenotypes, including T3S, motility, aggregation and biofilm formation on *Caenorhabditis elegans via* quorum sensing (QS). This involves two LuxR/I type QS systems termed YpsR/I and YtbR/I and multiple *N*-acylhomoserine lactone (AHL) signal molecules. Recently, the T3S system was shown to promote biofilm formation on the surface of the nematode *Caenorhabditis elegans* at 22ºC and in turn QS and flagellar regulatory components were found to repress the T3S system *via* a regulatory hierarchy.

To further expand this network, this study then set out to investigate the regulation of the aggregation phenotype at 37ºC. Aggregation experiments using the parent and QS mutants revealed that aggregation is downregulated by QS, since aggregation was enhanced in strains lacking *ypsI/ytbI* and *ypsR/ytbR*. Cultures lacking pYV or treated with proteinase K did not aggregate, suggesting that QS is regulating aggregation by repressing a pYV encoded and surface located aggregation factor(s). Tn*5* transposon mutagenesis of pYV together with targeted mutagenesis of the key structural T3S injectisome components YscF and YscV revealed that aggregation is attenuated when cells are unable to express the injectisome, demonstrating that the aggregation factor is the T3S injectisome and that QS reduces its expression.

N-Acetyl-D-glucosamine (GlcNAc) is known to play diverse roles in bacterial cells for signalling pathways that impact on virulence and is the major component of the extracellular matrix of *Yersinia* biofilms. The repressor of the GlcNAc metabolism, NagC, may also impact on QS in *Y. pseudotuberculosis* since it was found to bind to the *ypsR* promoter in pull-down experiments. By using QS gene

and *nagC* promoter *lux*-fusions, NagC was revealed as a repressor of both *ytbI* and *ypsI*, as an early activator of *ypsR* and as an activator of *ytbR* at 37ºC. It was further demonstrated that there is reciprocal regulation by QS. These observations were extended by phenotypic assays showing that NagC represses the aggregation phenotype and may affect T3S and motility by regulating QS. In contrast the biofilm phenotype appears to be directly regulated by NagC. Given that QS promotes biofilm formation, the upregulation of *ypsI* and *ytbI* in a *nagC* mutant should not lead to reduction of biofilm. However, biofilm formation in the *nagC* mutant was attenuated and addition of 5 mM GlcNAc could restore this phenotype, suggesting that attenuation of biofilm formation is caused by an enhanced degradation of external GlcNAc by the *nag*-operon.

Addition of GlcNAc or *N*-acetylmuramic acid to strains containing QS gene promoter *lux*-fusions repressed QS gene expression, revealing that *Y. pseudotuberculosis* may modify its behaviour in presence of other bacteria by sensing cell wall turnover.

Taken together these results demonstrate that GlcNAc and/or NagC impacts on T3S and injectisome mediated aggregation by regulating QS and that the injectisome of the T3SS is the aggregation factor, which may help and protect the bacteria in the early stages of host infection.

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Abbreviations

Abbreviation	Description
AHL	*N*-acyl-homoserine lactone
AIP	Autoinducing peptide
AI-2	Autoinducer-2
Amp	Ampicillin
bp	Base pair
Ca^{2+}	Calcium
°C	Degrees Celsius
Cm	Chloramphenicol
DAPI	4′,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSF	Diffusible signal molecule
EHEC	Enterohaemorrhagic *Escherichia coli*
g	Gram(s)
g/l	Grams per litre
GFP	Green fluorescence protein
GlcNAc	*N*-acetyl-D-glucosamine
GlcNAc	*N*-acetyl-D-glucosamine-6-phosphate
Gm	Gentamicin
h	Hour
hms	Hemin storage locus
HSL	Homoserine lactone
IM	Inner membrane
kb	Kilobase
kDa	Kilodalton
Km	Kanamycin
kV	Kilovolt
L	Litre

LB	Lysogeny broth
M	Molar concentration
min	Minute
mg	Milligram
ml	Millilitre
mM	Millimolar concentration
MurNAc	*N*-acetylmuramic acid
μM	Micromolar concentration
nM	Nanomolar concentration
μF	Microfaraday
μl	Microlitre
μg	Microgram
Nal	Nalidixic acid
ng	Nanogram
NGM	Nematode growth medium
Ω	Ohm
OD	Optical density
OM	Outer membrane
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	Potenz (power) of hydrogen
PQS	Pseudomonas quinolone signal
pYV	*Yersinia* virulence plasmid
QS	Quorum sensing
RNA	Ribonucleic acid
RLU/OD_{600}	Relative light units per cell
rpm	Revolutions per minute
s	Second
SAM	*S*-adenosylmethionine
SDS	Sodium dodecyl sulphate
Sm	Streptomycin

Spp.	Species
TAE	Tris-acetate-EDTA buffer
Tc	Tetracycline
TCA	Trichloroacetic acid
Tm	Trimethoprim
Tris	Tris hydroxymethyl aminomethane
T3SS	Type-III-secretion system
V	Volts
v/v	Volume to volume
WGA	Wheat-germ-agglutinin
w/v	Weight to volume
Yop	Yersinia outer protein
YpIII	*Yersinia pseudotuberculosis III* strain
%	Percent
x	Times
x *g*	Times gravitational force

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1 Introduction

1.1 Bacterial Communication is Wide-spread

The first person who wondered if bacteria work in communities rather than as single cells was Erwin Frink Smith saying "a multiple of bacteria are stronger than a few" (Smith, 1905). The proof that bacteria communicate was discovered in the middle of the last century when it was reported that *Pneumococcus* and *Streptococcus* spp. release 'hormone'-like molecules into their environment at a certain phase of growth, making them more competent to be transformed (Pakula and Walczak, 1963, Tomasz, 1965). Nealson then used the term 'autoinduction' after the discovery that *Vibrio fischeri* could be induced to luminesce when cells in the lag-phase were mixed with the supernatant of cells from the later exponential phase of growth (Nealson *et al.*, 1970). 10 years after this discovery the molecule responsible for bioluminescence was found to be *N*-(3-oxo-hexanoyl)homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981). After 10 more years of further investigation, it was also found that other bacteria also produce acyl-homoserine lactones (AHLs) and that their use is widespread (Bainton *et al.*, 1992, Swift *et al.*, 1993). 13 years later, following a lot of research into this topic, Fuqua *et al.*, 1994 established the term 'quorum sensing'. Quorum sensing (QS) summarised the then known findings on bacterial communication and described it as a mechanism important for adaptation to environmental changes in which a population of bacteria can modify gene expression after a certain population density is reached. This autoinducing system works by the release of signal molecules which will be received by surrounding bacteria and in turn when a specific concentration of

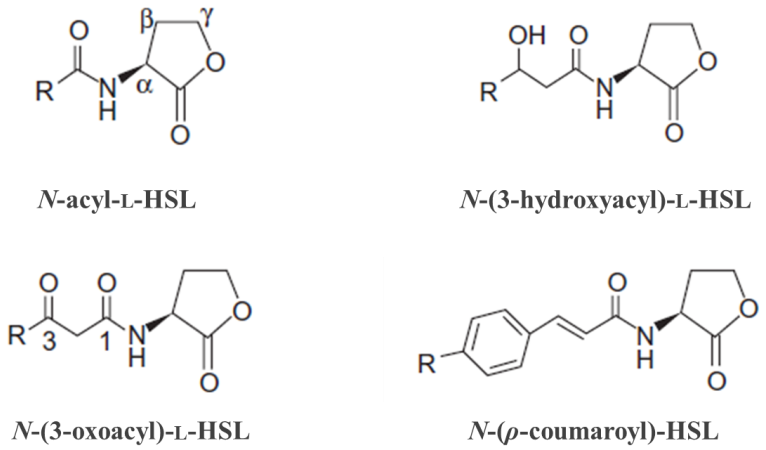


Figure 1.1: Structures of the most common AHLs produced by Gram negative bacteria. The figure was adapted from (Atkinson and Williams, 2009).

signal molecule is sensed by the population, each bacterium will change its behaviour. Usually these systems work by having response regulators that bind the signal directly and induce a signal cascade which will impact on gene expression (Fuqua *et al.*, 1994).

A number of these autoinducing molecules were found to be AHLs which vary in their side-chain length, saturation and oxidation state. The side-chain length varies between 4 and 18 C-atoms. Usually AHLs contain an even number of C-atoms but in rare cases an odd number can also be present. In addition, AHLs belong to the *N*-acyl, *N*-(3-oxoacyl) or *N*-(3-hydroxyacyl) classes of compounds (Williams *et al.*, 2007, Gray *et al.*, 1996, Horng *et al.*, 2002, Chhabra *et al.*, 2005)(Figure 1.1). The substrate for the AHL synthesis is *S*-adenosylmethionine (SAM) and together with an acyl-acyl carrier protein (acyl-ACP, or also acyl-CoA) it forms a homoserine-lactone (HSL) ring with an acyl group (Val and Cronan, 1998, Jiang *et al.*, 1998). This occurs by the binding of the *N*-acyl fatty acid at the α-position

of the HSL ring, which is unsubstituted at the *β*- and *γ*-positions [reviewed by (Williams *et al.*, 2007)]. The first synthase described was LuxI of *V. fischeri* (Fuqua *et al.*, 1994) The gene for the synthase is often located next to the gene coding for the QS response regulator LuxR, and both genes can be transcribed in opposite directions, in the same direction and either divergently or convergently (Fuqua *et al.*, 1994, Fuqua *et al.*, 1996). Generally, an AHL synthase produces the autoinducers which will be released into the cell's surrounding environment and will be taken up by the bacterial cell. The more molecules that are in the environment, the more will be taken up by the bacterial cell to bind to the promoters of target genes so activating or repressing the gene, leading to a change of phenotype/behavior (Williams, 2007)(Figure 1.2).

There are several different LuxI and LuxR homologues found in Gram negative bacteria, such as CepI/R of *Burkholderia cepacia*, producing molecules that

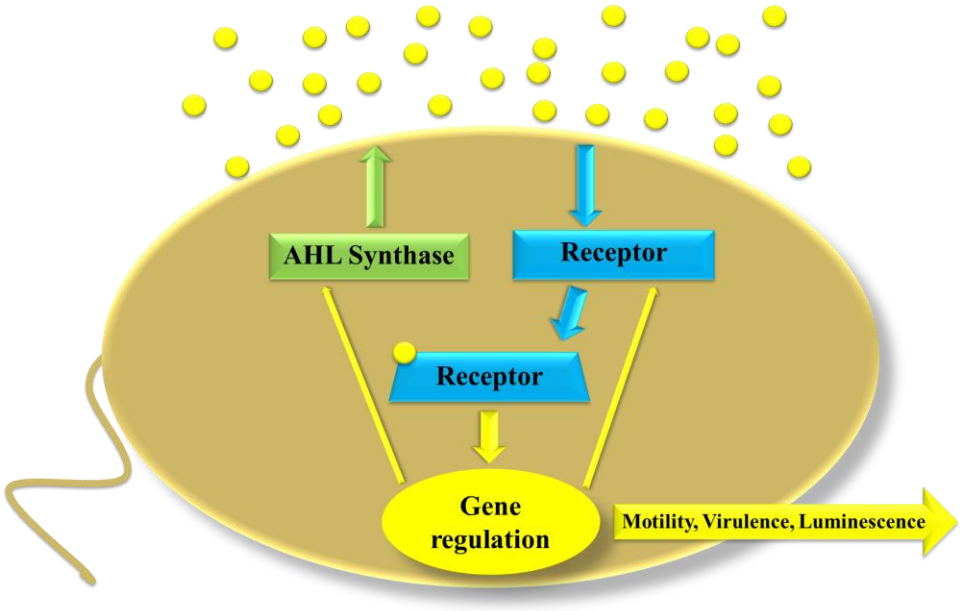


Figure 1.2: Simplified presentation of AHL mediated QS in Gram negative bacteria.

regulate virulence, and CviI/R of *Chromobacterium violaceum* important for the production of exoenzymes, cyanide and pigments (Lewenza *et al.*, 1999, McClean *et al.*, 1997, Williams *et al.*, 2007).

Some species might even have more than one homologous locus in their chromosome, such as *Pseudomonas aeruginosa* possessing *lasI/R* and *rhlI/R* which function hierarchically and regulate biofilm formation, cyanide and exoenzyme production and secretion (Latifi *et al.*, 1996, Williams *et al.*, 2007) or the pathogen *Burkholderia mallei* which was found to have two QS loci named *bmaR1/I* and *bmaR3/I3* important for virulence (Ulrich *et al.*, 2004). Interestingly, there are also orphan LuxR-type transcriptional regulators found in various species which do not act together with a synthase. It is assumed that bacteria which have just one orphan regulator and no other QS locus use those to sense the AHL signal molecules of other bacteria (Williams, 2007). This was shown for an enterohaemorrhagic *Escherichia coli* (strain O157:H7), since its LuxR homologue SdiA is a negative regulator for virulence and allows the bacteria to colonise humans (Kanamaru *et al.*, 2000) and the addition of AHLs caused activation of SdiA by a conformational change of this receptor (Yao *et al.*, 2006). In addition, other species possess both functioning QS genes and additional orphan receptors, such as *Sinorhizobium meliloti* which possesses the *luxR/I* loci *sinR/I* and *traR/M* and the one orphan *expR*, encoding for ExpR, which was shown to be important for the production of extracellular polysaccharides that are important for symbiosis (Marketon and González, 2002, Pellock *et al.*, 2002). However there is no evidence for orphan AHL synthases in bacterial genomes.

As mentioned above, there are several structurally different AHLs. *V. fischeri*, *P. aeruginosa*, *Serratia marcescens* and *Yersinia pseudotuberculosis* for example all produce a 3-oxo-C6-HSL but also others such as 3-hydoxy-C6-HSL and C6-HSL (Pearson *et al.*, 1994, Horng *et al.*, 2002, Ortori *et al.*, 2007). For species that possess more than one QS locus it has been shown that each AHL synthase might produce the same or different AHL signal molecules and the response regulator might just respond to signal molecules produced by its counterpart (Latifi *et al.*, 1996, Ulrich *et al.*, 2004).

However there are other AHLs which do not possess fatty-acyl side chains such as *p*-coumaroyl-HSL. Those are produced by acyl-HSL synthases which are recognised by a receptor homologous to fatty acyl-HSL receptors, and can be found in *Rhodopseudomonas palustris* or *Bradyrhizobium* spp. (Hirakawa and Tomita, 2012, Schaefer *et al.*, 2008)(Figure 1.1).

1.2 Other QS Systems of Gram Negative Bacteria

Other Gram negative bacterial QS signalling systems are also present in addition to the AHL-based systems and one example is the PQS signalling system produced by *P. aeruginosa*. PQS is the quinolone signal of *P. aeruginosa* belonging to the family of 2-alkyl-4-quinolones (Lépine et al., 2004) synthesised *via* the *pqsABCDE* operon (Gallagher et al., 2002). For biosynthesis of PQS, cells firstly produce and release the signal molecule 2-heptyl-4-quinolone (HHQ) which is taken up by neighbouring cells and subsequently converted to PQS by PqsH

(Déziel *et al.*, 2004). The PQS and AHL systems are linked by the activity of LasR on *pqsH* expression (Gallagher *et al.*, 2002). PQS induces the production of LecA and LecB (galactophilic lectin), pyocyanin and elastase important for biofilm formation and virulence and rhamnolipids that are important surfactants of *P. aeruginosa* (Diggle *et al.*, 2006, Winzer *et al.*, 2000, Jarvis and Johnson, 1949). Another QS signal molecule, 3-OH PAME (3-hydroxypalmitic acid methyl ester), is produced by *Ralstonia solanacearum* and its role in autoregulation was discovered in the middle of the 1990s by Flavier *et al.*, 1997a and was shown to be important for regulation of virulence regulation on plants. Linked to the species-specific 3-OH PAME pathway is the AHL mediated pathway SolI/R and the orphan AidA. It was further shown that there is a hierarchy between the two QS systems since 3-OH PAME regulates the expression of the AHL mediated pathway (Flavier *et al.*, 1997).

Vibrio harveyi even has three parallel working QS systems, two of which are also found in the human pathogen *Vibrio cholerae* (Miller et al., 2002). The first system, solely found in *V. harveyi*, produces the 3-hydroxy-octanoyl-HSL HAI-1 (*harveyi* autoinducer 1, AI-1) with the synthase LuxM (Bassler *et al.*, 1997). AI-1 is transduced by LuxN which activates the bioluminescence repressor LuxO (Bassler *et al.*, 1994). At low cell density LuxO also downregulates the expression of *luxR* (Lenz *et al.*, 2004). A second system produces the non-borate containing precursor of 3A-methyl-5,6-dihydro-furo(2,3D)(1,3,2)dioxaborole-2,2,6,6A-tetraol (autoinducer 2, AI-2) *via* the LuxS synthase, which is bound by the LuxP/LuxQ receptor inducing a complex system required for regulation of several phenotypes

together with the other QS systems (Surette *et al.*, 1999, Chen *et al.*, 2002, Williams *et al.*, 2007). Which are important for bioluminescence (Bassler *et al.*, 1993) and type-III-secretion (Henke and Bassler, 2004). The third system of *V. harveyi* acts parallel to the other two QS systems and regulates the same phenotypes. The autoinducer here is CAI-1 (*cholerae* autoinducer-1) which is produced by the synthase CqsA (Henke and Bassler, 2004).

Homologous autoinducer-2 (AI-2) molecules are produced by several bacterial species and have been proposed to be used as an intra- and inter-species communication system between Gram negative and Gram positive bacteria as *luxS* was found in over 60 species (Williams *et al.*, 2007, Vendeville *et al.*, 2005). AI-2 is a diffusible molecule, which is produced by LuxS cleaving *S*-ribosyl-L-homocysteine resulting in the AI-2 precursor, 4,5-dihydroxy-2,3-pentanedione (DPD) (Chen *et al.*, 2002). The receptors of bacteria other than *Vibrio* sp., e.g. *Enterobacteriaceae* and *Bacillaceae* use an ABC-transporter (Lsr receptor complex) for uptake of AI-2 (Xavier and Bassler, 2005, Auger *et al.*, 2006). However several studies showed that although *luxS* can be found in various genomes it may not act as a signal molecule synthase in all of them since it also plays an enzymatic role in the activated methyl cycle which accounts for the regeneration of the methyl donor *S*-adenosylmethionine (Winzer *et al.*, 2003) the major substrate for AHL synthases (Val and Cronan, 1998). This might be a reason for metabolic changes in the bacteria which are not due to AI-2 signalling. This hypothesis was underpinned by a genomic study demonstrating that not all bacteria possessed AI-2 receptors and proposed that phenotypic changes that

occurred in *luxS* mutants without known AI-2 receptors may be caused by changes in sulfur metabolism and that LuxS signalling is reserved for *Vibrionales* and pathogenic bacteria possessing an Lsr-receptor (Rezzonico and Duffy, 2008, Rezzonico *et al.*, 2012).

DFS ('diffusible factor'; methyl dodecenoic acid) was firstly described in *Xanthomonas* sp. (Boon *et al.*, 2007) and is another factor which is produced by several species of Gram negative bacteria such as *Stenotrophomonas maltophilia* and *P. aeruginosa* which can induce each other's DSF-regulated QS systems (Fouhy *et al.*, 2007, Nadal Jimenez *et al.*, 2012). DSF diffuses passively through cell membranes and was shown to regulate virulence systems, cyclic glycan and biofilm dispersal in *Xanthomonas* (Dow *et al.*, 2003, Vojnov *et al.*, 2001) and motility and cell aggregation in *S. maltophila* (Fouhy et al., 2007). It is produced by a long-chain fatty acyl coenzyme A ligase and an enoyl-CoA hydratase-like enzyme (Barber *et al.*, 1997). Homologues of DSF were shown to also take part in cross-species, even cross-kingdom signalling. This was shown by the dispersion of biofilms of *P. aeruginosa*, other Gram negatives, Gram positive bacteria and of biofilms of the yeast *Candida albicans* when incubated with *cis*-2-decenoic acid of *P. aeruginosa*. In addition BDSF produced by *B. cepacia* also disperses biofilms of *P. aeruginosa* and other species (Davies and Marques, 2009).

1.3 Signalling in Gram Positive Bacteria

Most Gram positive signal molecules are autoinducing peptides (AIPs) mediated by a two-component regulatory system using a membrane-located receptor histidine protein kinase and an intracellular response regulator as a signal transduction system (Parkinson, 1995, Grebe and Stock, 1999). These were first described for *Lactococcus lactis* and *Streptococcus pneumoniae* (Kuipers *et al.*, 1995, Håvarstein *et al.*, 1995). AIP precursors are synthesised on the ribosome and will be post-translationally modified to the functional AIP while being transported out of the cell (Sturme *et al.*, 2002). Signal transduction takes place after the release of AIPs into the environment by ABC exporters and AIPs activate the receptor kinases after reaching a certain threshold concentration in the area surrounding the cell. On binding the AIP, the receptor kinase auto-phosphorylates and transfers the phosphate to the response regulator leading to upregulation of a feedback loop which in turn activates the autoinducer, the genes for the regulatory two-component system and often the ABC exporter genes (Sturme *et al.*, 2002).

In *Staphylococcus aureus* (Saenz et al., 2000) AgrB processes AgrD to generate the AIP which activates the sensor kinase AgrC which autophosphorylates and then transfers the phosphate to the response regulator AgrA (Zhang et al., 2002, Zhang et al., 2004). The *agr*-operon is induced by the binding of phosphorylated AgrA to the the *agrP2* promoter so inducing the *agrACDB* operon and autoamplifying AIP production as well as the *agrP3* promoter. This drives the regulatory RNA RNAIII, the effector of the agr response affecting for example exotoxin production and metabolic genes [reviewed in (Atkinson and Williams,

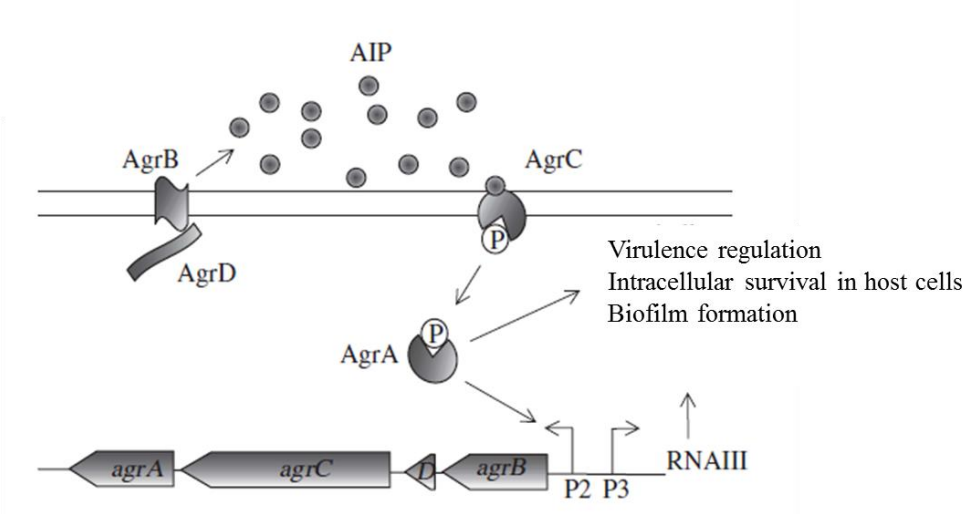


Figure 1.3: Regulation of AIP mediated QS of *S. aureus* encoded in the *agr*-operon (RNAII) and RNAIII system. The figure was adapted from (Atkinson and Williams, 2009).

2009)] (Figure 1.3). However, AgrA also binds directly to other promoters, such as the promoters of genes encoding for phenol-soluble modulins important for immune invasion (Queck *et al.*, 2008).

Homologues of the *agr* system can be found in several Gram positive bacteria such as *Clostridium perfrin*gens, *Clostridium acetobutylicum*, *Clostridium botulinum* and *Listeria monocytogenes* and regulate e.g. sporulation, granulose formation, toxin production and virulence (Steiner *et al.*, 2012, Williams *et al.*, 2005). Another system similar to the *agr* system is the *fsr* system of *Enterococcus faecalis*, which uses the cyclic lactone-peptide GBAP (gelatinase biosynthesis-activating pheromone) as a signal molecule that is produced and transduced by proteins encoded on the *frs*-operon. This QS system regulates gelatinase serine protease production important for virulence of *E. faecalis* but does not have a RNAIII unit, but instead has a unit encoding the gelatinase gene (*gelE*) and the

serine protease (*sprE*) (Nakayama *et al.*, 2001, Sturme *et al.*, 2002, Qin *et al.*, 2001).

Another QS system of Gram positive bacteria employs response regulators of the RNPP family (Rap, NprR, PlcR, and PrgX) (Novick *et al.*, 1995, Ohtani *et al.*, 2009, Rocha-Estrada *et al.*, 2010), which has been well-studied in *Bacillus subtilis*, *E. faecalis* and *Bacillius cereus* (Pottathil and Lazazzera, 2003, Gohar et al., 2008, Rocha-Estrada et al., 2010). In this case the AIPs bind directly to the response regulator of the recipient cell (Rocha-Estrada *et al.*, 2010). Furthermore, cassettes encoding for export proteins, signal molecules and response regulators can be found in several copies in the genome, e.g. the genome of *B. subtilis* harbours seven of the *rap-phr* cassettes (Pottathil and Lazazzera, 2003). In addition Rap proteins and the histidine kinases pathway of *B. subtilis* can work in opposition, as demonstrated for sporulation (Jiang *et al.*, 2000).

In contrast to the signalling peptides, the A-factor (2-isocapryloyl-3-hydroxymethyl-*γ*-butyrolactone) of *Streptomyces* sp. is a diffusible molecule built on a lactone backbone. Generally A-factor and various homologues are produced by enzymes AfsA and BprA *via* an 8-methyl-3-oxononanoyl-acyl carrier protein and dihydroxyacetone phosphate and is sensed by AdpA which regulates secondary metabolism and morphogenesis in *Streptomyces* sp. (Kato *et al.*, 2007, Ohnishi *et al.*, 2005).

1.4 Inhibition of Quorum Sensing as Antibacterial Target

Many of the bacterial species mentioned in the previous sections use their QS system for regulation of virulence. As the demand for novel antibacterial treatments is high due to pathogenic species becoming more resistant to established antibiotics and quickly gaining resistance to novel antibiotics, other approaches are needed to overcome this danger towards chronic and lethal infections which can be community or hospital acquired (WHO, 2014). However, research on QS of various species can help to develop new approaches against their virulence. There are natural compounds synthesised by all life forms that can inhibit bacterial QS as well as synthetic compounds produced to target specific bacteria (Kalia, 2013). Inhibition mechanisms attempt to interrupt or degrade the QS signal molecules or inhibit the activity of the synthase or receptor. For example synthetic compounds were synthesised that mimic the structure of the signal molecules and can directly inhibit QS (Kalia and Purohit, 2011, Rasmussen and Givskov, 2006). The criteria for a successful QS inhibitor in the case of AHL mediated QS are: The inhibitor must be a small molecule which efficiently reduces QS-regulated gene expression. It should be at least as long as the native AHL signal; however molecules with 1-2 more C-atoms than the native AHL were found to inhibit AHL signalling more efficiently. Furthermore the QS inhibitor should be highly specific for a QS regulator with no adverse effect on the bacteria or host and it must be stable and resistant to degradation (Hentzer and Givskov, 2003, Kalia, 2013). Some bacteria have both AHL production and degradation activities, such as the marine bacterium *Shewanella* sp. which degrades its own

long chain AHLs in stationary phase using AHL specific degrading enzymes (Tait *et al.*, 2009). Furthermore, some bacteria produce acylases which degrade AHLs with different side chain lengths, such as the marine bacteria *Tenacibaculum discolor*, *Halomonas* sp. and *Alteromonas* sp. (Romero *et al.*, 2011).

Antibiotic QS inhibiting compounds are also produced by fungi such as the well-known *Penicillium* spp., and these include patulin and penicillic acid produced by *Penicillium* spp. (Rasmussen *et al.*, 2005).

Some plants and animals also produce compounds which inhibit QS related phenotypes. Extracts of fruits, such as *Ananas comosus* and *Manilcara zapota* reduce pyocyanin and elastase production and biofilm formation of *P. aeruginosa* (Musthafa *et al.*, 2010, Kalia, 2013) and human airway epithelia cells were shown to inactivate the 3-oxo-C12-HSL, produced by *P. aeruginosa* (Stoltz et al., 2007).

1.5 The Three Human Pathogenic *Yersinia* species

The genus *Yersinia* includes eleven species with four being animal pathogens (Figure 1.4) (Chen *et al.*, 2010). Out of those four, three are human pathogens and *Yersinia ruckeri* is a fish pathogen, which causes redmouth disease in rainbow trout (*Oncorhynchus mykiss*) and other salmonids (Ewing *et al.*, 1978). *Y. pseudotuberculosis* and *Yersinia enterocolitica* are enteropathogens and *Yersinia pestis*, the causative agent of plague.

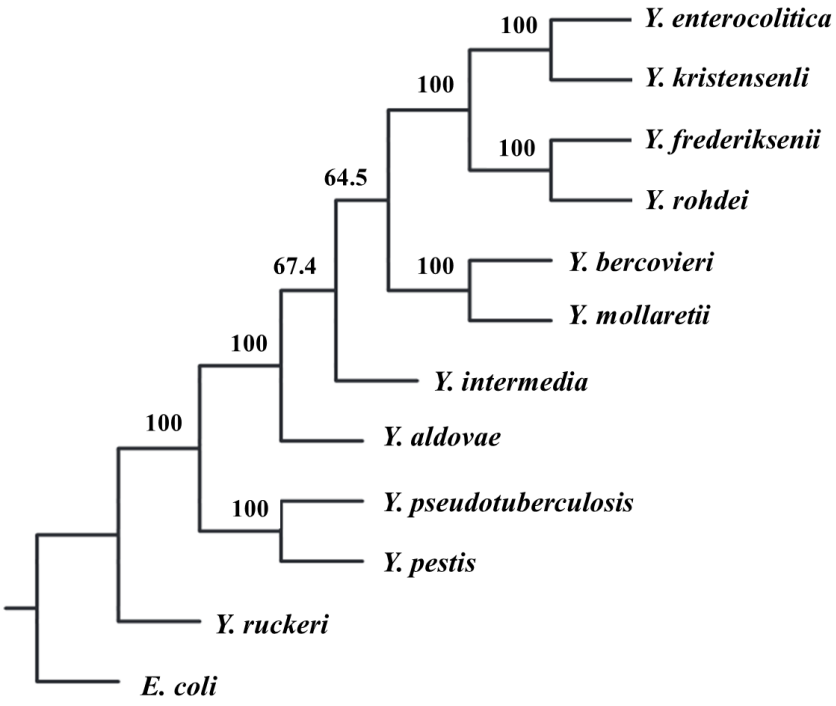


Figure 1.4: Phylogenetic tree of all eleven *Yersinia* species proteomes. The phylogeny was adapted from (Chen *et al.*, 2010) who constructed this tree with the software PHYLIP by conducting a parsimony analysis using 1000 bootstrap replicates.

Y. enterocolitica is relatively distant from the other two human pathogenic *Yersinia* (Kotetishvili *et al.*, 2005), whereas *Y. pestis* and *Y. pseudotuberculosis* are closely related to each other, as *Y. pestis* is derived from *Y. pseudotuberculosis* (Achtman *et al.*, 1999).

Y. enterocolitica and *Y. pseudotuberculosis* are transmitted *via* the faecal-oral route. Since they can infect other mammals, either domestic or free-living, *Y. enterocolitica* infections often occur after consumption of undercooked pork meat and *Y. pseudotuberculosis* is found on vegetables such as carrots and lettuce where illness is caused if the food has not been properly washed or cooked [reviewed in (Heroven and Dersch, 2014)]. Infections caused by *Y. enterocolitica*

and *Y. pseudotuberculosis* are in most cases not lethal. An infection by either of these two bacteria is called yersiniosis and starts from the gut, where the pathogens invade the lymph system. In the majority of cases, yersiniosis results in fever, and gut-associated symptoms such as diarrhoea and abdominal pain for 1-3 weeks. In rare cases, infections can also result in complications such as adenitis and/or terminal ileitis, reactive arthritis and septicaemia (Butler, 1994, Koornhof *et al.*, 1999).

Y. pestis evolved from *Y. pseudotuberculosis* 1,500 years - 20,000 years ago by acquisition of chromosomal and extrachromosomal DNA, degeneration of the chromosome by mutations and by deletions and frameshifts (Derbise *et al.*, 2010, Skurnik *et al.*, 2000, Achtman *et al.*, 1999). In contrast to *Y. pseudotuberculosis*, *Y. pestis* needs a vector to be transported and to get through the host's skin. The most common vectors are human fleas which distribute the bacteria between rodents and humans [reviewed in (Brubaker, 1991)]. *Y. pestis* led to the death of millions of people during three recorded pandemics (Justinian's plague: 541-767 AD, the Black Death: 1346 AD - early 19th century, Modern Plague: start 1894 in China – early 20th century) and is still causing the death of many people every year (Perry and Fetherston, 1997, Silver, 2015, Riehm *et al.*, 2015, Ge *et al.*, 2015, Achtman *et al.*, 1999). There are three forms of plague: bubonic, septicaemic and pneumonic plague [reviewed in (Perry and Fetherston, 1997)]. The form of plague which mostly occurred during the three pandemics was bubonic plague which causes tender and swollen lymph nodes, often referred to as buboes. In this form of plague, bacteria are distributed throughout the lymph

system and it is often seen together with septicaemic plague, in which bacteria are distributed in the patient's blood (Perry and Fetherston, 1997, Butler *et al.*, 1976). The third and most deadly form of plague is the pneumonic plague. In rare cases (2% in the U.S.) the bacteria are spread between hosts, which are often cats and humans, by respiratory droplets and directly infect the respiratory system (Doll *et al.*, 1994, Perry and Fetherston, 1997). However, patients who develop secondary plague septicaemia and pneumonia and who are not treated with antibiotics usually do not survive the infection (Crook and Tempest, 1992, Perry and Fetherston, 1997). Recently it has been suggested that the acquisition of the Pla protease together with the virulence plasmid pPCP1 made the bacterium able to infect mammals and causes pneumonic plaque, with a further amino acid change in Pla enabling the bacterium to infect the lymph system and cause the bubonic form of the disease (Zimbler *et al.*, 2015).

1.6 Virulence of *Y. pseudotuberculosis*

Once ingested *via* the faecal-oral route *Y. pseudotuberculosis* invades M-cells (microfold cells) of the outer layer of Peyer's patches (Clark *et al.*, 1998). These cells normally transport bacteria in vesicles to antigen-presenting cells that are located inside the gut-associated lymphoid tissues. Peyer's patches belong to these tissues and are lymphoid follicles similar to lymph nodes located in the mucosa and submucosa of the small intestine in mammals. These small lymphoid nodes mostly contain B-cells (Nagler-Anderson, 2001). However, *Yersinia* are not

enclosed by a vesicle when transported into the Peyer's patches instead they produce an outer membrane protein, termed invasin, which is able to attach to β1-integrins on the surface of M-cells promoting transfer into the lymphatic system (Marra and Isberg, 1997, Autenrieth and Firsching, 1996, Pepe and Miller, 1993). Expression of *inv* is mediated by the transcriptional activator RovA which itself is negatively regulated by the virulence regulator YmoA (Nagel *et al.*, 2001, Ellison *et al.*, 2003). *Y. pseudotuberculosis* also possesses other adhesion factors. YadA, the gene for which is located on the virulence plasmid pYV, is expressed at 37°C. YadA is important for attachment independent of *inv* and is activated in the absence of invasin (Eitel and Dersch, 2002). A recent study showed that YadA of *Y. enterocolitica* can bind to several surface proteins, such as β_1-chain integrins and αV integrins and mediates tight binding to the host cells (Keller *et al.*, 2015, Marra and Isberg, 1997). A third adhesin of *Y. pseudotuberculosis* is the pH6 antigen which is expressed at 37°C under acidic conditions and was shown to facilitate attachment to cultured mammalian cells in the absence of both *inv* and the virulence plasmid (Yang and Isberg, 1997).

1.7 The *Yersinia* Type-III-Secretion System

To enter the host's lymph system, the pathogenic *Yersinia* species possess a type-III-secretion system (T3SS). This system is encoded on a virulence plasmid (pYV), consisting of collections of genes and regulators which encode for the membrane-bound structure of an injectisome which translocates the plasmid

encoded *Yersinia*-outer-protein (Yop) effectors into host cells, especially macrophages to interfere with their actin cytoskeleton dynamics preventing phagocytosis and causing apoptosis (Galán and Collmer, 1999, Cornelis *et al.*, 1998). By circumventing the immune response, the T3SS allows *Y. pseudotuberculosis* to disseminate through the intestinal lymphoid follicles to spread around the body and after 24 h the bacteria can be found in the mesenteric lymph nodes [reviewed in (Barnes *et al.*, 2006)].

The assembly of the injectisome starts by production of the peptidoglycan embedded outer membrane secretin YscC on which YscD and YscJ discretely attach. The inner membrane export apparatus is composed of the multimeric ring-

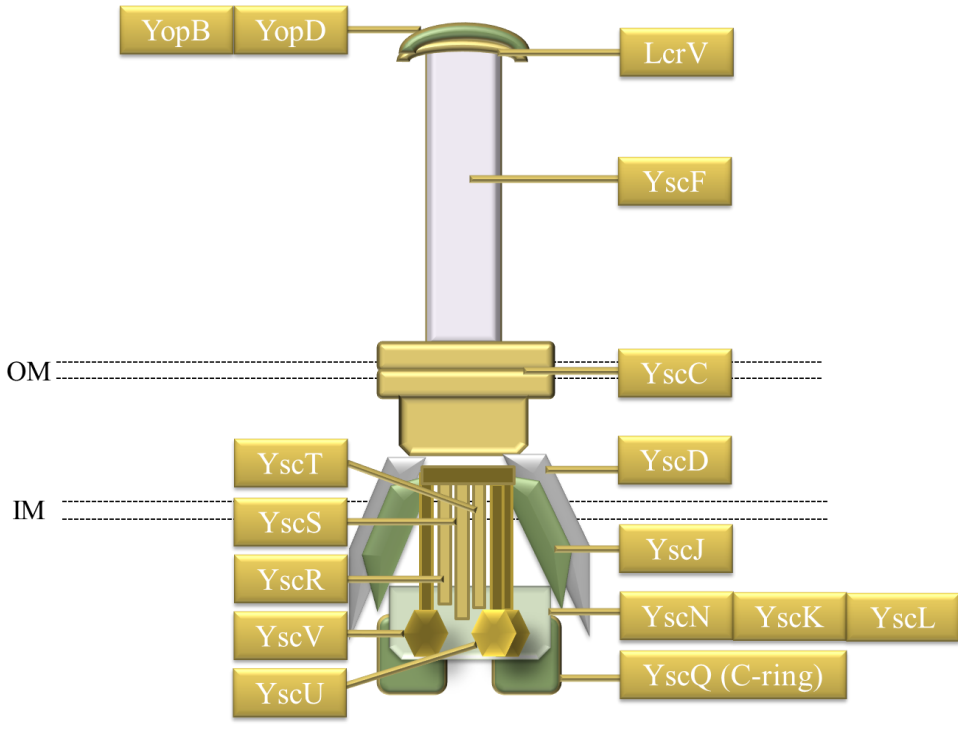


Figure 1.5: The assembled injectisome of *Y. pseudotuberculosis.* The figure was adapted from (Diepold *et al.*, 2011, Dewoody *et al.*, 2013).

structured YscV, the oligomerisation of which is promoted by YscR, YscT and YscS, and the transmembrane protein YscU (Diepold *et al.*, 2011). The export apparatus links to YscJ, YscD and the ATPase complex formed by YscN, YscK and YscL which are important in chaperone removal and necessary for a functional injectisome (Akeda and Galan, 2005, Bozue *et al.*, 2012, Diepold *et al.*, 2010, Diepold *et al.*, 2011). The needle itself is a hollow tube which is assembled by helical polymerisation of approximately 150 copies of YscF (Deane *et al.*, 2006). The needle length is determined by YscP which is stretched during injectisome assembly and when it reaches its full length, needle building YscF proteins will not be further exported and YscP will be shed (Journet *et al.*, 2003). The tip of the injectisome is formed by YopB and YopD together with LcrV, enabling the bacteria to form a pore in the host cell membrane to release effector Yops (Deane *et al.*, 2006, Mueller *et al.*, 2005) (Figure 1.5).

Y. pseudotuberculosis synthesises six effector Yops which are released into the host cell after attachment to host cells (Cornelis *et al.*, 1998, Cornelis and Wolf-Watz, 1997). Several Yop effectors are bound to chaperones in the cytosol which keep Yops in an inactive state and aids translocation *via* the injectisome (Cornelis et al., 1998).

YopE is a GTPase activating protein that is involved in the disruption of actin microfilaments in the target cell by the introduction of an ‘arginine finger’ motif into the GTPases catalytic site, which results in efficient GTP hydrolysis and deactivation of GTPases (Black and Bliska, 2000, Cornelis and Wolf-Watz, 1997). Its chaperon is SycE which is encoded in the region upstream of YopE (Forsberg

and Wolf-Watz, 1990). YopM has the structure of a scaffolding protein due to its 20 leucine-rich-repeats and is horseshoe shaped forming a hollow cylinder when 4 monomers attach. Although its function is not clearly understood, it has recently been suggested that YopM has multiple functions which are important for virulence of *Yersinia* such as colonisation and dissemination within the host (Höfling *et al.,* 2015). Furthermore, YopM interacts with proteins inside the host cell suggesting an anti-inflammatory function (Cornelis and Wolf-Watz, 1997, Höfling *et al.*, 2015). YopM does not require a chaperone for its release (Trulzsch *et al.,* 2003). YopO (YpkA) is a multi-domain protein with kinase domains located at the cytosol side of the host cell plasma membrane after injection. It was recently shown to bind actin in a complex to phosphorylate it for inhibition of actin polymerisation and to dysregulate host-actin regulating proteins leading to inhibition of phagocytosis (Lee *et al.*, 2015, Cornelis and Wolf-Watz, 1997). The activity of YopO is mediated by the chaperone SycO (Letzelter *et al.*, 2006). YopJ is a serine/threonine acetyl-transferase associated with increased death of the host cell by blocking MAP kinase pathways, especially by inhibiting of dTAK1, the critical MAP3 kinase of the innate immune NF-κB signalling pathway (Cornelis and Wolf-Watz, 1997, Paquette *et al.*, 2012). YopH is a tyrosine phosphatase, which is believed to have originated from eukaryotes and takes part in inactivation of host cell signal transduction pathways by dephosphorylation of several proteins, leading, for example, to an inhibition of the neutrophil calcium response. YopH was also shown to take part in disruption of actin-filaments by targeting the Rho GTPase and taken together with its phosphatase function inhibits phagocytosis

(Trosky *et al.*, 2008, Rolan *et al.*, 2013). YopH activity is mediated by SycH (Wattiau *et al.*, 1994). YopT is a cysteine protease which also inhibits phagocytosis by targeting the Rho GTPase (Aepfelbacher *et al.*, 2005), a mechanism in which YopT cleaves the posttranslationally modified Rho GTPase at its C-terminus and releases it from the cell membrane, leading to a disruption of the actin skeleton (Shao *et al.*, 2002). SycT is the chaperone important for release of YopT (Iriarte and Cornelis, 1998).

In general, T3SSs are not confined to *Yersinia* spp. but are also found in many other gram-negative bacteria such as *Salmonella enterica* spp., *Shigella* spp., enteropathogenic *E. coli* and *Pseudomonas* spp. [reviewed in (Mota and Cornelis, 2005, Diepold *et al.*, 2011)]. Furthermore, the T3SS injectisome has the same origin as the flagella apparatus since both appear to have the same basic structures (Macnab, 2003).

1.7.1 Regulation of the T3SS

The T3SS is temperature and Ca^{2+} dependent *in vitro*. For expression of the injectisome, a temperature of 37ºC is required and for the release of Yops a low amount of Ca^{2+} in the medium is necessary, a phenomenon referred to as the 'low-calcium response' (Perry and Brubaker, 1987, Cornelis, 1998).

Several regulators are known to be important for the activation of the T3SS in *Yersinia*. LcrF (VirF for *Y. enterocolitica*) is a virulence regulatory transcription

factor which belongs to the MAR proteins (multiple adaptational response) of the AraC transcription factor family and is pYV encoded (Garrity-Ryan *et al.*, 2010, Gallegos *et al.*, 1997). Inactivation of *lcrF* results in avirulence in a murine model (Flashner *et al.*, 2004) which is due to the fact that LcrF is a transcriptional activator of *ysc* and *yop* genes at 37°C (Cornelis and Wolf-Watz, 1997). It was further shown that *lcrF* expression is higher at 37ºC, due to its Shine-Dalgarno sequence being inside a stem-loop which is more stable at 37ºC (Hoe and Goguen, 1993). It was proposed that pYV has a conformation which represses virulence gene expression at lower temperatures *via* DNA bending stabilised with histone-like proteins. At 37ºC the bends will melt and the structure will collapse. This change in formation is believed to promote formation of the *lcrF* promoter, leading to expression of Yops (Rohde et al., 1999). Several studies showed that *lcrF* is induced at 37°C by the temperature regulated protein YmoA which works in combination with an intergenic RNA thermosensor (Böhme *et al.*, 2012). However, *virF* expression is also controlled by a protein that displays 79 % identity with the *E. coli* iron-sulfur cluster regulator, IscR (Miller *et al.*, 2014) and RcsB, a response regulator of the Rcs phosphorelay system (Li *et al.*, 2015). YmoA (*Yersinia* modulator A) belongs to the family of Hha (haemolysin expression regulating) proteins which are similar to H-NS, a histone-like DNA binding protein responsible for transcriptional repression and genome organisation (Cruz *et al.*, 1992, Ellison and Miller, 2006, Falconi *et al.*, 1993). It was recently shown that YmoA is important for virulence in *Yersinia* by binding to the upstream region of *lcrF*, in the 5'-UTR of *yscW*, causing a blockage of *lcrF*

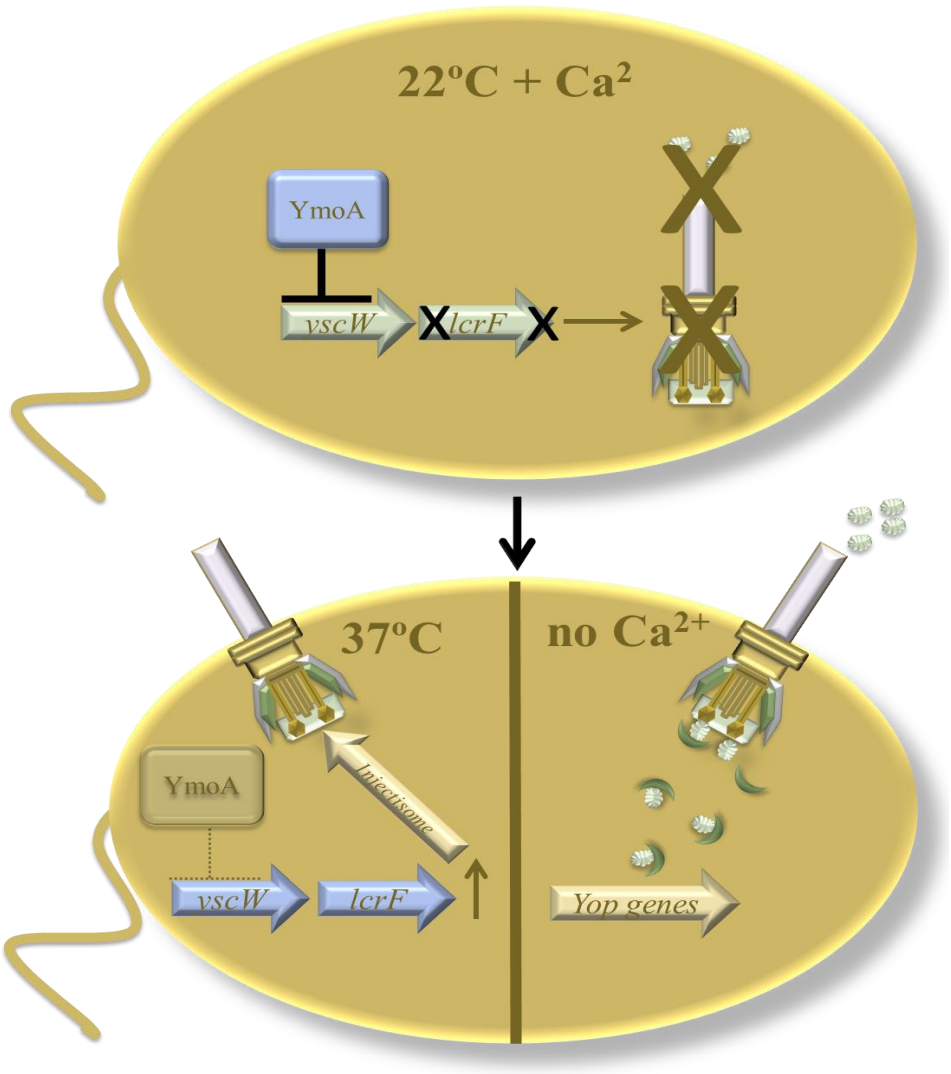


Figure 1.6: Simplified illustration of regulation of the T3SS. Expression of the T3SS is repressed by YmoA which blocks the expression of the T3SS regulator LcrF at 22ºC. A temperature shift to 37ºC causes degradation of YmoA which enables the expression of *lcrF* and leads further to expression of the genes encoding for the T3SS. A lack of Ca^{2+} in the environment then switches on the release of Yops *via* the injectisome.

translation. This is due to occurrence of a temperature shift upon entering the host which will slowly degrade YmoA with the help of ClpP and Lon proteases and leads to the transcription of *lcrF* for activation of the virulence system (Böhme *et al.*, 2012, Jackson *et al.*, 2004). Furthermore, in *Y. enterocolitica* YmoA was found to regulate *inv* expression and also controls production of the YadA adhesin at 37ºC (Ellison *et al.*, 2003, Madrid *et al.*, 2007). In addition, activation of the T3SS of *Y. pestis* is also dependent on other chromosomally encoded genes. Using Mu-transposon mutagenesis in the *Y. pestis* chromosome, several genes including a gene for the LPS biosynthesis (*rfaL*) and genes important for the synthesis and integrity of the cell membrane (*pgsA, pssA*) were found which stop the bacteria from exporting Yops (Houppert *et al.*, 2012).

1.8 Quorum Sensing in *Yersinia* spp.

Pathogenic *Yesinia* species possess QS loci which encode for AHL synthases and LuxR-type response regulators and regulate the expression of virulence associated genes.

Y. enterocolitica 8081 possesses one QS locus, encoding for YenR/I and the orphan receptor YcoR (Throup *et al.*, 1995, Ng, 2010). The synthase YenI produces at least 15 AHLs, including C6-HSL and 3-oxo-C6-HSL (Throup *et al.*, 1995, Atkinson *et al.*, 2006, Ng, 2010). YenI was shown to be important for motility in some strains since mutagenesis of *yenI* delayed swimming and abolished swarming motility (Atkinson *et al.*, 2006). Using microarray-based

transcriptome analysis of *yenI* and *yenR* mutants virulence, plasmid replication and partition genes were found to be regulated by QS (Ng, 2010). A further study implied that adherence is regulated by QS in *Y. enterocolitica* since a lack of AHLs and *yenR* reduced the ability of the bacteria to attach to Caco-2 cells (Grasso, 2013).

Two QS loci (*yspI/R* and *ypeI/R*) are present in the *Y. pestis* genome. YspI is a homologue of the YtbI synthase and YpeI of the YpsI synthase of *Y. pseudotuberculosis* (Kirwan *et al.*, 2006, Atkinson *et al.*, 1999). It was demonstrated that YspI mainly produces 3-oxo-C8-HSL and 3-oxo-C6-HSL and also 3-oxo-C10-HSL, C6-HSL and C8-HSL. The role of YpeI for AHL production has not been clarified since YpeI expressed in *E. coli* showed the same AHL profile as wild type *Y. pestis* (Kirwan et al., 2006). Several studies indicated that QS of *Y. pestis* is important for different phenotypes: One study showed that addition of the C8-HSL and 3-oxo-C8-HSL to *Y. pestis* cultures reduced expression of the virulence factor (LcrV) important for the low-calcium-response for expression of the type-III-secretion system (Gelhaus *et al.*, 2009). Furthermore, deletion of QS genes decreases biofilm formation in *Y. pestis*, suggesting that biofilms are QS regulated (Bobrov *et al.*, 2007). In contrast, another study showed that formation of biofilms on glass surfaces and inside the flea's digestive tract are QS independent (Jarrett *et al.*, 2004).

Since *Y. pestis* evolved from *Y. pseudotuberculosis* and their QS systems are nearly identical, it may be possible that understanding the QS regulated phenotypes of *Y. pseudotuberculosis* may shed light on those of *Y. pestis*.

Y. pseudotuberculosis possesses two QS loci, encoding for YpsR/I and YtbR/I (Atkinson *et al.,* 1999), which were shown to regulate each other since YpsI/R positively regulates its own expression and the expression of *ytbI/R* and YtbI/R positively regulates the expression of *ytbI* and *ypsI/R* suggesting that these QS loci do not function in a hierarchical manner (Patrick, 2010). LC-QqQLIT-MS (liquid chromatography coupled to hybrid quadrupole–linear ion trap mass spectrometry) revealed that 24 AHLs are produced by *Y. pseudotuberculosis* which differ in side chain lengths of C4 to C15 with or without 3-oxo or 3-hydroxy substitutions out of which the 3-oxo-C6-HSL and 3-oxo-C8-HSL are mainly synthesised (Ortori *et al.*, 2007). It was further shown that YtbI is able to produce all detected AHLs whereas YpsI only synthesises the 3-oxo-C6-HSL, C8-HSL and 3-oxo-C7-HSL (Ortori *et al.*, 2007) and that YtbR and YpsR are unable to distinguish AHL molecules but prefer HSLs with an acyl side-length of at least C10 (Patrick, 2010).

1.9 QS Regulated Phenotypes in *Y. pseudotuberculosis*

1.9.1 Flagellar-Mediated Motility

For assembly of the flagella, firstly *flhDC* a class-I master regulator of all flagellar genes is expressed in the cascade for the organelle assembly from the inner membrane to the outer cell surface. Building on the tetrameric structure of FlhDC, Class II gene products are responsible for the assembly of the motor and hook of the flagella. After assembly, the Class-II flagellar-specific RNA polymerase sigma

factor, FliA, activates the Class-III genes. These genes code for the major flagellar structural component, flagellin, that forms a long filamentous propeller (Scott A. Minnich, 2004). *flhDC* expression is regulated by OmpR (Hu *et al.*, 2009), a response regulator for osmoregulation and production of outer membrane proteins (Rudd, 2000), which is also a regulator of the flagellar master operon in many bacterial species (Kim *et al.*, 2003, Shin and Park, 1995).

Early research on QS of *Y. pseudotuberculosis* revealed that flagellar-mediated motility is regulated by both QS loci at 22ºC (Atkinson *et al.*, 1999). Mutants of *ypsI* and *ypsR* show flagellar-mediated swimming motility at 22ºC, suggesting that the YpsI/R system of *Y. pseudotuberculosis* represses swimming motility at 22ºC (Atkinson *et al.*, 1999). A later study confirmed these results by further assays on swim agar plates using mutants of all QS genes and concluded that the YpsI/R system is repressing and the YtbI/R system is activating motility of the wild-type (Atkinson *et al.*, 2008). The same study showed that this is due to regulation of the flagellar master regulon *flhDC* and flagellar specific sigma factor *fliA* by both quorum sensing loci, since it was found that mutation of single QS genes or both synthases and regulators repress *flhDC* but increase *fliA* expression (Atkinson *et al.*, 2008). Furthermore, FlhDC regulates the expression of *fliA* which is a link with the regulatory hierarchy of flagellar assembly (Atkinson *et al.*, 2008). In contrast, for *Y. enterocolitica* it was shown that mutation of *yenI* does not affect *flhDC and fliA* expression but down-regulates expression of *fleB*, the flagellin structural gene (Atkinson *et al.*, 2006). In addition, similar results as for *Y. pseudotuberculosis* were obtained when investigating the links between QS and

flagellar motility in other bacteria. It was found that a deletion of *tofI*, homologous to *luxI*, also decreased expression of *flhC* in the rice pathogen *Burkholderia glumae* (Jang et al., 2014). In contrast to the results obtained for *Y. pseudotuberculosis*, *flhDC* expression is negatively regulated by QS response regulators of the plant pathogen *Erwinia carotovora* subsp. *carotovora* (Chatterjee et al., 2010).

1.9.2 Biofilm Formation on *Caenorhabditis elegans*

Biofilms are bacteria surrounded by an extracellular matrix (ECM) (Costerton, 1995). Generally a biofilm is built after planktonic bacteria settle and attach to a surface where they firstly form a monolayer of cells that then develops into a microcolony due to bacterial cell division. Cells in this microcolony alter gene expression to produce exopolysaccharides which form the extracellular matrix (ECM) of the biofilm which shelters the bacteria from a hostile environment. Some biofilms might grow into mushroom-like structures and when they reach a certain size, bacteria can be released from the biofilm *via* physical detachment or shear forces and become planktonic again and will seek to settle elsewhere [reviewed in (Costerton, 1995) and (Costerton *et al.*, 1999)]. Research on the regulation of biofilm development is very important since several human pathogenic bacteria form biofilms during human infections. These are difficult to treat because biofilms are very resistant to antibiotics, for example due to their surrounding matrix retarding the diffusion of antibiotics to the enclosed cells.

Furthermore bacteria inside the biofilm are often starved or in a slow-growing state which makes them less susceptible to antibiotics (Costerton *et al.*, 1999).

Y. pseudotuberculosis and *Y. pestis* form biofilms on the head, especially around the mouth region of the nematode *Caenorhabditis elegans* (Darby *et al.*, 2002, Joshua *et al.*, 2003). *Y. pestis* also forms biofilms in the proventriculus and gut of fleas (Darby *et al.*, 2002). The ECM of *Yersinia* biofilms mainly consists of *β*-1,6-*N*-acetyl-D-glucosamine (GlcNAc) (Bobrov *et al.*, 2008, Hinnebusch and Erickson, 2008). By using *C. elegans* as model organism it was shown that a mutation of the *hmsHRFS* locus (hemin storage locus) of *Y. pestis* and *Y. pseudotuberculosis* leads to the inability to form biofilms on the mouth region of the nematode (Darby *et al.*, 2002). The *hmsHRFS* locus encodes a polyGlcNAc-like polysaccharide (Bobrov *et al.*, 2008). For example, polyGlcNAc produced by *Staphylococcus epidermidis* is essential for surface and cell-cell adherence during biofilm formation (Mack et al., 1996). In addition, the *nagE-nagBACD* and *glmUS* operons are important for degradation and production of GlcNAc in *E. coli* and were shown to be regulated by NagC (Plumbridge, 1991, Plumbridge, 1995). Homologues of these genes are also found in *Yersinia* (Deng et al., 2002).

Recently it was shown that the QS system of *Y. pseudotuberculosis* is required for biofilm formation on *C. elegans* since the double AHL synthase mutant (Δ*ypsI*/Δ*ytbI*) and the double mutant of AHL response regulators (Δ*ypsR*/Δ*ytbR*) are severely attenuated or delayed in their biofilm formation. AHLs were also detected in the biofilms on *C. elegans*, suggesting that QS may promote biofilm formation (Atkinson *et al.*, 2011). The same study demonstrated that deletion of

the key genes involved in motility *flhDC*, *fliA* and *flhA*, of which *flhDC* and *fliA* are QS regulated, also resulted in the loss of biofilm development on *C. elegans*. However, non-motile bacteria without flagella after mutation of *fliC*, did not show any reduction of biofilm (Atkinson *et al.*, 2011, Atkinson *et al.*, 2008). Furthermore, expression of the T3SS of *Y. pseudotuberculosis* appeared to attenuate biofilm development since the wild-type grown under T3SS inductive conditions inhibited biofilm formation which could be restored in the QS and motility mutants by curing the pYV virulence plasmid. In addition, secretion of Yops in Δ*ypsI*/Δ*ytbI*, Δ*flhDC* and Δ*fliA* occurred under non-permissive conditions for the T3SS. This observation was supported by the introduction of a *yscJ* deletion in the Δ*ypsI*/*ytbI* mutant. This triple mutant was unable to assemble the T3S injectisome and showed restoration of biofilm formation (Atkinson *et al.*, 2011).

1.9.3 Regulation of the T3SS by QS

It was recently shown that QS is an important regulator for the T3SS since mutation of flagellar components (*flhDC*, *fliA* and *flhA*) and several QS genes (including *ypsR*, *ytbR*, *ytbI* and the double mutants *ypsI*/*ytbI*, *ypsR*/*ytbR*) lead to the release of Yops into the supernatant *in vitro* at 30°C and in the presence of Ca^{2+} (Atkinson *et al.*, 2011). Interestingly, both T3SS related regulators LcrF and YmoA negatively influence QS whereas QS up-regulates expression of *ymoA* which then causes a decrease of *lcrF* expression (this laboratory, unpublished

data). Other bacteria also have been reported to regulate the T3SS *via* QS. The release of the T3S effector ExoS and the expression of the 'translocon' operon in *P. aeruginosa* was shown to be upregulated in an C4-HSL negative Δ*rhlI* mutant indicating that the T3SS is negatively controlled by QS (Bleves *et al.*, 2005). In addition OpaR, a LuxR homologue, that controls colony opacity, was identified as a repressor of the T3S regulon in *V. parahaemolyticus* from microarray transcriptome studies (Gode-Potratz and McCarter, 2011).

1.9.4 Cell-Cell Aggregation

Aggregation of *Y. enterocolitica* and *Y. pseudotuberculosis* cells after growth at 37ºC was first observed in the 1980s and was linked to a plasmid encoded protein P1 which was later assumed to be YadA. Interestingly, the same studies showed that YadA is a homotrimer of 45 kDa subunits and P1 consisted of parts with a size of 24 kDa and 52.5 kDa (Skurnik *et al.*, 1984, Balligand *et al.*, 1985, Roggenkamp *et al.*, 1995), demonstrating that P1 and YadA may be not the same. Furthermore, the attachment factor YadA was shown to be expressed mainly in minimal medium (Eitel and Dersch, 2002).

Another study investigated whether aggregation was QS dependent and revealed that mutation of *ypsR* in *Y. pseudotuberculosis* caused enhanced aggregation (Atkinson *et al.*, 1999). Goldstone, (2011) then demonstrated that mutation in both AHL synthases and response regulators also enhanced aggregation and that curing cells of pYV abolished this phenotype. The same study confirmed the link

between T3S in aggregation since mutation of *yscJ* in Δ*ypsI*/*ytbI* also stopped *Y. pseudotuberculosis* from aggregating (Goldstone, 2011).

Other studies have linked T3S with aggregation and *Salmonella typhimurium* was shown to aggregate and produce biofilms only in the presence of a T3SS (Jennings *et al.*, 2012). *P. aeruginosa* was found to aggregate only when attaching to host-cells (Tran *et al.*, 2014), which indicates that biofilm formation and T3SS aggregation are linked.

1.10 Previous Work and Aims of this Study

Several investigations of the QS dependent phenotypes in *Y. pseudotuberculosis* have been previously conducted (Atkinson *et al.*, 1999, Atkinson *et al.*, 2008, Atkinson *et al.*, 2011). In particular the QS regulation of swimming motility is well understood since Atkinson *et al.*, (2008) showed that QS regulates *flhDC* and *fliA*, which in turn control the expression of the flagellar operon (Goldstone, 2011). It was also shown that there is be a link between T3S and biofilm formation on *C. elegans* which are both regulated by QS (Atkinson *et al.*, 2011). It has also been proposed that biofilms and the T3SS are important for cell-cell aggregation since aggregation appears to depend on pYV and mutation of *yscJ* abolished aggregation in an AHL deficient mutant (Goldstone, 2011).

To determine whether there are any other regulators which control QS, a previous study conducted QS gene promoter pull-down experiments and identified the GlcNAc regulatory protein NagC bound to the *ypsR* promoter (Robert Goldstone,

unpublished data). NagC functions as repressor of the *nagE-nagBACD* operon, which is important for GlcNAc metabolism in *E. coli* and is derepressed in presence of GlcNAc-6P (Plumbridge, 1991, Plumbridge, 1990). NagC and the *nagE-nagBACD* operon are also present in *Yersinia* (Deng et al., 2002).

The finding that NagC is a potential QS regulator is important given its role in controlling GlcNAc metabolism and the contribution of GlcNAc to biofilms as it is a major component of the ECM of *Yersinia* biofilms (Bobrov *et al.,* 2008). Furthermore, GlcNAc was shown to be an important factor for virulence related mechanisms in other bacteria. For example, GlcNAc is needed for virulence and biofilm formation in *Streptococcus mutans* and for colonisation of the mouse intestine by *E. coli* (Kawada-Matsuo et al., 2012, Chang et al., 2004). In *P. aeruginosa* GlcNAc and peptidoglycan were found to enhance production of antimicrobial molecules and virulence factors suggesting that *P. aeruginosa* can sense its surrounding microbial community (Korgaonkar and Whiteley, 2011, Korgaonkar *et al.*, 2013). In addition, NagC is a regulator of other genes in *E. coli*, such as the chitobiose operon and the fimbrial protein *fimB* (Plumbridge and Pellegrini, 2004, Sohanpal et al., 2004).

Assuming that these mechanisms might also be influenced by GlcNAc in *Yersinia* and given that NagC is the regulator of GlcNAc metabolism, it may well be that NagC regulates QS and QS related phenotypes.

This study set out to explore the findings described above to obtain a deeper understanding of *Yersinia* QS regulated phenotypes and to study the role of NagC in QS regulation.

For investigation of the aggregation phenotype the following aims were established:

- Characterisation of the role of QS in cell aggregation.
- Investigation of the roles of T3S and biofilms in aggregation.
- Identification of the aggregation factors by *in vitro* Tn*5* transposon mutagenesis of pYV.

For investigation of NagC, GlcNAc and QS, the following aims were pursued:

- Determination of the impact of NagC on QS gene expression and *vice versa*.
- Determination of effects of GlcNAc and MurNAc on *nagC* and QS gene expression.
- Characterisation of the *nagC* mutant phenotype.
- Elucidation of the effect of GlcNac on *nagC* mutant phenotypes.

2 Materials and Methods

2.1 Growth Media and Growth Conditions

Unless otherwise stated, *Y. pseudotuberculosis* strains were grown at 30°C in liquid low-salt lysogeny broth (LB) (Luria and Delbrück, 1943) – 10 g/l tryptone (Oxoid™), 5 g/l NaCl, 5 g/l yeast extract (Oxoid™) whilst shaking with aeration at 250 rpm. If grown on plates, 15 g/l agar was added to the media. *E. coli* strains were grown at 37°C. To maintain a stable pH of 6.8, 3-(*N*-morpholino)propanesulfonic acid (MOPS), with a final concentration of 50 mM was added to the media. For conjugations and Yop extraction assays (2.4.2 and 2.5.1) *Y. pseudotuberculosis* strains were grown in brain-heart-infusion broth (BHI) (Oxoid™) over night at 22°C or on BHI agar plates at 30°C. To induce Yop expression, 20 µM $Na_2C_2O_4$ and 20 µM $MgCl_2$ for production of MgC_2O_4 were added to the medium. Swimming motility plates contained 10 g/l tryptone (Difco-Bacto™) 5 g/l NaCl and 3 g/l agar (Difco-Bacto™) (Atkinson *et al.*, 1999). *C. elegans* were maintained on nematode growth medium (NGM) at 22°C, containing 3.0 g NaCl, 2.5 g peptone (Difco-Bacto™), 17.0 g agar and 975 ml distilled H_2O. After autoclaving 1 ml cholesterol (5 mg/ml), 1 ml $CaCl_2$ (1 M), 1 ml MgS (1 M) and 25 ml KH_2PO_4 (1 M, pH 6) was added (Epstein, 1995). For assays with *N*-acetyl-D-glucosamine (GlcNAc), 0.3 mM, 1.0 mM or 5 mM GlcNAc was added to the agar. Antibiotics and their concentrations were ampicillin (Amp) at 100 µg/ml, chloramphenicol (Cm) at 10 µg/ml (liquid culture) and 15 µg/ml (agar plates), gentamicin (Gm) at 10-20 µg/ml, kanamycin (Km) at 50 µg/ml, nalidixic acid (Nal) at 15 µg/ml, streptomycin (Sm) at 50 µg/ml, tetracycline (Tc) at 10 µg/ml and trimethoprim (Tm) at 100 µg/ml.

2.2 Bacterial Strains

Bacterial strains that were used in this study are listed in Table 2.1

Table 2.1: Bacterial strains used in this study.

Bacterial strain	Description	Source
<u>*E. coli*</u>		
DH5α	*E. coli* K-12 cloning strain, [Fφ80*lacZ*ΔM15Δ (*lacZYA-argF*) U169 *recAI endAI hsdR17* (r_K- m_K^+) *supE44 thiI gyrI relAI*]	(Hanahan, 1983)
OP50	Uracil auxotroph nutrient source for *C. elegans* and control for uninfected worms / Tc^R	(Epstein, 1995)
S17-1 λ-pir	λ *-pir* lysogen of S17-1 [*thi pro hsdR– hsd*M+ *recA* RP4 2-Tc::Mu-Km::Tn*7*], permissive host capable of transferring suicide plasmids requiring the Pir protein by conjugation to recipient cells / Sm^R	(Simon *et al.*, 1983)
JM109 p*xylA*p	*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17* Strain harbouring p*xylA*p, a plasmid harbouring GFP	NEB
ER2915	*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)* Tc^S *endA1 rspL136* (Sm^R) *dam13::Tn9* (Cam^R) *xylA-5 mtl-1 thi-1 mcrB1 hsdR2* Strain lacking *dam*/*dcm* for DNA methylation	NEB
S17.1 pDM4	S17.1 containing the suicide vector pDM4 (Cm^R)	(Milton *et al.*, 1996)

Bacterial strain	**Description**	**Source**
S17.1 pDM4.2	S17.1 containing the suicide vector pDM4.2 (Gm^R)	Joanne Purves (unpublished)
S17.1 pUC18R6KTminiTn*7*::Tc	S17.1 harbouring pUC18R6KT, a vector containing the Tn*7* transposon (Tc^R)	(Choi *et al.*, 2005)
S17.1 $P_{ypsR:}$:lux	S17.1 containing a lux fusion of the *ypsR* promoter of YpIII on pKNG10 (Sm^R)	(Atkinson *et al.*, 2008)
S17.1 $P_{ypsI:}$:lux	S17.1 containing a lux fusion of the *ypsI* promoter of YpIII on pKNG10 (Sm^R)	(Atkinson *et al.*, 2008)
S17.1 P_{ytbR}::lux	S17.1 containing a lux fusion of the *ytbR* promoter of YpIII on pKNG10 (Sm^R)	(Atkinson *et al.*, 2008)
S17.1 P_{ytbI}::lux	S17.1 containing a lux fusion of the *ytbI* promoter of YpIII on pKNG10 (Sm^R)	(Atkinson *et al.*, 2008)
<u>*Y. pseudotuberculosis*</u>		
YpIII pLB1	Parent strain of YpIII harbouring the virulence plasmid pYV. Serotype O:3 (Nal^R)	(Rosqvist *et al.*, 1988)
YpIII pYV-	YpIII parent cured of pYV (Nal^R)	this study
YpIII pAJD434	YpIII harbouring pAJD434, a plasmid containing the λ-red recombinase (Tm^R)	(Derbise *et al.*, 2003)
YpIII p*xylA*p	YpIII harbouring the GFP plasmid p*xylA*p (Amp^R)	this study

Bacterial strain	**Description**	**Source**
YpIII Δ*nagC*	YpIII lacking the repressor of the GlcNAc-operon NagC (Km^R)	this study
YpIII Δ*nagC* pYV-	YpIII Δ*nagC* cured of pYV(Km^R)	this study
YpIII Δ*nagC* p*xylA*p	YpIII Δ*nagC* harbouring the GFP plasmid p*xylA*p (Amp^R)	this study
YpIII Δ*nagC* complemented	Complementation of YpIII lacking NagC with a functional copy of *nagC* in the *glmS* region of Δ*nagC* (Tc^R)	this study
YpIII Δ*nagC* complemented p*xylA*p	YpIII Δ*nagC* complement harbouring the GFP plasmid p*xylA*p (Amp^R)	this study
YpIII Δ*nagE*	YpIII lacking the phosphotransferase of the GlcNAc metabolism NagE (Gm^R)	this study
YpIII Δ*ypsR*	YpIII lacking the AHL receptor YpsR (Km^R)	(Atkinson *et al.*, 1999)
YpIII Δ*ypsR* pYV-	YpIII Δ*ypsR* cured of pYV(Km^R)	this study
YpIII Δ*ypsI*	YpIII lacking the AHL synthase YpsI (Km^R)	(Atkinson *et al.*, 1999)
YpIII Δ*ypsI* pYV-	YpIII Δ*ypsI* cured of pYV(Km^R)	this study
YpIII Δ*ytbR*	YpIII lacking the gene for the AHL receptor YtbR (Cm^R)	(Atkinson *et al.*, 2008)

Bacterial strain	**Description**	**Source**
YpIII Δ*ytbR* pYV-	YpIII Δ*ytbR* cured of pYV(Cm^R)	this study
YpIII Δ*ytbI*	YpIII lacking the AHL synthase YtbI (Cm^R)	(Atkinson *et al.*, 2008)
YpIII Δ*ytbI* pYV-	YpIII Δ*ytbI* cured of pYV(Cm^R)	this study
YpIII Δ*ypsI*/ Δ*ytbI*	YpIII lacking both AHL synthases YpsI and YtbI (Km^R, Cm^R)	(Atkinson *et al.*, 2008)
YpIII Δ*ypsI*/ Δ*ytbI* pYV-	YpIII Δ*ypsI*/ Δ*ytbI* cured of pYV(Km^R, Cm^R)	this study
YpIII Δ*ypsR*/ Δ*ytbR*	YpIII lacking both AHL receptors YpsR and YtbR (Km^R, Cm^R)	(Atkinson *et al.*, 2008)
YpIII Δ*ypsR*/ Δ*ytbR* pYV-	YpIII Δ*ypsR*/ Δ*ytbR* cured of pYV(Km^R, Cm^R)	this study
YpIII Δ*ymoA*	YpIII lacking expression modulator YmoA (Km^R)	unpublished
YpIII Δ*virF*	YpIII lacking the transcriptional activator of the *Yersinia* virulence regulon VirF (Km^R)	unpublished
YpIII Δ*yscF*	YpIII lacking the injection needle of the T3SS YscF (Tc^R)	this study
YpIII Δ*yscF*/ Δ*ypsI*/ Δ*ytbI*	Triple mutant lacking both AHL synthases YpsI , YtbI and the injectisome needle YscF (Tc^R, Km^R, Cm^R)	this study

Bacterial strain	**Description**	**Source**
YpIII Δ*yscV*	YpIII lacking part of the export apparatus of the T3SS YscV (Gm^R)	this study
YpIII Δ*yscV*/ Δ*ypsI*/ Δ*ytbI*	Triple mutant lacking both AHL synthases YpsI , YtbI and part of the export apparatus YscV (Gm^R, Km^R, Cm^R)	this study
YpIII Δ*yscJ*	YpIII lacking part of the injectisome of the T3SS YscV (Tc^R)	(Atkinson *et al.*, 2011)
YpIII Δ*yscJ*/ Δ*ypsI*/ Δ*ytbI*	Triple mutant lacking both AHL synthases YpsI , YtbI and part of the injectisome YscV (Tc^R, Km^R, Cm^R)	(Atkinson *et al.*, 2011)
YpIII Δ*ypsI*/ Δ*ytbI* pYVTn*5*::Tc	YpIII lacking both AHL synthases with a Tn*5* transposon insertion in its virulence plasmid (Km^R, Cm^R, Tc^R)	This study
<u>Promoter fusions</u>		
YpIII P_{ypsR}::*lux*	Lux fusion of the *ypsR* promoter of YpIII integrated into YpIII by pKNG10 (Km^R, Sm^R)	this study
YpIII P_{ypsI}::*lux*	Lux fusion of the *ypsI* promoter of YpIII integrated into YpIII by pKNG10 (Km^R, Sm^R)	this study
YpIII P_{ytbR}::*lux*	Lux fusion of the *ytbR* promoter of YpIII integrated into YpIII by pKNG10 (Cm^R, Sm^R)	this study

Bacterial strain	**Description**	**Source**
YpIII P_{ytbI}::*lux*	Lux fusion of the *ytbI* promoter of YpIII integrated into YpIII by pKNG10 (Cm^R, Sm^R)	this study
YpIII Δ*nagC* P_{ypsR}::*lux*	Lux fusion of the *ypsR* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Km^R, Sm^R)	this study
YpIII Δ*nagC* P_{ypsI}::*lux*	Lux fusion of the *ypsI* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Km^R, Sm^R)	this study
YpIII Δ*nagC* P_{ytbR}::*lux*	Lux fusion of the *ytbR* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Km^R, Sm^R)	this study
YpIII Δ*nagC* P_{ytbI}::*lux*	Lux fusion of the *ytbI* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Km^R, Sm^R)	this study
YpIII Δ*nagC* complemented P_{ypsR}::*lux*	Lux fusion of the *ypsR* promoter of YpIII integrated into the Δ*nagC* complemented by pKNG101 (Amp^R, Sm^R)	this study
YpIII Δ*nagC* complemented P_{ypsI}::*lux*	Lux fusion of the *ypsI* promoter of YpIII integrated into the Δ*nagC* complemented by pKNG101 (Amp^R, Sm^R)	this study
YpIII Δ*nagC* complemented P_{ytbR}::*lux*	Lux fusion of the *ytbR* promoter of YpIII integrated into the Δ*nagC* complemented by pKNG101 (Amp^R, Sm^R)	this study

Bacterial strain	**Description**	**Source**
YpIII Δ*nagC* complemented P_{ytbI}::*lux*	Lux fusion of the *ytbI* promoter of YpIII integrated into the Δ*nagC* complemented by pKNG101 (Amp^R, Sm^R)	this study
YpIII P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into YpIII by pDM4 (Nal^R, Cm^R)	this study
YpIII Δ*ypsR* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ypsR* by pDM4 (Km^R, Cm^R)	this study
YpIII Δ*ypsI* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ypsI* by pDM4 (Km^R, Cm^R)	this study
YpIII Δ*ytbR* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ytbR* by pDM4.2 (Cm^R, Gm^R)	this study
YpIII Δ*ytbI* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ytbI* by pDM4.2 (Cm^R, Gm^R)	this study
YpIII Δ*ypsI*/ Δ*ytbI* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ypsI*/ Δ*ytbI* by pDM4 (Km^R, Cm^R)	this study
YpIII Δ*ypsR*/ Δ*ytbR* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*ypsR*/ Δ*ytbR* by pDM4 (Km^R, Cm^R)	this study

Bacterial strain	**Description**	**Source**
YpIII Δ*nagC* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*nagC* by pDM4.2 (Km^R, Cm^R)	this study
YpIII Δ*nagE* P_{ypsR}::*lux*	Lux fusion of the *ypsR* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Gm^R, Sm^R)	this study
YpIII Δ*nagE* P_{ypsI}::*lux*	Lux fusion of the *ypsI* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Gm^R, Sm^R)	this study
YpIII Δ*nagE* P_{ytbR}::*lux*	Lux fusion of the *ytbR* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Gm^R, Sm^R)	this study
YpIII Δ*nagE* P_{ytbI}::*lux*	Lux fusion of the *ytbI* promoter of YpIII integrated into Δ*nagC* by pKNG101 (Gm^R, Sm^R)	this study
YpIII Δ*nagE* P_{nagC}::*lux*	Lux fusion of the *nagC* promoter of YpIII integrated into Δ*nagE* by pDM4 (Gm^R, Cm^R)	this study

2.3 General Genetic Modifications

2.3.1 Molecular Cloning

2.3.1.1 Handling of Plasmids and Restriction Digests

Plasmids were extracted using the Sigma GenElute™ Plasmid Miniprep Kit. For extraction of pYV, the Qiagen Plasmid Midi Kit™ was used. Unless otherwise stated, plasmids were eluted in H_2O and stored at -20°C.

Restriction enzymes (Promega) for digestion of plasmids were used according to the supplier's instructions as described by Schagat (Schagat, 2007).

2.3.1.2 Gel Electrophoresis and DNA Extraction

Gel electrophoresis was performed using agarose gels (1.0 % (w/v) agarose, 1x Tris-acetate-EDTA [TAE] buffer [50x stock solution: 2.0 M Tris-acetate pH 8, 0.05 M ethylene-diamine-tetraacetic acid - EDTA]). Gels contained ethidium bromide at a final concentration of 10 µg/ml were loaded with 9 µl – 20 µl of DNA in 6x loading dye (Promega) and were run next to a DNA ladder (1 kb ladder - Promega) in 1x TAE buffer at 80 V for 55 min.

For purification and cleaning of DNA fragments, excised from agarose or for purification of open vectors and PCR products, the Promega Wizard® SV Gel and PCR Clean-Up System was used and the supplier's instructions were followed.

2.3.1.3 Ligation of Vector and Fragment

Ligation of vector and digested DNA fragments / PCR products was performed in a 1:3 ratio using T4 DNA ligase (Promega). Ligations were either incubated at room temperature for 2 h or at 4°C overnight.

2.3.1.4 Transformation of DNA into Competent Cells

Electro-competent cells were produced by diluting the appropriate overnight culture to OD_{600} 0.1 into a 250 ml flask containing 100 ml LB. In case of λ-red recombination, arabinose was added to the medium with a final concentration of 0.8 % (w/v). Cultures were grown until early exponential phase (OD_{600} 0.6 - OD_{600} 0.9) and were then harvested by centrifugation at 4°C at 10,000 x *g* for 5 min in 50 ml falcon tubes. Afterwards cells were washed in 40 ml 10 % (v/v) glycerol and centrifuged under the same conditions as in the first step. Those steps were repeated with 20 ml and 5 ml of 10 % (v/v) glycerol. Cells were then pelleted, resuspended in 40 µl of 10 % (v/v) glycerol and stored in 20 µl aliquots at -80°C until needed.

Prior to electroporation (section 2.3.1.2 and 2.3.1.3) DNA was dialysed over H_2O for 0.5 h using 0.025 µm pore Millipore® filters (Fisher Scientific). The dialysed product was then mixed with the competent cells and transferred into a 2 mm electroporation cuvette and electroporated using a Gene Pulsar (BioRad) set to 2.5 kV, 200 Ω, 25 µF. Cells were recovered in 1.0 ml of LB and left for 1 h – 3 h

at the appropriate temperature before plating the cells onto LB agar containing the appropriate antibiotics.

2.3.1.5 Plasmids Used in this Study

All plasmids, which were used in this study, are listed in Table 2.2.

Table 2.2: Plasmids used in this study.

Plasmid	Description	Source
pYV	Virulence plasmid of *Y. pseudotuberculosis*	This study
pYVTn*5*::Tc	Virulence plasmid harbouring EZ-Tn*5*™ Transposons (Tc^R)	This study, Epicentre®, Illumina
pBlueTet	pBluescript II KS+ encoding *tetA* gene from pBR322, maintained in *E. coli* DH5α (Amp^R, Tet^R)	(Atkinson *et al.*, 2008)
pBlue*lux*	pBluescript II KS+ Vector containing the *luxCDABE* operon (Amp^R)	(Atkinson *et al.*, 2008)
pUC4K	pUC 4 containing a kanamycin resistance gene from pML21 (Km^R)	(Vieira and Messing, 1982)
pEX18Gm	Gm^R, oriT +, sacB+, gene replacement vector, MCS fromn pUC18 (Gm^R)	(Hoang *et al.*, 1998)
pAJD434	Contains the gene of a λ-red recombinase inducible with arabinose, maintained in *E. coli* DH5α (Tm^R)	(Derbise *et al.*, 2003)

Plasmid	**Description**	**Source**
pUC18R6KT::Tc mini Tn*7*	R6K *Ori*; mini-Tn*7* transposition suicide vector, allows for single copy chromosomal insertion at *att*Tn7 sites (Tc^R)	(Choi *et al.*, 2005)
pAW1	Tn7 suicide vector including the construct for complementation of *nagC*	This study
pTNS2	pUX-BF13 *Ori*; mini-Tn*7* helper plasmid, encodes TnsABC +D transposase complex to catalyze high frequency insertion (Amp^R)	(Choi *et al.*, 2005)
pGEM-T-easy	Cloning vector (Amp^R)	Promega
pGEM-T	Cloning vector (Amp^R)	Promega
pHG327	Complementation vector, low copy number (Amp^R)	(Stewart *et al.*, 1986)
pKNG101	Suicide vector that contains promotor fusions, maintained in *E. coli* DH5α (Sm^R)	(Kaniga *et al.*, 1991)
pKNG101:: P_{ypsI}::*lux*	Suicide vector containing a promoter::*lux* fusion of *ypsI*	(Atkinson *et al.*, 2008)
pKNG101:: P_{ypsR}::*lux*	Suicide vector containing a promoter::*lux* fusion of *ypsR*	(Atkinson *et al.*, 2008)
pKNG101:: P_{ytbI}::*lux*	Suicide vector containing a promoter::lux fusion of *ytbI*	(Atkinson *et al.*, 2008)
pKNG101:: P_{ytbR}::*lux*	Suicide vector containing a promoter::*lux* fusion of *ytbR*	(Atkinson *et al.*, 2008)
pDM4	Suicide vector: *mob*RK2, *ori*R6K (*pir* requiring). *sacBR* of *Bacillus subtilis* (Cm^R)	(Milton *et al.*, 1996)

Plasmid	Description	Source
pDM4::P_{nagC}::*lux*	Suicide vector containing a promoter::*lux* fusion of *nagC* (Cm^R)	This study
pDM4::*yscV*-Gm	Suicide vector containing the *yscV* knock-out construct	This study
pDM4.2	Suicide vector: *mob*RK2, *ori*R6K (*pir* requiring). *sacBR* of *Bacillus subtilis*, contains Gm-resistance cassette of pEX18Gm instead of Cm-resistance cassette (Gm^R)	Joanne Purves (unpublished)
pDM4.2:: *yscF*-Tc	Suicide vector containing a promoter::*lux* fusion of *nagC*	This study
pDM4.2::P_{nagC}::*lux*	Suicide vector containing a promoter::*lux* fusion of *nagC*	This study
p*xylA*p	Vector harbouring GFP, (Amp^R)	Stephan Heeb

2.3.2 Polymerase Chain Reaction

Polymerase chain reactions (PCRs) were performed by either using Go Taq® DNA polymerase (Promega) or Phusion® High-Fidelity DNA polymerase (NEB). A standard 50 µl PCR consisted of 0.2 µl DNA polymerase, 1.0 µl of sample (~1 ng DNA), 5 µl of reaction buffer (Promega or NEB), 1 µl of each 20 µM forward and reverse primer, 1 µl of 20 mM nucleotides (dNTPs). H_2O was added to the mix to obtain a final concentration of 50 µl. If necessary, 1 µl of DMSO was also added.

The standard PCR conditions varied depending on the length of the final product and the melting temperature of the primers and were performed according to the polymerase supplier's instructions (for Go Taq®: Promega / for Phusion®: NEB). Primers used in this study are listed in Table 2.3.

Table 2.3: Primers that were used in this study. Underlined parts of the primer sequences are restriction enzyme recognition sites.

Primer name	Sequence (F: 5'-3') (R: 5'-3')	Restriction enzyme	Source
yscU F	AAAAGCAAGCGTCGTCAGTT	-	(Goldstone, 2011)
yscU R	GCTGTGGCCTCTATTTGCTC	-	(Goldstone, 2011)
nagC-ko-F	GGCGGACAAGCACAAATTGG TAACGTGGATCTGGTAAAACA ACTCAATGGAGAAAGCCACG TTGTGTCTCAA	-	This study
nagC-ko-R	AGTTTCTAGCAAACGTTGCAG CAAGACACCATTGAGCATAG CGCGCTTAGCTTAGAAAAACT CATCGAGCAT	-	This study
nagC-ko-ch-F	GGCGTGGATAAACAGTTAGG	-	This study
nagC-ko-ch-R	CCAGCATCCTGAATACGG	-	This study
nagC-comp-F	CTCGAGCGGTATCCGATTTGC GACC	*Xho*I	This study
nagC-comp-R	GGATCCGGTCACTATAACATG GCTGTGG	*Bam*HI	This study
nagC-lux-F	CTCGAGCGGTATCCGATTTGC GACC	*Xho*I	This study
nagC-lux-R	GGATCCGGTACCCCTCGTTGC CATTAACGATGG	*Kpn*I, *Bam*HI	This study

Primer name	**Sequence**	**Restriction enzyme**	**Source**
lux RV	CTACAACATCATAAAGGCC	-	(Grasso, 2013)
pst-up-2-F	GCTATACGTGTTTGC	-	Joanne Purves (unpublished)
glmS-dn-2-R	ACGCCACCGGAAGAACCG	-	Joanne Purves (unpublished)
Gm F	GGATCCACGTCAGGTGGCACTTTTCGG	*Bam*HI	This study
Gm R	CCCGGGGAACGAATTGTTAGGTGGCG	*Xma*I	This study
yscF-up-F	CTCGAGCGCCAAGAGTTTGGCAATCG	*Xho*I	This study
yscF-up-R	GGTACCTGATCTAGAAGCCACCGCATCTAAGTCTGC	*Kpn*I, *Xba*I	This study
Tc-no-prom-F	ATTCTAGACGCAGTCAGGCACCG	*Xba*I	Joanne Purves (unpublished)
Tc-no-term-R	AAGGTACCGAGTGGTGAATCCGTTAG	*Kpn*I	Joanne Purves (unpublished)
yscF-dn-F	CTCGAGATCGGTACCGGCATCCTACAGAAGTTCC	*Xho*I, *Kpn*I	This study
yscF-dn-R	ACTAGTCCCGTGTCTTTTCAGCGG	*Spe*I	This study
yscF-ko-ch-F	GGTAATCAACGCAAGCGGC	-	This study
yscF-ko-ch-R	CCCACCGCTTACCAAACG	-	This study
yscF-comp-F	GGATCCGGTGAGCTGATTGAATTACGC	*Bam*HI	This study
yscF-comp-R	CCCGGGGCAATCTCTGCTAACAGTACG	*Xma*I	This study
yscV-up-F	CTCGAGCGAGCTCAAGATTATCGGCG	*Xho*I	This study

Primer name	Sequence	Restriction enzyme	Source
yscV-up-R	GGTACCTGATCTAGACAGCATGATATCTTTACGC	*Kpn*I, *Xba*I	This study
Gm-no-prom-F	CGTCTAGAGTCGATGTTAGTTGTTATG	*Xba*I	Joanne Purves (unpublished)
Gm-no-term-R	ACGGTACCTTAGGTGGCGGTACTTG	*Kpn*I	Joanne Purves (unpublished)
yscV-dn-F	CTCGAGATCGGTACCGGCTTGCCGGTACTTTCATAC	*Xho*I, *Kpn*I	This study
yscV-dn-R	ACTAGTGGGAAGATTTCATAATCTACC	*Spe*I	This study
yscV-ko-ch-F	CCTGTGGCTTGCCTGAACG	-	This study
yscV-ko-ch-R	GGCGTTAAGCCGATATCAGC	-	This study
yscV-comp-F	CTGCAGCCTATGGGACTTTGCTTCTCC	*Pst*I	This study
yscV-comp-R	CCCGGGGCCAAGCCATGTTCGGTAAGCC	*Xma*I	This study
nagE up F	CTCGAGGCCCAGAATAAACGGACGTTC	*Xho*I	This study
nagE up R	CCCGGGGATGGATCCCCAACCGACTGGGTCTATCCAG	*Xma*I, *Bam*HI	This study
nagE dn F	CTCGAGAGCCCCGGGCGGTGTTAGAACTGGATCTGG	*Xho*I, *Xma*I	This study
nagE dn R	TCTAGAGTATTCTTGTATCCTGGCCG	*Xba*I	This study
nagE ko ch F	CGTAACTCAGATTCTGACC	-	This study
nagE ko ch R	GATCGGTTATTCAGTACACC	-	This study
nagE comp F	GGATCCGCCCAGAATAAACGGACGTTC	*Bam*HI	This study

Primer name	Sequence	Restriction enzyme	Source
nagE comp R	CCCGGGCCTGAATCTCAAACG GAATACG	*Xma*I	This study
M13 F	TGTAAAACGACGGCCAGT	-	Joanne Purves (unpublished)
M13 R	CAGGAAACAGCTATGACC	-	Joanne Purves (unpublished)
Tc-Tn*5*-F	GGGTGCGCATGATCCTCTAGA GT	-	Epicentre®, Illumina
Tc-Tn*5*-R	TATTTCTAGATTTCAGTGCAA TTTA	-	Epicentre®, Illumina

2.3.3 DNA Sequencing

DNA was checked for purity and quantity using a Nano Drop® 1000 spectrophotometer (Fisher Scientific).

Sanger sequencing (Sanger and Coulson, 1975) was carried out at the BSAU, Queen's Medical Centre, Nottingham, UK or Source Bioscience, Science Park, Nottingham, UK was contracted for sequencing analysis.

Sequences were analysed using BioEdit® version 7.2.2 to view .ab1 files and SeqBuilder® version 8.1.4 of the DNAStar® LaserGene software for virtual cloning strategies and for viewing .seq files.

2.4 Genetic Modifications

2.4.1 Gene Mutations

2.4.1.1 Gene Mutations Using the λ-red Recombinase System

Mutations using the λ-red recombinase system described by Derbise (Derbise *et al.*, 2003) were performed as follows. Primers were used to amplify an antibiotic cassette. The resulting PCR product contained flanking regions of the gene of interest. Purified PCR product was electroporated into *Y. pseudotuberculosis* YpIII cells (Table 2.1) harbouring the arabinose inducible λ-red recombinase plasmid pAJD434 (Table 2.2) (Derbise *et al.*, 2003). Cells were first shaken for 3 h in LB containing 0.8 % (w/v) arabinose at 30°C to allow recombination to occur and were then plated out onto LB containing the appropriate antibiotic. Colonies of potential mutants were screened by PCR (2.3.2).

2.4.2 Gene Mutations Using the Suicide Plasmids pDM4 and pDM4.2

Mutations using the sucrose sensitive suicide plasmids pDM4 and pDM4.2 (Table 2.1) were performed by exchanging the major part of the gene of interest with a gentamicin or tetracycline cassette as previously described (Atkinson *et al.*, 2008). Primers containing different restriction enzyme recognition sites were used to amplify the upstream region and downstream regions of the gene of interest and

the chosen antibiotic cassette. Each product was ligated into pGEM-T-easy (Table 2.2) and transformed into *E. coli* DH5α. Using the appropriate restriction enzymes the antibiotic cassette was excised from pGEM-T-easy and cloned into a similarly digested pGEM-T-easy containing the upstream region. This construct was again excised from pGEM-T-easy and cloned into the vector containing the downstream region of the target gene, resulting in an upstream-antibiotic-downstream insert. The construct was then excised from pGEM-T-easy, subcloned into the suicide vector pDM4 or pDM4.2 (Table 2.2) and transformed into *E. coli* S17-1 (Table 2.1) .

For mutation of the gene of interest in *Y. pseudotuberculosis* YpIII, *E. coli* S17-1 harbouring the suicide plasmid with the mutation construct and YpIII wild-type cells were mated as described by Milton, 1996 (Milton *et al.*, 1996). In summary, overnight cultures of *E. coli* S17-1 containing the suicide plasmid were mixed in a 9:1, 3:1 and 1:1 ratio with *Y. pseudotuberculosis* YpIII in a 1.5 ml tube. Cultures were pelleted at 16.000 x *g* for 1.5 min, resuspended in 30 µl of BHI medium and spotted onto a BHI agar plate. After an incubation time of 6 h or 24 h, bacteria cells were removed, resuspended in 0.5 ml of BHI and plated. The plates were incubated at 30°C for several days until single colonies were visible. Those colonies were then cross streaked onto several BHI agar plates, containing 12 % (w/v) sucrose and/or the appropriate antibiotics. Colonies that were able grow on sucrose and the antibiotic that was part of the mutation construct, but unable to grow on the antibiotic encoded on the suicide plasmid were screened for mutation by PCR.

2.4.3 *lux*-Gene Promoter Fusions

Promoter-*lux* fusions into the *Y. pseudotuberculosis* YpIII strains (Table 2.1) were constructed as previously described (Atkinson *et al.*, 2008). Briefly, *E. coli* S17-1 donor cells containing the appropriate vector and the YpIII recipient were mixed in ratios 1:1, 1:4 and 1:10, pelleted at 14,000 x g for 90 sec at room temperature. The cell mixture was resuspended in 30 µl of LB, pipetted into the middle of a LB plate and incubated at 37°C for 6 h and 24 h. Cells were taken from the plate, resuspended in 0.5 ml of LB, spread onto a fresh plate containing the appropriate antibiotics and incubated at 30°C until light colonies were visible using a photon detecting imaging software Wasabi® of the photon detecting imaging camera (Hamamatsu Photonics Germany GmbH).

2.4.3.1 Visualisation of Promoter Activity by Using *lux*-Gene Promoter Fusions

For analysis of gene activity of the promoter-*lux* fusions in several YpIII strains (Table 2.1) the protocol adapted from Atkinson *et al.* (2008) was used. The promoter fusions were grown over night at 30°C in 10 ml LB. Bacterial cultures were then diluted 1:100 in 10 ml LB and were incubated until mid-log phase for 3 h – 4 h at 30°C. Cells were washed twice in 1 ml of fresh LB and were diluted to OD 0.006. 300 µl of the sample was transferred into a 96-well microtiter plate (Greiner Chimney black, GreinerBioOne). The assay was performed in triplicates of each culture.

2.4.3.2 Luminescence and Cell Density Measurement Using a Tecan® Luminometer/Spectrophotometer

For luminescence and cell density measurements, bacterial samples in a 96-well microtitre plate (Greiner Chimney black, GreinerBioOne) were placed into a Tecan Infinite®, Infinite Pro® or Genius Pro® luminometer/spectrophotometer (Tecan). Triplicates of each sample were pipetted into the same microtitre plate. Using the plate reader, luminescence and optical density of the samples were measured every 30 min for 23 h at 22°C or 37°C.The data was evaluated using Microsoft Excel® (Microsoft Cooperation).

2.4.4 Tn*5* Mutagenesis of the *Yersinia* Virulence Plasmid

Tn*5* transposon mutagenesis was carried out, using the Epicentre® EZ-Tn*5*™ <TET-1> Insertion Kit (Illumina). The transposons were inserted by using the supplier's protocol, which was adapted from Goryshin, 1998 (Goryshin and Reznikoff, 1998), in which 0.2 µg of plasmid DNA was mixed with 1 µl reaction buffer, 1 µl transposase and 0.0043 pmol of the transposon. Sterile water was added to the mixture to achieve a total volume of 10 µl. The reaction was incubated for 2 h at 37°C, followed by a heat inactivation of the enzyme for 10 min at 70°C and transformation into target cells.

2.5 Analysis of Proteins

2.5.1 Extraction of *Yersinia*-Outer-Proteins

Yops of *Y. pseudotuberculosis* were extracted by using the method described by Trülzsch, 2004 (Trülzsch *et al.*, 2004), in which cells grown overnight in 10 ml BHI at 22°C were diluted to an OD_{600} of 0.1 in BHI containing 0.2 % (w/v) glucose, 20 mM sodium oxalate (NaOx) and 20 mM magnesium chloride ($MgCl_2$) and were incubated for 2 h at 22°C. To induce the expression and release of Yops into the media, cells were then shifted to 37°C for 3 h. Bacteria cells were removed by centrifugation and finally the supernatant was taken off and filter sterilised using a 0.2 µM filter. Proteins were extracted from 10 ml of supernatant by incubation with 1 ml of 100 % (w/v) trichloroacetic acid (TCA) for 30 min, followed by centrifugation at 10,000 x *g* for 10 min at 4°C. Proteins were resuspended in 0.5 ml of 10 % (w/v) sodium dodecyl sulphate (SDS) and re-precipitated in 1 ml of ice cold acetone for 30 min on ice. Proteins were pelleted at 16,000 x *g* for 30 min at 4°C. The resulting pellet was air dried and resuspended in 30 µl phosphate buffered saline (PBS).

2.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by boiling protein samples in 2x SDS loading buffer (1 ml Tris pH 6.8, 4 ml 10 % (w/v) SDS, 1 ml of 1 % (w/v) bromophenol blue, 2 ml glycerol, 560µl 2-mercaptoethanol) before loading onto an SDS-PAGE gel (containing 12 % (w/v) acrylamide) in 1x SDS running buffer (3.0 g Tris, 14.4 g glycine and 1 g SDS in 1000 ml H_2O). The sample was run next to the ColorPlus™ Prestained Protein Ladder (NEB) for 80 min at 150 V. Proteins were visualised by staining with Coomassie Blue. Sequencing analysis of proteins that were previously excised off the SDS-PAGE gel was carried out by MALDI-TOF at the Proteomics facility of the University of York, York.

2.6 Phenotypic Assays

2.6.1 Swimming Motility Assays

For detection of swimming motility at 22°C, 1 µl of bacterial overnight cultures grown in 10 ml LB at 30°C were pipetted into the middle of a swimming agar plate (2.1) and were left static at room temperature for 48 h.

2.6.2 Maintenance of *C. elegans* and Biofilm Assays

For maintenance of *C. elegans* (wild-type N2 Bristol, obtained from the *Caenorhabditis* Genetics Centre, University of Minnesota, USA), 500 µl of *E. coli* OP50 overnight culture (Table 2.1) were spread onto NGM agar plates (2.1). *C. elegans* was seeded onto the plates by transferring a 1 cm x 1 cm agar block from a 2 -3 day-old worm plate onto a fresh plate. The worms were kept at 22°C until needed.

C. elegans biofilm infection assays as described by Joshua *et al.*, 2003 and Atkinson *et al.*, 2011 involved a 10 ml LB overnight culture of the appropriate culture being grown at 30°C. 2 ml of the culture was diluted to OD_{600} 1.0 and was plated onto a fresh NGM plate with or without GlcNAc. 20 - 30 nematodes were picked from a *C. elegans* stock plate onto the seeded plate using a micro-lance and after 6 h and 24 h at 22°C, the biofilm severity index (BSI) was determined by scaling the size of the biofilm (scale: 0 - 3) and was calculated as follows:

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

0 - no biofilm, 1- a small biofilm around the buccal cavity, 2 - large, mono-focal biofilm, 3 - a very large, mono-focal biofilm (Atkinson *et al.*, 2011).

To examine the developed YpIII biofilm by phase and confocal microscopy (2.6.4), infected *C. elegans* were transferred to a cavity microscope slide. Worms were washed with H_2O, immobilised with 30 mM sodium azide (NaN_3) and the extracellular matrix (polyGlcNAc) of the biofilm was stained with a 1/30 dilution of rhodamine-labelled wheat germ agglutinin (WGA) in H_2O,

(Vectorlaboratories). For detection of the bacteria inside the biofilm, YpIII strains were transformed with p*xylA*p, a plasmid containing the gene encoding GFP (Table 2.1).

2.6.3 Aggregation Assay

As described by Atkinson *et al.*, 1999, bacteria were grown for 17 h in LB at 37°C, cultures diluted to OD_{600} 2.0 - 3.0 in fresh medium and left static in a 1.0 mm cuvette for up to 45 min at 22°C.

For assays using different pYV proportions, colonies were firstly checked for the existence of pYV by PCR (2.3.2) using the primer combination of *yscU* F and *yscU* R (Table 2.3). Colonies found to contain pYV (100 % pYV) were then mixed with cultures which did not show an *yscF* PCR product (0 % pYV) to the appropriate ratio.

2.6.3.1 Enzyme Treatment and Staining of Aggregated Bacteria

To determine if surface proteins are involved in the cell aggregation, bacteria were washed, resuspended in 100 µL PBS and treated with 1.5 µL of 20 mg/ml proteinase K for 30 min at 22°C. Cells were then washed three times with PBS before being left to settle for 45 min.

To find out, if cells excrete factors into the supernatant, causing aggregation, filter-sterilised supernatant was heat treated at 100°C for 30 min. The supernatant was cooled to room temperature, and used to resuspend cells in clumping assays.

To investigate, if the YpIII cells are forming biofilms when aggregating, several assays were performed:

Firstly, DNAse-I (NEB) was directly added to the cell suspension or PBS washed bacterial cells to a final concentration of 40 u/ml up to 200 u/ml to degrade any extracellular DNA and cells were left to settle at 37°C for 45 min (Conover *et al.*, 2011).

For detection of extracellular DNA (eDNA), cell suspension was stained by addition of 4',6-diamidino-2-phenylindole (DAPI) to a final concentration of 0.5 µg/ml - 1 µg/ml before or after settlement for 45 min.

In a third assay, rhodamine-labelled WGA (20 µg/ml final concentration) was added to the aggregated cells to visualise the extracellular matrix of the biofilm (Allen *et al.*, 1973).

2.6.4 Microscopy

For examination of bacterial colonies or for transfer of *C. elegans* between NGM plates (2.6.2), a dissecting microscope (Nikon SMZ1000, Nikon) was used. For verification of fluorescent bacteria and biofilms as well as for analysis of bacterial aggregation the Nikon Eclipse 50i fluorescence microscope (Nikon) was used with

4,000-fold magnification. Images were taken by using the Nikon picture acquisition tool (Nikon).

Confocal microscopy was carried out using the Zeiss LSM700 and image analysis performed with the Zen software (Zeiss). For biofilm on *C. elegans* lenses of 1,000-fold magnification were used.

The excitation and emission wavelengths for DAPI stained DNA were 300 nm - 420 nm and 380 nm - 600 nm and for rhodaminelabelled WGA on biofilms the excitation and emission wavelengths were 450 nm - 600 nm and 550 nm - 700nm.

3 The Relation Between T3S and Cell-Aggregation in *Y. pseudotuberculosis*

3.1 Introduction

The QS system of *Y. pseudotuberculosis* is known to influence several phenotypes which include cell-cell aggregation and virulence (type-III-secretion) (Atkinson *et al.*, 1999, Atkinson *et al.*, 2008).

Y. pseudotuberculosis was found to aggregate and settle to the bottom of a container when grown at 37°C. This aggregation phenotype was reported to be related to QS, since a mutation of *ypsR* resulted in enhanced settlement (Atkinson *et al.*, 1999). A recent observation showed that there might be a relationship between the virulence plasmid encoded T3SS and this phenotype, since curing the *ypsI/ytbI* mutant of the plasmid or mutation of *yscJ*, a structural component of the T3S injectisome stopped aggregation (Goldstone, 2011). However, previous studies on aggregation of *Yersinia* described the plasmid-encoded YadA as an aggregation factor probably due to YadA binding to itself in a zipper or velcro-like structure as a host cell adhesion factor (El Tahir and Skurnik, 2001, Hoiczyk *et al.*, 2000). YadA was also shown to bind to several surface proteins, such as β_1-chain integrins, αV integrins and other, as yet unknown factors in *Y. enterocolitica* (Keller *et al.*, 2015). Furthermore, YadA mediates tight binding to the host cell, which facilitates the multiplication of the bacteria in the mucus at the surface of Peyer's patches (Marra and Isberg, 1997).

Aggregation was also seen for antigen 43 of uropathogenic *E. coli*, which is an autotransporter, similar to YadA (Heras et al., 2014). One study suggested that the autotransporter YapC of *Y. pestis*, which lacks YadA, would cause aggregation (Felek *et al.*, 2008). Earlier studies showed that there is an aggregation factor on

the surface of *Y. pseudotuberculosis* and *Y. enterocolitica* which caused aggregation when the bacterium is grown at 37°C. This factor was identified by a Tn*5* transposon mutagenesis of pYV and was named P1 (Bölin *et al.*, 1982, Skurnik *et al.*, 1984, Balligand *et al.*, 1985). Generally, it is assumed that P1 is YadA. However, it was shown that YadA is a homotrimer of 45 kDa subunits and P1 consisted of two components of 24 kDa and 52.5 kDa. This indicates that P1 may not be YadA and that further studies, such as a in-frame deletion of *yadA* together with phenotypic assays and microscopy are needed to verify that P1 is YadA (Skurnik *et al.*, 1984, El Tahir and Skurnik, 2001). Furthermore, it was discovered that expression of YadA is maximally induced in minimal medium but repressed when grown in LB which in addition shows that YadA might not be the aggregation factor in nutrient-rich media (Eitel and Dersch, 2002, Skurnik *et al.*, 1984) Hence, it is unlikely that the previously identified aggregation factor P1 is YadA. This further supports the suggestion that other factors, such as the injectisome may be involved in promoting aggregation in complex media (Goldstone, 2011).

Yersinia possesses several other adherence factors for attachment to host cells. For invading the host, the cells firstly pass through the epithelial layer, then they attach to macrophages in the Peyer's patches inside the intestinal epithelium *via* M-cells (Grützkau *et al.*, 1990). To attach to host cells, *Y. pseudotuberculosis* uses the surface protein invasin (Inv) which binds to β_1-chain integrins on host cell surfaces (Isberg and Leong, 1990, Marra and Isberg, 1997). Expression of *inv* is mediated by the transcriptional activator RovA depending on environmental

changes and is negatively regulated by the virulence regulator YmoA (Nagel *et al.*, 2001, Ellison *et al.*, 2003). However in the absence of invasin, YadA is the main adhesin (Eitel and Dersch, 2002).

While being attached and transported by M-cells through the Peyer's patches, the main virulence determinant of *Y. pseudotuberculosis*, the T3SS, encoded on the pYV virulence plasmid is switched on. This system is important for transduction of effector proteins (Yops) into host immune cells including, macrophages (Galán and Collmer, 1999).. The T3S injectisome of the T3SS is a hyperdermic needle, which is able to inject Yop effectors into the host cell. Those effector proteins inhibit phagocytosis, disrupt actin filaments and block the innate immune response so that the bacteria cannot be detected and will be distributed within the host body through the lymph system (Diepold *et al.*, 2010, Diepold *et al.*, 2011, Torruellas *et al.*, 2005, Gamez *et al.*, 2012, Cornelis, 2002).

Several regulators of the T3S are known in *Yersinia*, such as the virulence regulatory transcription factor LcrF (VirF for *Y. enterocolitica*) and the temperature-depended protein YmoA (Garrity-Ryan *et al.*, 2010, Gallegos *et al.*, 1997, Cornelis *et al.*, 1991). *lcrF* expression at 37°C is induced by YmoA in combination with an intergenic RNA thermosensor (Böhme *et al.*, 2012). LcrF and YmoA were shown to negatively influence QS, whereas QS is upregulating expression of *ymoA* which then causes a decrease of *lcrF* expression (Amy Slater, personal communication).

It was recently revealed that QS is an important regulator for T3S since a mutation of flagellar components (*flhDC*, *fliA* and *flhA*) and several QS genes (including

ypsR, *ytbR*, *ytbI* and the double mutants *ypsI*/*ytbI*, *ypsR*/*ytbR*) lead to a release of Yops into the supernatant *in vitro* at 30°C and in presence of Ca^{2+} (Atkinson *et al.*, 2011).

3.1.1 Aims of this Study

The mechanism(s) which underpin the aggregation phenotype (1.9.4) and its relation to QS in *Y. pseudotuberculosis* are unknown. So far, it has been established that an aggregation factor is encoded on the virulence plasmid, (Skurnik *et al.*, 1984, Goldstone, 2011) that this factor is possibly YadA (El Tahir and Skurnik, 2001) and that supernatant factors may be involved (Goldstone, 2011).

Atkinson *et al.*(1999) showed that a deletion of *ypsR* leads to an enhanced aggregation phenotype, revealing that QS regulates aggregation.

This chapter aims to determine

- how QS mediates aggregation,
- the role of the virulence plasmid in promoting aggregation,
- identification of the plasmid encoded factor responsible for aggregation.

3.2 Results

3.2.1 The Role of pYV in *Y. pseudotuberculosis* Aggregation

Initially this study was directed towards clarifying the role of the pYV plasmid for *Y. pseudotuberculosis* aggregation and to investigate whether plasmid encoded genes are responsible for this phenotype. Cultures containing either 100 % pYV or 0 % pYV were prepared and mixed to investigate the aggregation behaviour of cultures with varying levels of pYV.

Aggregation assays and microscopy revealed that cells containing 100 % pYV began to aggregate immediately and the aggregates became more dense on further incubation (Figures 3.1 and 3.2). After 45 min the density of the aggregates was highest. However, the assay and microscopy revealed that aggregation falls with decreasing pYV proportion of the population and that there is a mixture of

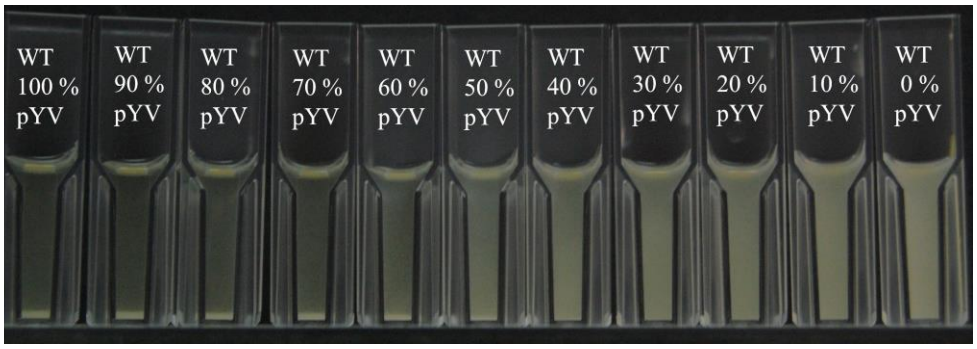


Figure 3.1: Aggregation of the *Y. pseudotuberculosis* YpIII wild-type (WT) after growth at 37°C. 100 % pYV^+ cells were mixed with pYV^- cells to obtain ratios of 100 % until 0 % pYV in the population. After incubation for 45 min, cultures containing 60 % to 100 % pYV have almost completely settled out. The culture with 50 % pYV has partially settled and cultures containing less than 50% of pYV or no pYV do not aggregate.

aggregates and planktonic cells within the culture. The numbers of planktonic cells also rise with decreasing pYV levels.

However, it was observed that cells were aggregated at time-point 0 min, before being transferred into a cuvette (Figure 3.2), suggesting the cells start aggregating while growing in liquid culture at 37°C.

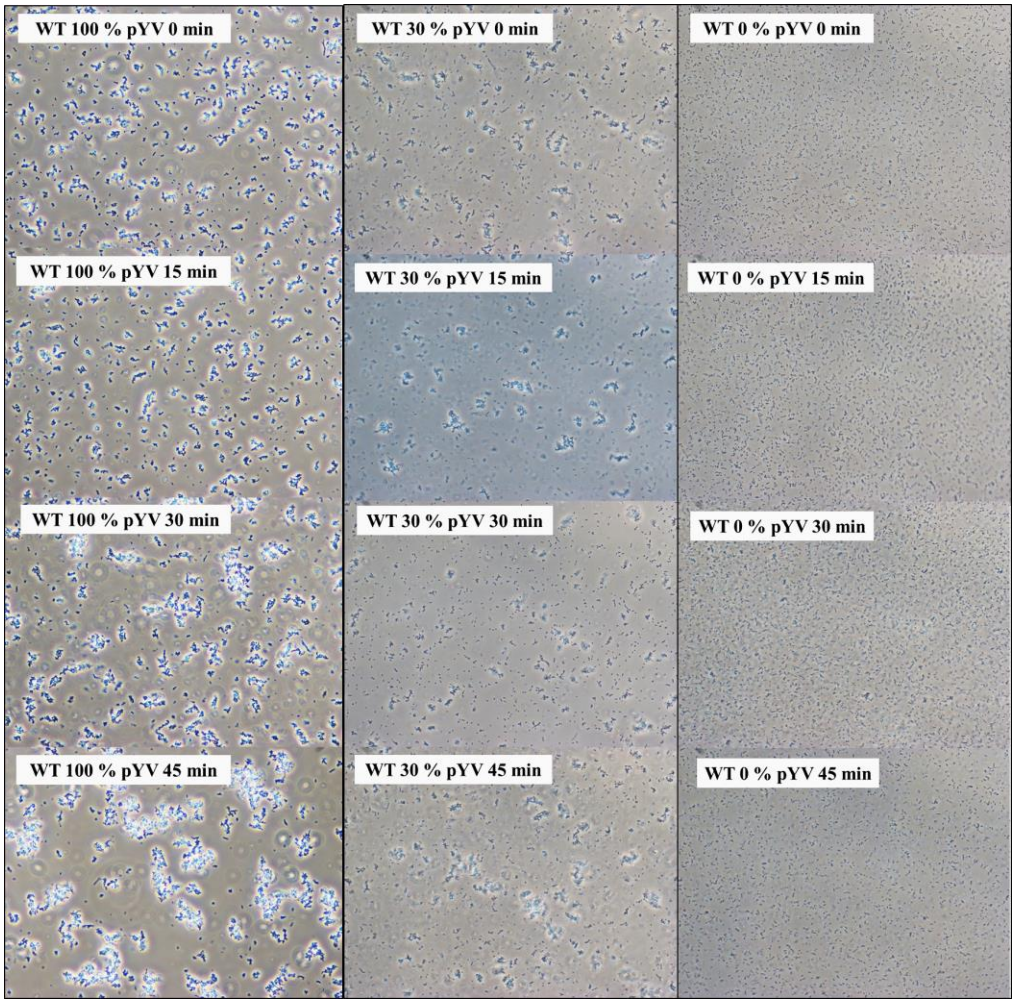


Figure 3.2: Microscopy showing aggregation of *Y. pseudotuberculosis* YPIII containing 100 %, 30 % or 0 % pYV over time. Images were taken at the start of the experiment and after 15 min, 30 min and 45 min of incubation. Cells containing 100 % pYV show strongest aggregation after 30 min and 45 min and populations containing 30 % pYV show similar minimal aggregation over the time period. Cultures lacking pYV do not show any aggregation.

It must be noted that when repeating these assays, differences in the aggregate density were observed. This was due to a variation in population density since the assays were performed between OD_{600} 2.0 and OD_{600} 3.0.

3.2.2 The Role of QS in Aggregation

Previous studies showed that QS of *Y. pseudotuberculosis* has an effect on aggregation since mutation of the QS response regulator, *ypsR* or the AHL synthase genes *ypsI/ytbI* lead to enhanced aggregation (Atkinson *et al.*, 1999, Goldstone, 2011).

Since the previous section revealed that pYV influences aggregation, it was important to examine all the QS mutants; including those previously reported by Atkinson *et al.* (1999) and Goldstone (2011), while taking into account the role of pYV. All single QS mutants (Δ*ypsI*, Δ*ypsR*, Δ*ytbI*, Δ*ytbR*) and two of the double mutants (Δ*ypsI*/Δ*ytbI*, Δ*ypsR*/Δ*ytbR*) were compared with the *Y. pseudotuberculosis* wild-type in their aggregation behaviour. 100 % pYV (pYV^+) populations and 0 % pYV (pYV^-) populations were prepared and observed at different time points. While there was no difference between the WT and any of the single mutants (data not shown), the experiments revealed that Δ*ypsI*/Δ*ytbI* aggregated faster than the wild-type (Figure 3.3 A and B). The same result was obtained when comparing the Δ*ypsR*/Δ*ytbR* with the wild-type (data not shown). These findings confirmed a role for QS in the aggregation phenotype.

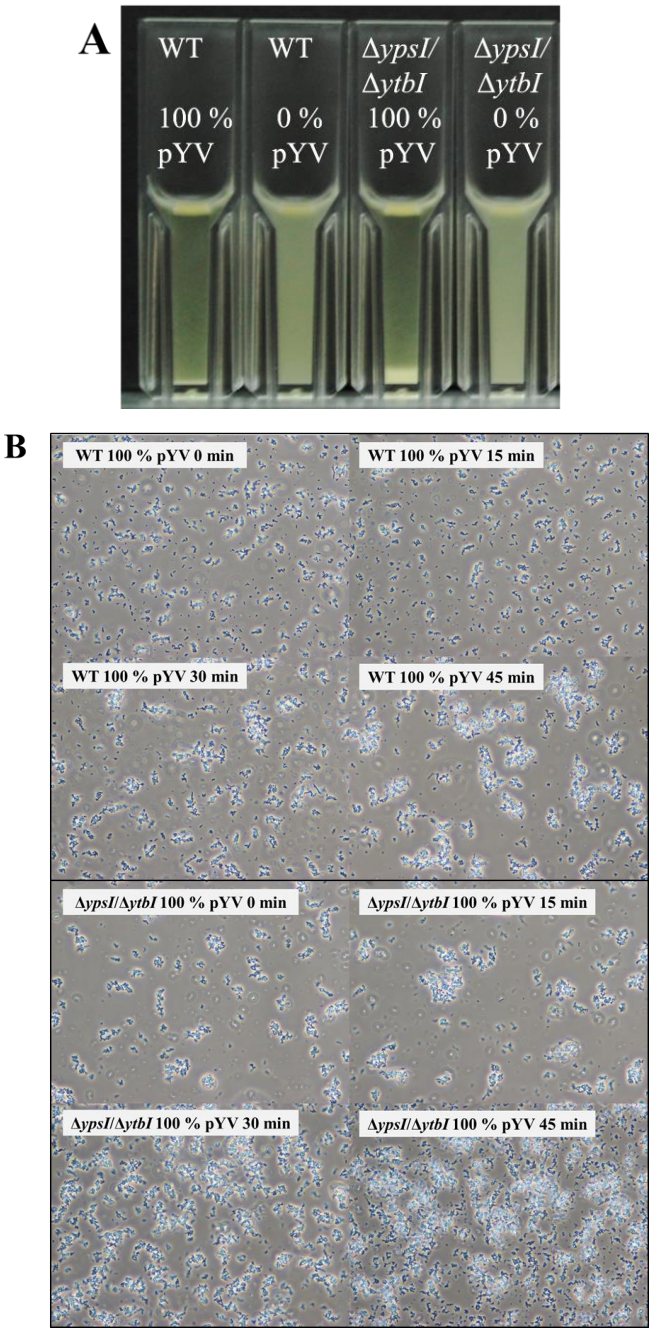


Figure 3.3: (A) Aggregation of the *Y. pseudotuberculosis* YpIII wild-type and the Δ*ypsI*/Δ*ytbI* after 45 min, containing either 100 % pYV or no pYV in the population, respectively. (B) Micrographs of the wild-type and Δ*ypsI*/Δ*ytbI* aggregates taken at different time points.

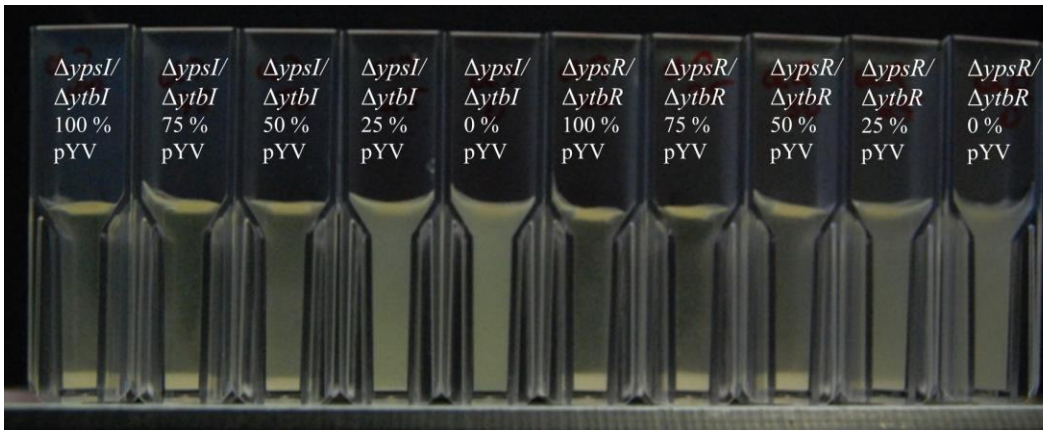


Figure 3.4: Aggregation of Δ*ypsI*/Δ*ytbI* and Δ*ypsR*/Δ*ytbR*, containing different percentages of pYV in their population. Both QS mutants aggregate at the same density and rate. Aggregation cannot be seen when there is 0 % of pYV in the population.

To clarify the role of pYV for the QS double mutants (Δ*ypsI*/Δ*ytbI*, Δ*ypsR*/Δ*ytbR*), cultures containing a range of pYV levels were examined. Figure 3.4 shows that aggregation is dependent on pYV in both YpIII QS double mutants. These results are similar to those obtained for the wild-type in 3.2.1, however after 45 min, the wild-type culture containing 50 % pYV is far less aggregated than the QS mutants containing the same percentage of pYV (Figure 3.4), which suggests that QS is involved in regulation of aggregation.

3.2.3 Biofilms are Not Required for Aggregation

Studies on *S. enterica* ser. Typhimurium and *P. aeruginosa* have indicated that biofilms are produced by aggregated cells for enhanced survival in the host at early stages of infection and for more efficient infection (Jennings *et al.*, 2012,

Tran *et al.*, 2014). Since QS regulates *Y. pseudotuberculosis* biofilms on *C. elegans* (Atkinson et al., 2011), it was important to investigate whether the QS mediated aggregation phenotype was also biofilm dependent.

Since biofilms of *Y. pseudotuberculosis* are known to produce an ECM containing polyGlcNAc (Bobrov *et al.*, 2008), rhodamine-labelled wheat germ agglutinin (WGA) was used to stain the aggregates. Since the pYV^{+} Δ*ypsI*/Δ*ytbI* aggregated more than the wild-type, aggregates from this strain were incubated with WGA and analysed under the fluorescence microscope. No ECM matrix indicative of biofilm formation was apparent after 45 min of incubation suggesting that there is no polyGlcNAc in the aggregates (data not shown).

Although GlcNAc does not appear to be present other biofilm-related substances may be found in the surrounding region of the aggregates. Biofilms usually contain eDNA which is important for connection with other biofilm matrix components to form network-like structures and provide stability for the biofilm architecture (Allesen-Holm *et al.*, 2006, Conover *et al.*, 2011). Aggregates were stained with DAPI to determine whether any eDNA was present. Aggregates of pYV^{+} Δ*ypsI*/Δ*ytbI* fluoresce blue, suggesting that there is eDNA surrounding the cells. However, pYV^{-} bacteria which were not aggregated or settled to the bottom of their container could also be shown to possess eDNA on their surface (data not shown). These data suggest that eDNA might generally be a surrounding substance of *Y. pseudotuberculosis* grown at 37°C rather than a biofilm compound. Nevertheless these data do not unequivocally establish the presence of eDNA in the aggregates.

To determine whether disruption of eDNA disperses the aggregates, they were treated with DNAse-I, since this enzyme was reported to be very efficient for disruption of biofilms of *Bordetella* sp. (Conover *et al.*, 2011). After treatment cells were stained with DAPI and Figure 3.5 shows that there is no difference in aggregation of the Δ*ypsI*/Δ*ytbI* when incubated with different concentrations of DNAse-I. However, when using the fluorescence microscope, a reduction of DAPI-stained eDNA is visible when cells were treated with 200 u/ml of DNAse-I but not when treated with less (20 u/ml or 60 u/ml) DNAse-I, suggesting that only high concentrations of DNAse-I disrupt *Y. pseudotuberculosis* eDNA. Since cells are aggregating independently of the existence of eDNA, it can be assumed that eDNA is not required for aggregation but that aggregation might be required for release of eDNA.

Taken together, results using WGA, DNAse-I and DAPI suggest that the aggregation phenotype is unlikely to be a biofilm-mediated process.

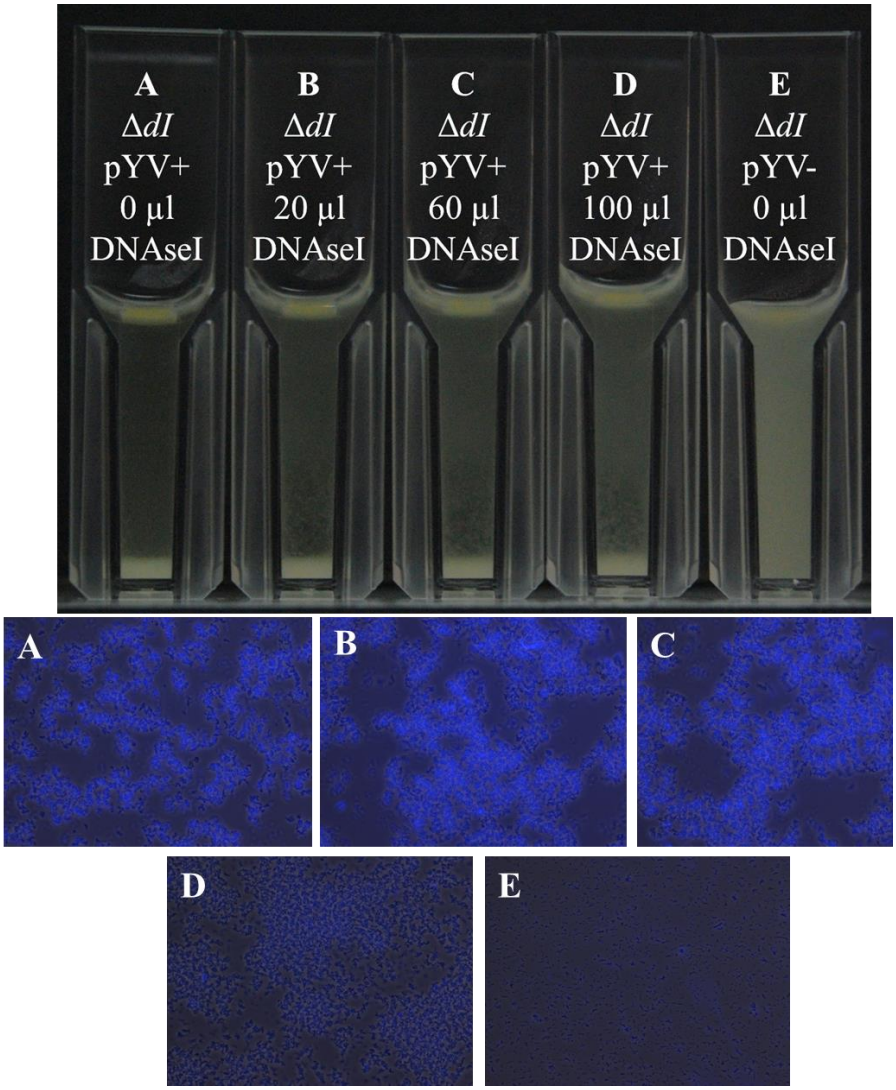


Figure 3.5: Aggregation assay of the *Y. pseudotuberculosis* Δ*ypsI*/Δ*ytbI* treated with different concentrations of DNAse-I (0 u/ml -A, 40 u/ml -B, 60 u/ml -C and 200 u/ml -D) as well as pYV$^-$ negative control -E. After treatment cells were stained with DAPI and observed by phase contrast/fluorescence microscopy. Incubation with 200 u/ml DNAse-1 causes a degradation of eDNA but has no effect on aggregation (D).

3.2.4 Is there a Role for Both Supernatant and Surface Factors in Aggregation?

Since the work described in the last section suggests that the cellular aggregates are not suspended biofilms, it was important to identify supernatant and surface factors that may be important for the aggregation phenotype.

To determine whether a supernatant factor was responsible for aggregation two assays were performed. Firstly pellets of 100 % pYV and 0 % pYV wild-type and Δ*ypsI*/Δ*ytbI* cultures were each resuspended in spent supernatant from either 100 % pYV or 0 % pYV wild-type and Δ*ypsI*/Δ*ytbI* cultures, respectively. In a second experiment supernatants of the wild-type and Δ*ypsI*/Δ*ytbI* were boiled to denature any heat sensitive substances. The supernatant was then used to resuspend the pellet of the culture it was taken from.

The results from both experiments revealed that the wild-type does not aggregate faster when resuspended with Δ*ypsI*/Δ*ytbI* supernatant and the Δ*ypsI*/Δ*ytbI* does not aggregate more slowly when resuspended in wild type supernatant (data not shown). Furthermore, addition of supernatant from pYV^- cultures did not inhibit aggregation and boiling the supernatant did not affect aggregation (data not shown). This indicates that no aggregation factor is present in the supernatant of *Y. pseudotuberculosis*.

It was also important to investigate the surface factors involved in aggregation since several studies showed that components of the bacterial envelope, such as YadA and YapC of *Y. pseudotuberculosis* and OmpX in *Y. pestis* promote this

phenotype (El Tahir and Skurnik, 2001, Felek et al., 2008, Kolodziejek et al., 2007).

Figure 3.6 shows that after treatment with proteinase K, both the wild-type and the Δ*ypsI*/Δ*ytbI* stop aggregating and show a similar phenotype to the pYV$^-$ wild type. However, controls resuspended in LB as well as controls washed and resuspended in PBS aggregate normally (Figure 3.6). The fact that PBS washed pellets still aggregate provides additional confirmation that no supernatant factor is involved in aggregation. The proteinase K treatment strongly suggests that a surface associated protein(s) promotes this phenotype.

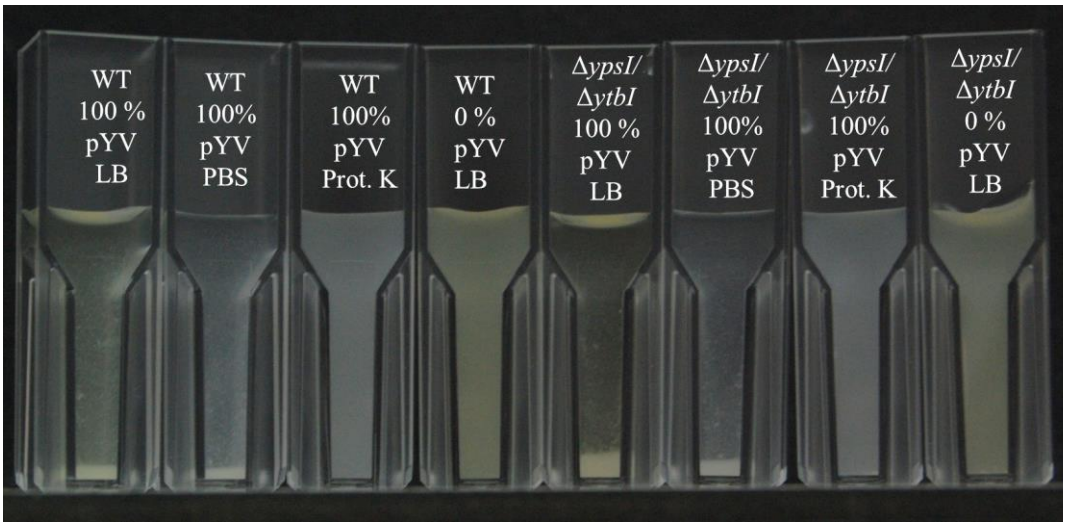


Figure 3.6: Treatment of the *Y. pseudotuberculosis* wild-type and Δ*ypsI*/Δ*ytbI* with proteinase K abolishes aggregation. The pYV$^+$ wild-type and Δ*ypsI*/Δ*ytbI* resuspended in LB as well as washed and resuspended in PBS aggregate whereas treatment with proteinase K inhibits aggregation and cultures shows the same phenotype as a pYV$^-$ strain.

3.2.5 Transposon Mutagenesis of the Virulence Plasmid

Early *Yersinia* research revealed that the virulence plasmid is important for aggregation of *Y. enterocolitica* and *Y. pseudotuberculosis* and suggested that the pYV encoded P1, was an important aggregation factor. Studies with a Tn*5* mutant of P1 showed that a mutation of the *p1* gene leads to a non-aggregating phenotype (Skurnik *et al.*, 1984). Later studies linked this protein with other phenotypes such as host cell attachment and virulence and it was renamed YadA (Skurnik, 2007). Results of this study confirmed that a surface protein(s) encoded on the virulence plasmid is the aggregation factor.

To unequivocally identify the surface proteins responsible for the aggregation phenotype *in vitro,* Tn*5* transposon mutagenesis (Goryshin and Reznikoff, 1998) of the pYV extracted from hyper-aggregating Δ*ypsI*/Δ*ytbI* was performed. After random insertion of the transposons, the mutated pYV plasmids were introduced back into a pYV$^-$ Δ*ypsI*/Δ*ytbI* and were screened for loss of aggregation. In total pYV plasmids from 16 non-aggregating cultures were extracted and sequenced using the Tn*5*-primers Tc-Tn*5*-F and Tc-Tn*5*-R (Table 2.3). Results were analysed using the Artemis® tool (Sanger) by comparing the sequences obtained with the known pYV sequence of *Y. pseudotuberculosis* pB1/+ (Challacombe, unpublished data, NCBI reference: NC_010635.1). Out of the 16 insertions sequences, 4 had fragments of 8 - 14 genes deleted and these were disregarded. The other 12 transposons had inserted in the individual genes listed in Table 3.1.

Table 3.1: Results of the sequence analysis of the Tn*5* transposon insertions into pYV that result in loss of the aggregation phenotype.

Inserted Gene	Function of Gene Product	Transposon Insertion Position	In Frame?
3 x *yscN*	ATPase of the T3SS	1- 45/54 of 1320 bp 2- 303/312 of 1320 bp 3- 303/312 of 1320 bp	Yes Yes Yes
1 x *yscT*	Part of export apparatus of T3SS	147/156 of 786 bp	Yes
2x *yscU*	Part of export apparatus of T3SS	1- 257/266 of 1065 bp 2- 367/376 of 1065 bp	No No
1 x *yscV*	Part of export apparatus of T3SS	561/570 of 2115 bp	Yes
1 x *yscR*	Part of export apparatus of T3SS	313/322 of 654 bp	No
1 x *yscG*	Yop chaperone of the T3SS	231/240 of 448 bp	Yes
1 x *yopH*	Yop effector of the T3SS	174/183 of 1407 bp	Yes
1 x *sycH*	YopH chaperone of the T3SS	96/105 of 426 bp	Yes
1 x YPTS_4263	Function not clear	116/125 of 618 bp	No

11 of 12 genes contained transposon insertions that are important for a functioning T3SS. 8 of the 12 genes are injectisome structural proteins, of which *yscN* encodes for the ATPase and *yscT*, *yscU*, *yscV* and *yscR* encode for 4 of the 5 structural genes of the export apparatus. The other remaining genes encode the Yop effector YopH, its targeting protein SycH and the Yop chaperone YscG. YPTS_4263 belongs to the TraT superfamily, important for complement resistance by conjugal transfer.

3.2.6 Mutations in *yscF* and *yscV*

Although the Tn*5* mutagenesis revealed that the injectisome was involved in aggregation, it was assumed that exposed structural proteins were more likely to be contributing factors to aggregation, since proteinase K treatment stopped aggregation of pYV^{+} cells (3.2.4).

The assembly of the T3S injectisome begins with the production of the inner membrane located export apparatus and the outer membrane components (YscC and YscD), which will be linked to each other by YscJ. Further components of the injectisome such as YscN and YscF will be assembled afterwards (Figure 3.7). It was shown that the absence of YscC/YscD will stop the production cascade to add YscJ and YscN, which then prevents the YscF polymerisation into the needle-like

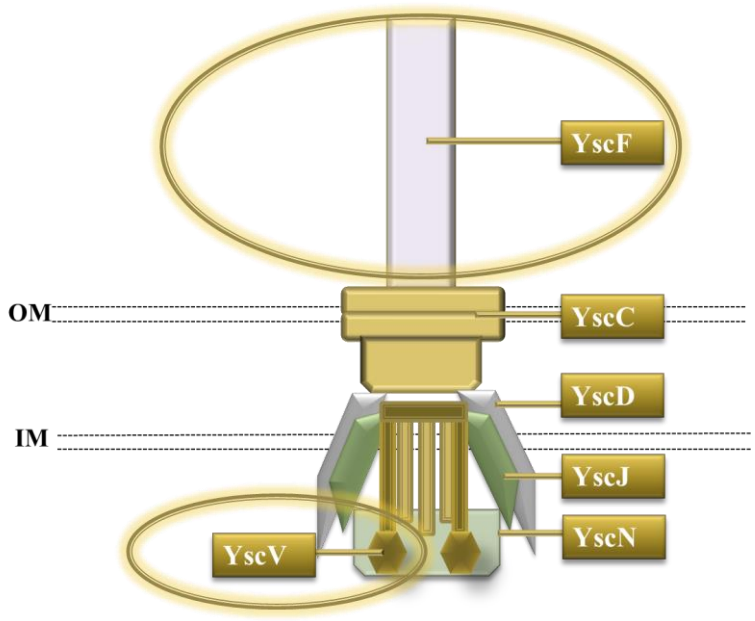


Figure 3.7: Simplified illustration of the assembled *Y. pseudotuberculosis* injectisome. Highlighted are the needle structure YscF and one export apparatus part YscV. The figure was adapted from Diepold *et al.*, 2011.

structure (Diepold *et al.*, 2010, Diepold *et al.*, 2011). Furthermore, a functioning injectisome is important for activation of Yop production, transport and secretion and hence there will be a lack of Yops after deletion of injectisome components (Allaoui *et al.*, 1995). Results in 3.2.4 show that a surface protein is involved in aggregation. This result together with the Tn*5* mutagenesis suggests that, despite the majority of the Tn*5* insertions being in genes encoding for inner membrane and cytoplasmic components of the T3S injectisome, the inactivation of these genes may have been indicative of a loss of later expressed, surface exposed proteins such as YscF.

Two T3SS proteins were therefore chosen for further study YscF and YscV. YscF, the injectisome needle is a surface exposed late export protein (Deane *et al.*, 2006), which was not represented among the Tn*5* insertion mutants. YscV is one of the early injectisome assembly proteins which acts as an anchor protein in the inner membrane. YscV was disrupted by a Tn*5* insertion and is known to abolish assembly of a functioning injectisome when inactivated (Diepold *et al.*, 2011).

3.2.6.1 Construction of the Needle Mutants Using Suicide Vectors

The mutations of *yscF* and *yscV* were constructed in the wild-type and Δ*ypsI*/Δ*ytbI* by using a suicide plasmid for allelic exchange.

Primers used for *yscF* mutagenesis were *yscF*-up-F and *yscF*-up-R (Table 2.3) to amplify a 539 bp fragment consisting of 458 bp of the upstream region of *yscF* and 59 bp of *yscF*, Tc-no-prom-F and Tc-no-term-R for amplification of the

1264 bp tetracycline cassette, as well as *yscF*-dn-F and *yscF*-dn-R for amplification of a 532 bp fragment consisting of 487 bp of the downstream region and 24 bp of *yscF*, which were then assembled into a 2335 bp fragment in pDM4 and were integrated into *Y. pseudotuberculosis* YpIII wild-type and the Δ*ypsI*/Δ*ytbI* pYV plasmids *via* allelic exchange resulting in Δ*yscF* and the triple mutant Δ*yscF*/Δ*ypsI*/Δ*ytbI*. Mutation of the gene was confirmed by sequence analysis using primers *yscF*-ko-ch-F and *yscF*-ko-ch-R (Table 2.3).

For mutation of *yscV* in the YpIII wild-type and Δ*ypsI*/Δ*ytbI* mutant, *yscV*-up-F and *yscV*-up-R (Table 2.3) were used to amplify a 564 bp fragment consisting of 483 bp of upstream region and 60 bp of *yscV*, Gm-no-prom-F and Gm-no-term-R for amplification of the 580 bp gentamicin cassette, as well as *yscV*-dn-F and *yscV*-dn-R for amplification of a 587 bp fragment containing 491 bp of the downstream region and 75 bp of *yscV*. For mutagenesis the total construct was cloned into pDM4 before being conjugated into both YpIII strains. The resulting mutants were termed Δ*yscV* and Δ*yscV*/Δ*ypsI*/Δ*ytbI*. Mutation of the gene was confirmed by sequence analysis using primers *yscV*-ko-ch-F and *yscV*-ko-ch-R (Table 2.3) (data not shown).

After mutagenesis of the two injectisome components, functional copies of *yscF* and *yscV* including the promoter region and open reading frame (ORF) were ligated into pHG324 and inserted into the relevant Δ*yscF*, Δ*yscF*/Δ*ypsI*/Δ*ytbI*, Δ*yscV* and Δ*yscV*/Δ*ypsI*/Δ*ytbI*. To achieve this a 836 bp fragment was amplified containing 533 bp of promoter region and 246 bp *yscF* ORF and for *yscV*, a

2686 bp fragment was amplified containing 529 bp of its promoter region and 2115 bp of the *yscV* ORF (data not shown).

3.2.6.2 Phenotypic Confirmation of Δ*yscF* and Δ*yscV*

After mutation of *yscF* and *yscV*, it was necessary to confirm the mutation in phenotypic assays. Since it is known that deletion of each gene inhibits Yop secretion (Diepold *et al.*, 2011, Torruellas *et al.*, 2005), supernatant protein assays in Yop inducing conditions were performed.

Figure 3.8 reveals that mutation of either *yscF* or *yscV* in the wild-type and in Δ*ypsI*/Δ*ytbI* abolish Yop secretion. After insertion of a functioning copy of the respective genes into the mutated bacteria on a low-copy plasmid, the secretion of

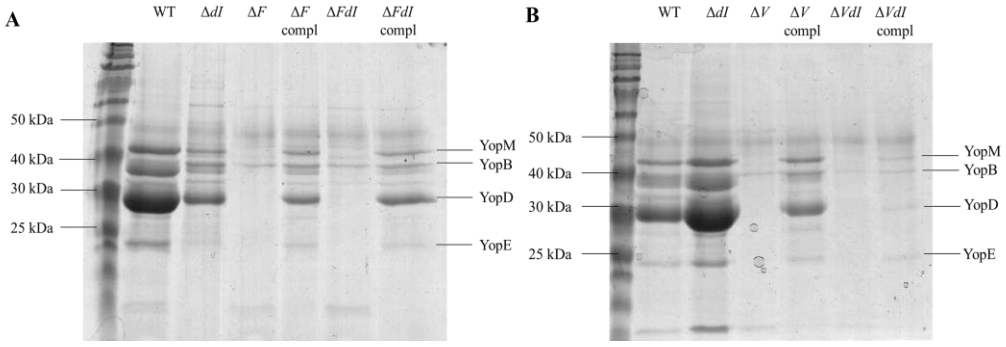


Figure 3.8: (A) SDS-PAGE of Yops secreted into the supernatants of the YpIII wild-type (WT), the Δ*ypsI*/Δ*ytbI* (Δ*dI*), the Δ*yscF* (Δ*F*), the complemented Δ*yscF* (Δ*F* compl), the Δ*yscF*/Δ*ypsI*/Δ*ytbI* (Δ*FdI*) and the complemented Δ*yscF*/Δ*ypsI*/Δ*ytbI* (Δ*FdI* compl) (B) Secreted Yop profiles of the WT and Δ*dI* in comparison with the Δ*yscV* (Δ*V*), the complemented Δ*yscV* (Δ*V* compl), the Δ*yscV*/Δ*ypsI*/Δ*ytbI* (Δ*VdI*) and the complemented Δ*yscV*/Δ*ypsI*/Δ*ytbI* (Δ*VdI* compl). Mutation of *yscF* and *yscV* abolishes extraction of Yops in the single and triple mutants, respectively. Insertion of a functioning plasmid-borne *yscF* or *yscV* into the relevant mutants restored Yop secretion.

Yops was restored. It is noticeable that the expression of Yops in the complemented triple mutant (Δ*yscV*/Δ*ypsI*/Δ*ytbI*) was lower than the expression of Yops in the wild-type and double mutant. However, there is a leakage of YopB into the supernatant when the needle and the export apparatus are not expressed.

3.2.7 Aggregation Assays of Δ*yscF* and Δ*yscV*

After confirming successful mutation of *yscF* and *yscV*, aggregation assays were performed to confirm the non-clumping phenotype detected for the Tn*5* mutants inserted into injectisome components (Table 3.1.)

Additionally, since QS also regulates aggregation, triple mutants (Δ*yscF*/Δ*ypsI*/Δ*ytbI* and Δ*yscV*/Δ*ypsI*/Δ*ytbI*) may help to identify the direct role of QS in promoting aggregation.

Since Atkinson *et al.* (2011) demonstrated that a deletion of both *ypsI* and *ytbI* led to a loss of T3SS regulation, enhanced aggregation might be caused by overexpression of the T3SS in the QS mutants.

Microscopy of the aggregates shown in Figure 3.9 and Figure 3.10 reveal that Δ*yscF* and Δ*yscV* do not aggregate and so behave like pYV$^-$ cultures and that complementation restored aggregation to wild-type levels. The Δ*yscV*/Δ*ypsI*/Δ*ytbI* was also unable to aggregate when compared to the Δ*ypsI*/Δ*ytbI*. However aggregation albeit reduced, still occurred in the Δ*yscF*/Δ*ypsI*/Δ*ytbI* mutant. Aggregation was partially restored by inserting a functional copy of *yscF* and *yscV* into the Δ*yscF*/Δ*ypsI*/Δ*ytbI* and Δ*yscV*/Δ*ypsI*/Δ*ytbI*, respectively.

These data suggest that the injectisome needle and the export apparatus both have contributing roles in aggregation. However, the Δ*yscF*/Δ*ypsI*/Δ*ytbI* mutant showed slight aggregation compared to the single *yscF* mutant which underpins the previous finding that QS affects aggregation and suggests that the injectisome needle is important for aggregation but that there are also other plasmid encoded factors, which promote aggregation, that are QS regulated.

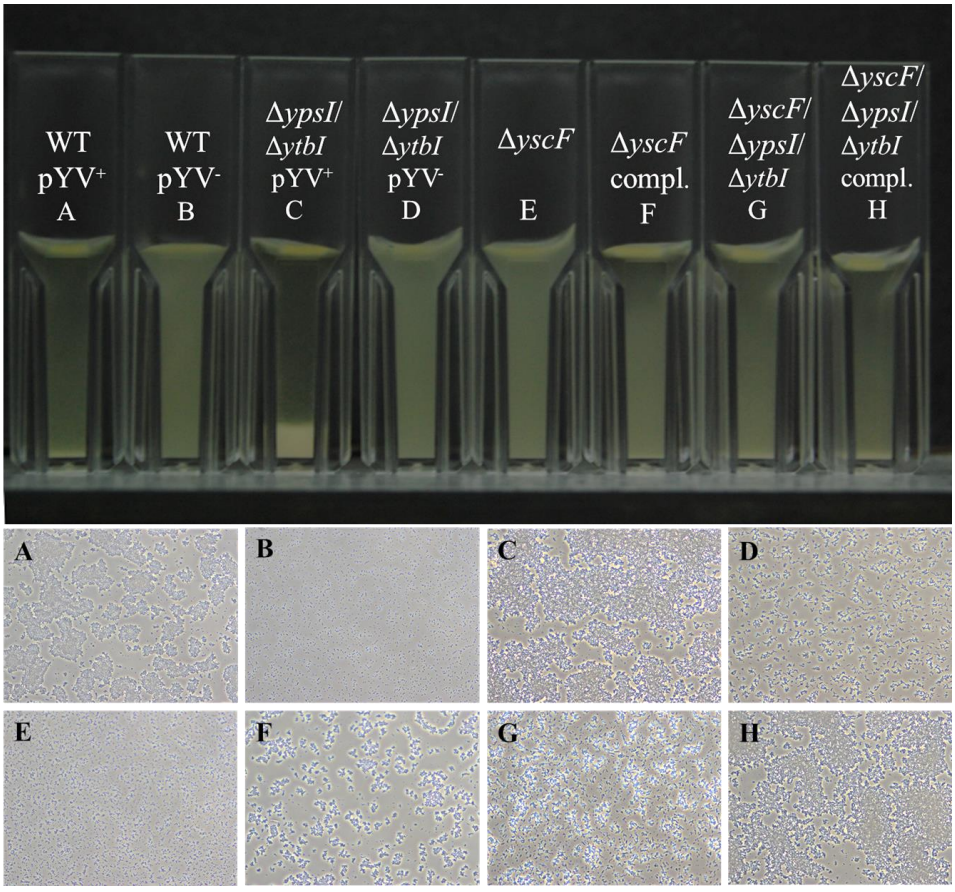


Figure 3.9: Aggregation assay of the pYV$^+$ and pYV$^-$ *Y. pseudotuberculosis* wild-type (A, B) and Δ*ypsI*/Δ*ytbI*(C, D) compared with Δ*yscF* (E), the complemented Δ*yscF* (F) and the triple mutant Δ*yscF*/Δ*ypsI*/Δ*ytbI* (G) and complemented Δ*yscF*/Δ*ypsI*/Δ*ytbI* (H). The pYV$^+$ wild-type shows aggregation, which is enhanced in the Δ*ypsI*/Δ*ytbI*. The pYV$^-$ controls do not aggregate. Mutation of *yscF* in the wild-type abolishes aggregation of the cells and the triple mutant shows slight aggregation. The aggregation phenotype reverts to the wild-type and Δ*ypsI*/Δ*ytbI* aggregation levels when complemented.

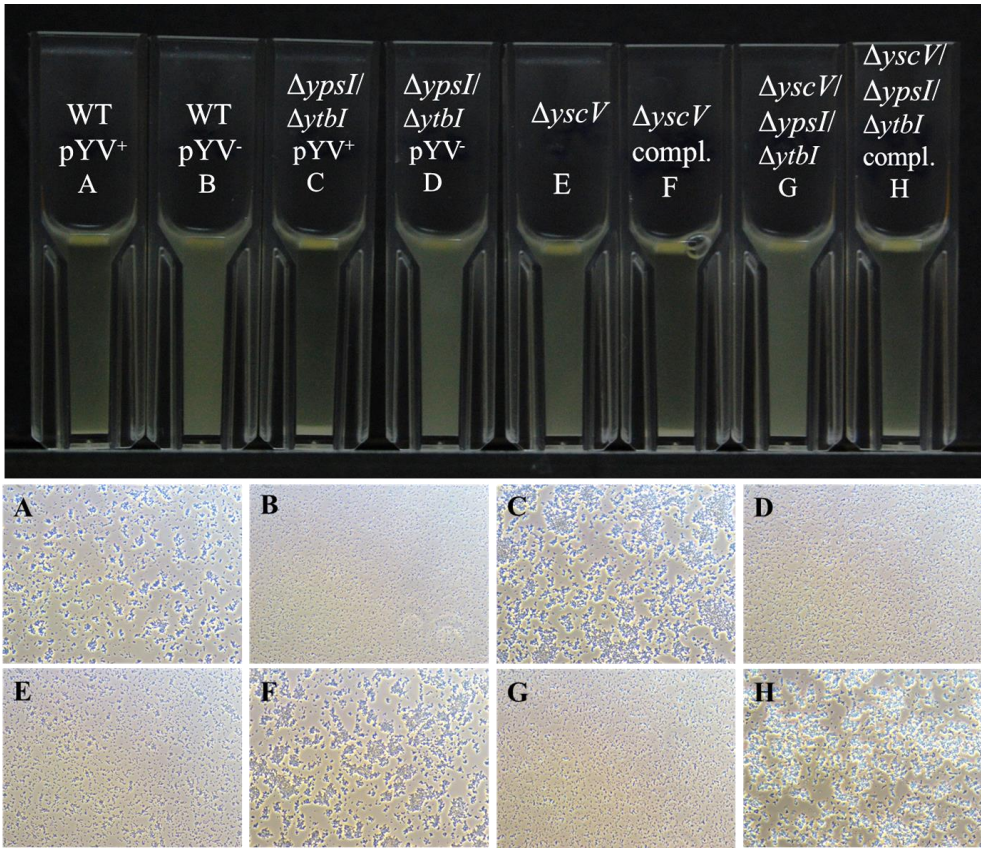


Figure 3.10: Aggregation of the pYV$^+$ and pYV$^-$ *Y. pseudotuberculosis* wild-type (A, B) and Δ*ypsI*/Δ*ytbI* (C, D) in comparison to Δ*yscV* (E), complemented Δ*yscV* (F) and the triple mutant Δ*yscV*/Δ*ypsI*/Δ*ytbI* (G) and complemented Δ*yscV*/Δ*ypsI*/Δ*ytbI* (H). The pYV$^+$ wild-type shows aggregation. This phenotype is enhanced in the Δ*ypsI*/Δ*ytbI* mutant. The pYV$^-$ controls do not aggregate. Mutation of *yscV* in both strains abolishes aggregation of the cells. The aggregation phenotype reverts to the wild-type and Δ*ypsI*/Δ*ytbI* aggregation levels when complemented.

3.2.8 Summary

In conclusion, aggregation of *Y. pseudotuberculosis* is mediated by a surface protein encoded on the virulence plasmid and regulated by QS. Results show that biofilms are not important for aggregation but that eDNA is surrounding the aggregates. Tn*5* transposon mutagenesis revealed that the T3S injectisome is

essential for aggregation. Further mutation of the export apparatus structure YscV and the injectisome needle YscF underpinned this results leading to the assumption that the injectisome might be the aggregation factor.

3.3 Discussion

Studies of *Y. pseudotuberculosis* and *Y. enterocolitica* already reported a role for pYV in aggregation (Skurnik *et al.*, 1984). Atkinson *et al.* (1999) showed that aggregation was QS dependent but did not identify the target(s) and Goldstone (2011) proposed that aggregation is a process mediated by supernatant and surface factors.

This study confirmed that the presence of pYV is necessary for *Y. pseudotuberculosis* aggregation, since there is a dose response of aggregation to the proportion of pYV in the population (3.2.1). There needs to be at least 50 % pYV in the population for the cells to aggregate. However, when cultures containing > 50 % pYV were observed under the microscope, after harvesting by centrifugation, they were already aggregated to some extent. This suggests that cells might already aggregate after each division, before cultures are left to settle and that it takes 45 min until all aggregated cells are settled.

The next step was to confirm the role of QS in aggregation. By testing the four QS single mutants of *Y. pseudotuberculosis,* Atkinson *et al.* (1999) showed that QS plays a role in the aggregation of YpIII. However, they did not consider that the

presence of pYV in the cells might be correlated with aggregation and showed that the wild-type in contrast to the Δ*ypsR* mutant failed to aggregate.

It was therefore necessary to examine the role of aggregation in all QS mutants and if the proportion of pYV in the population is important. There were no differences in aggregation between the YpIII wild-type and single QS mutants in contrast to the data shown by Atkinson *et al.* (1999), who possibly worked with a pYV^- wild-type. However, the double mutants Δ*ypsI*/Δ*ytbI* and Δ*ypsR*/Δ*ytbR* lacking either both AHL synthases or QS response regulators showed increased settlement and bigger aggregates. Furthermore, the Δ*ypsI*/Δ*ytbI* and Δ*ypsR*/Δ*ytbR* still aggregate with only 25 % pYV in the population.

This reveals that the expression of the aggregation factor is repressed under wild-type conditions but by mutation of both AHL synthases and QS response regulators, this repression is released and aggregation enhanced.

Aggregation of bacteria is often linked to subsequent biofilm formation which helps bacteria overcome environmental challenges, which was shown for example in *S. Typhimurium* and *P. aeruginosa* (Jennings *et al.*, 2012, Tran *et al.*, 2014). In contrast, a study using *Erwinia chrysanthemi* revealed aggregation was not biofilm related (Yap *et al.*, 2005). For *Yersinia,* a relationship between biofilms and aggregates has not yet been established.

Interestingly, when staining the cell aggregates with WGA, no polyGlcNAc could be detected, suggesting biofilms are not produced or the ECM consists of different exopolymers which have not yet been identified in *Yersinia*. This could be the case since this study showed that aggregated bacteria released eDNA into their

environment and eDNA is often found in biofilms (Allesen-Holm *et al.*, 2006, Goldstone, 2011). However, treatment with DNAse-I did not affect aggregation, which suggests that if biofilm is surrounding aggregated bacteria, it will be produced independently of aggregation and might even be produced afterwards. To find out if ECM forms around *Y. pseudotuberculosis* aggregates, other fluorescent labelled lectins or antibodies could be tried which bind to other potential extracellular polymeric substances that are known to contribute to the ECM such as alginate, Pel and Psl produced by *P. aeruginosa* or homopolysaccharides such as glucans and fructans which are sugars in the ECM of oral streptococci [reviewed in (Flemming and Wingender, 2010)].

After revealing that aggregation is not mediated by biofilms, it was determined whether extracellular or cell surface factors were involved in aggregation. The data obtained did not demonstrate a role for supernatant factors in experiments based on mixing supernatants from aggregated Δ*ypsI*/Δ*ytbI* and the wild-type cells or by heat treatment of the supernatant. However, it was shown that aggregation was pYV dependent and that treatment of the cells with proteinase K inhibited aggregation of both the wild-type and the Δ*ypsI*/Δ*ytbI*, revealing that surface protein factors encoded on the virulence plasmid are responsible for aggregation.

Tn*5* transposon mutagenesis was performed to identify the aggregation factor. 8 of 16 transposon insertions that resulted in the loss of the aggregation phenotype were incorporated in genes important for the expression of parts of the

injectisome, including *yscN*, the ATPase and parts of the export apparatus (*yscV*, *yscU*, *yscT* and *yscR*).

It was therefore necessary to identify which protein component of the injectisome was responsible for aggregation. Since the ATPase and the export apparatus proteins are not located at the bacterial surface, it can be assumed that deletion of the corresponding genes leads to inhibition of later injectisome assembly, such as the injectisome needle YscF which is located at the bacterial surface (Deane *et al.*, 2006, Diepold *et al.*, 2011).

This was confirmed by aggregation assays of a *yscJ* mutant which caused loss of aggregation in the Δ*ypsI*/Δ*ytbI* mutant (Goldstone, 2011). YscJ is a ring structure (MS ring) joining the outer membrane ring and the inner membrane export apparatus (Silva-Herzog *et al.*, 2008), hence it is essential for the development of the entire injectisome needle and for the secretion of Yops (Diepold *et al.*, 2011). The non-aggregating phenotype of Δ*yscJ*/Δ*ypsI*/Δ*ytbI* could be restored by complementing with plasmid-borne *yscJ* and suggests that the injectisome plays an essential role in cell-cell aggregation for *Y. pseudotuberculosis* (Goldstone, 2011).

After revealing that a functioning T3SS is important for aggregation of *Y. pseudotuberculosis*, two additional injectisome mutants were constructed, namely Δ*yscF* and Δ*yscV*, to identify which components of the injectisome are important for aggregation.

Aggregation was abolished by a lack of *yscV* in the wild-type and the Δ*ypsI*/Δ*ytbI*, showing that either a functioning export apparatus or ATPase and needle complex is necessary for aggregation. However mutation of *yscF* also abolished aggregation, leading to the assumption that the injectisome needle is the aggregation factor. Interestingly, aggregation could not be entirely abolished in the Δ*yscF*Δ*ypsI*/Δ*ytbI*, suggesting that aggregation can still occur when the export apparatus and ATPase are active and that there might be other plasmid encoded factors, not only the injectisome needle, which bind to other cells and contribute to aggregation. Further studies of site directed *yscF* mutants or mutagenesis of *yscP*, the needle-length determinant, could confirm if YscF is the aggregation factor. Since aggregation was still observed in the Δ*yscF*/Δ*ypsI*/Δ*ytbI* and not in Δ*yscF* it can be assumed that QS must have a regulatory effect on aggregation.

These results are in contrast to the reports that YadA is the aggregation factor of cells grown in rich medium (Skurnik *et al.*, 1984, El Tahir and Skurnik, 2001). A recent study demonstrated that aggregation of *Y. pseudotuberculosis* is independent of YadA when grown in complex medium. Aggregation experiments showed that a *yadA* mutant exhibits the same aggregation phenotype as the YpIII wild-type, even though it would be expected that deletion of *yadA* would inhibit aggregation (Joanne Purves, personal communication). This strongly suggests that YadA, in fact, is not the aggregation factor.

In conclusion, after performing all the assays described in this chapter it can be hypothesised that YscF is the aggregation factor, since this it is a surface protein and will not be expressed after deletion of genes by transposon insertions in *yscN*,

yscV, *yscU*, *yscT* or *yscR* or after deletion of *yscV* or *yscF*. Aggregation assays for Tn*5* mutants in these genes revealed that they do not aggregate. However, a study of *P. aeruginosa* suggested that aggregation is mediated by proteins located at the tip of the injectisome needle (Tran *et al.*, 2014). Genes building this translocon of *Yersinia* are YopB and YopD. A mutation in *yopD* was shown to attenuate virulence since it made the cells susceptible to the immune response and deficient for Yop effector secretion (Costa *et al.*, 2013). After deletion of *yscF* both Yop translocators will not be expressed, either. Hence, separate mutations in *yopD* and *yopB* could give more information of the roles of the injectisome surface components for aggregation.

3.3.1 Summary

The results of this chapter show that with respect to aggregation:

- The virulence plasmid is important,
- QS negatively affects aggregation,
- No supernatant factors are involved,
- Surface factors are required,
- Aggregation is independent of biofilms,
- The T3S injectisome, especially the injectisome needle is essential for aggregation.

When combining all these results, it can be assumed that the injectisome needles promote aggregation and that deletion of QS results in upregulation of the injectisome needles which causes enhanced aggregation of the bacteria cells (Figure 3.11)

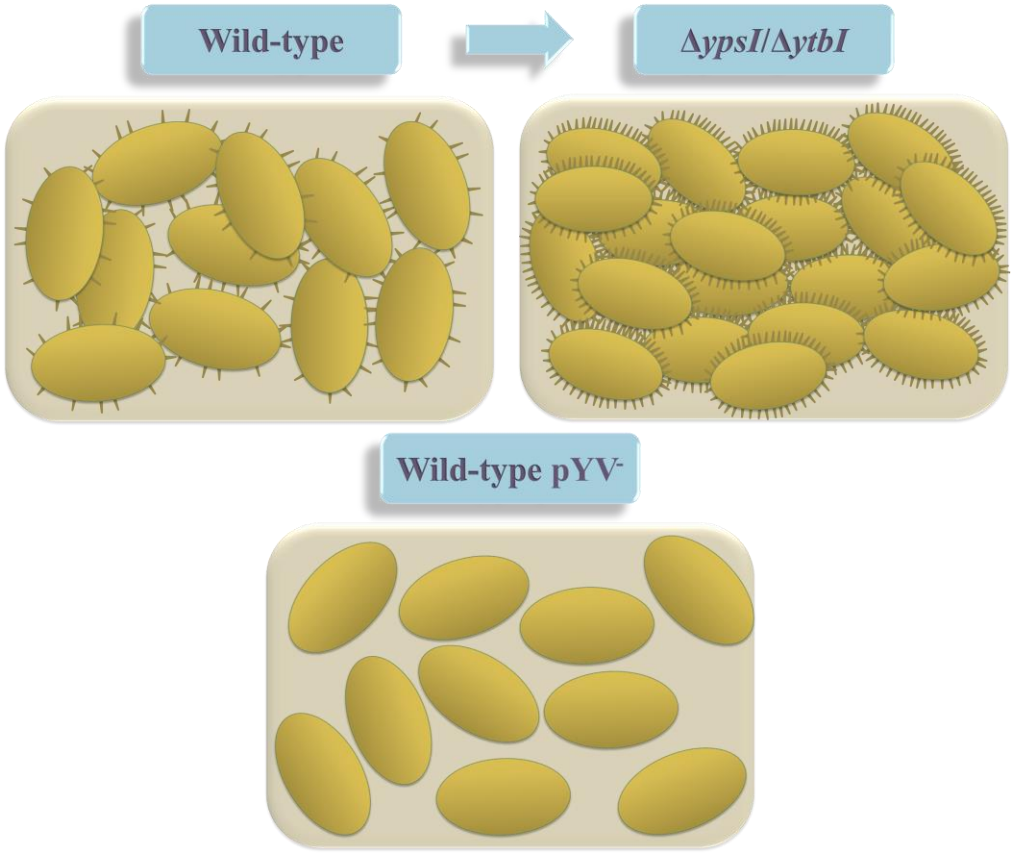


Figure 3.11: Proposed model demonstrating how aggregation is mediated by the T3S injectisome and how it is regulated by QS. It is assumed that a lack of QS is releasing the repression of injectisome expression on pYV which leads to enhanced aggregation of *Y. pseudotuberculosis* cells.

4 The Relationship Between NagC, *N*-acetyl-D-glucosamine and Quorum Sensing in *Y. pseudotuberculosis*

4.1 Introduction

The glucose derivative *N*-acetyl-D-glucosamine (GlcNAc) performs various functions in bacterial cells. GlcNAc together with *N*-acetylmuramic acid (MurNAc) are major components of the mesh-like peptidoglycan layer in the cell wall. These two sugars are connected by *β*-(1,4)-glycosidic bonds and cross-linked *via* peptide chains that are attached to the MurNAc. Peptidoglycan also defines one of the differences between Gram positive and Gram negative bacteria. Gram positive organisms possess a thick multi-layered peptidoglycan layer that forms 90 % of the dry weight, whereas in Gram negative organisms the peptidoglycan is not as thick and forms just 10 % of the dry weight of the bacteria (Madigan, 2012).

Additionally, for *Y. pseudotuberculosis*, GlcNAc in the form of polyGlcNAc is also incorporated in the extracellular matrix of biofilms, which can be observed forming on the nematode *C. elegans.* Interestingly one study showed that there are mechanisms promoting biofilm formation on the nematode which are regulated by both, *Y. pseudotuberculosis* and *C. elegans*. Biofilm formation did not occur after mutation of *srf-2, srf-3* or *srf-5*, respectively or after mutating the dauer pathway gene *daf-1* in the nematode. Alternatively mutation of the hemin storage locus and genes for lipopolysaccharide synthesis in *Y. pseudotuberculosis* also prevented biofilm formation on the nematode (Joshua *et al.*, 2003). *Y. pseudotuberculosis* biofilm formation on *C. elegans* is regulated by QS, since a mutation of genes for both AHL synthases (*ytbI/ypsI*) or of genes for both AHL

response regulators (*ytbR/ytbR*) severely reduces and delays biofilm formation. Mutation of the key QS-controlled flagellar regulators, Δ*flhDC* and Δ*fliA*, also attenuate biofilm development on *C. elegans* (Atkinson *et al.*, 2011).

GlcNAc also influences virulence related mechanisms in *E. coli*, *P. aeruginosa*, *V. cholerae* and *S. mutans* [reviewed in (Naseem and Konopka, 2015)]. In *E. coli* the *nagE-nagBACD* operon is responsible for the degradation and transport of extracellular GlcNAc and is repressed by NagC in the absence of GlcNAc-6-phosphate (Plumbridge, 1991). In contrast, NagC is an activator of the *glmUS* operon, responsible for production of GlcNAc (Figure 4.1) (Plumbridge, 1995). Since the *Yersinia nagC* is 78 % homologous to the *E. coli nagC*, it is assumed that it exhibits similar functions (Deng et al., 2002).

NagC is also a regulator of several other genes in *E. coli* such as those required for chitobiose degradation (Plumbridge and Pellegrini, 2004). NagC is also linked to negative regulation of *galP* which encodes a transporter of galactose (El Qaidi *et*

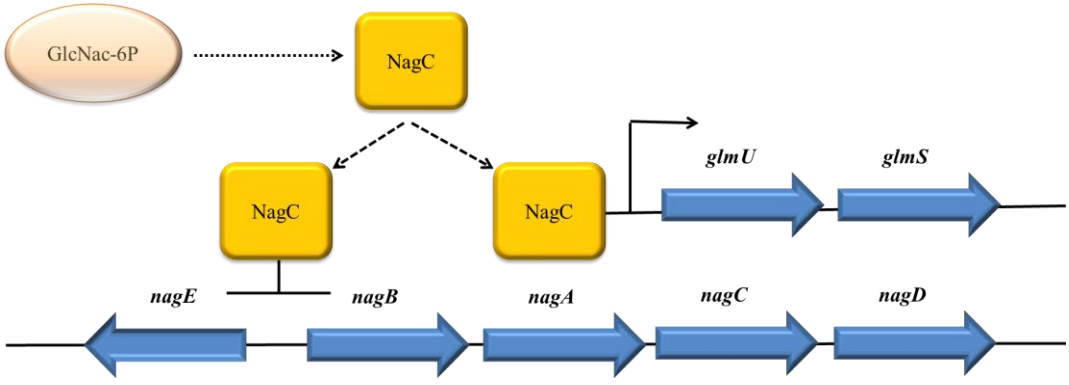


Figure 4.1: Activation of NagC expression in the absence of intracellular GlcNAc-6P. NagC activates the *GlmUS* operon for production of GlcNAc-6P and represses the *nagE-nagBACD* operon to prevent degradation of GlcNAc-6P in *E. coli*. This figure was modified from Konopka, 2012.

al., 2009), showing that NagC is a regulator for the transport and metabolism of several sugars in *E. coli*. In addition, it was reported that regulation of *fimB*, a recombinase assisting type-I-fimbrial expression, is inhibited by GlcNAc and is also regulated by NagC (Sohanpal *et al.*, 2007). Type-I-fimbriae are a known virulence factor in urinary tract infections for uropathogenic *E. coli* (Hannan et al., 2012).

In contrast to *Y. pseudotuberculosis*, *E. coli* does not possess any known LuxI homologues but the enterohaemorrhagic *E. coli* (EHEC O157:H7) possesses a homologue of the QS response regulator LuxR (SdiA) of *V. fischeri* which is important for adhesion and virulence of this bacterium (Kanamaru *et al.*, 2000).

4.1.1 Aims of this Study

The results of Chapter 3 present data showing that the QS regulated aggregation factor of *Y. pseudotuberculosis* is the injectisome of the T3SS which is essential for virulence.

GlcNAc is an important factor in the virulence of several different γ-proteobacteria (Naseem and Konopka, 2015) and its metabolism is regulated by NagC (Plumbridge, 1991). Previous promoter pulldown experiments revealed that NagC might have an influence on QS, since it binds to the *ypsR* promoter of *Y. pseudotuberculosis* (Goldstone, unpublished data). Plumbridge *et al.*, 1991 showed that NagC represses the expression of the GlcNAc *nagE-nagBACD* operon of *E. coli* in the absence of GlcNAc. A study using a *nagC* deletion mutant

was used to determine whether NagC carries out similar functions in *Y. pseudotuberculosis* YpIII. Furthermore, promoter *lux*-fusions on all QS genes and *nagC* were constructed to investigate whether,

- NagC regulates QS
- QS regulates NagC.

By addition of GlcNAc it was investigated, whether GlcNAc

- Impacts on QS gene expression directly or through derepression of NagC.

Addition of MurNAc was used to potentially indicate whether

- *Y. pseudotuberculosis* is able to indirectly sense bacteria in its vicinity by sensing extracellular cell wall peptidoglycan breakdown products.

4.2 Results

4.2.1 Construction of a *Y. pseudotuberculosis nagC* Mutant

To investigate whether NagC is a regulator of QS, an in-frame deletion of *nagC* was constructed by inserting a kanamycin cassette between base 60 and base 1176 of the 1227 bp *nagC* gene using the λ-red recombinase system (Derbise *et al.*, 2003). Figure 4.2 shows *nagC* in the *nagE-nagBACD* operon being exchanged for the kanamycin cassette. Primers *nagC*-ko-F and *nagC*-ko-R (Table 2.3) were used to amplify the 1029 bp kanamycin cassette of pUC4K containing flanking regions of *nagC* on each side (base -9 to -60 after the *nagC* start codon and base -9 to -60 before the stop codon of *nagC*). The PCR product was transformed into the

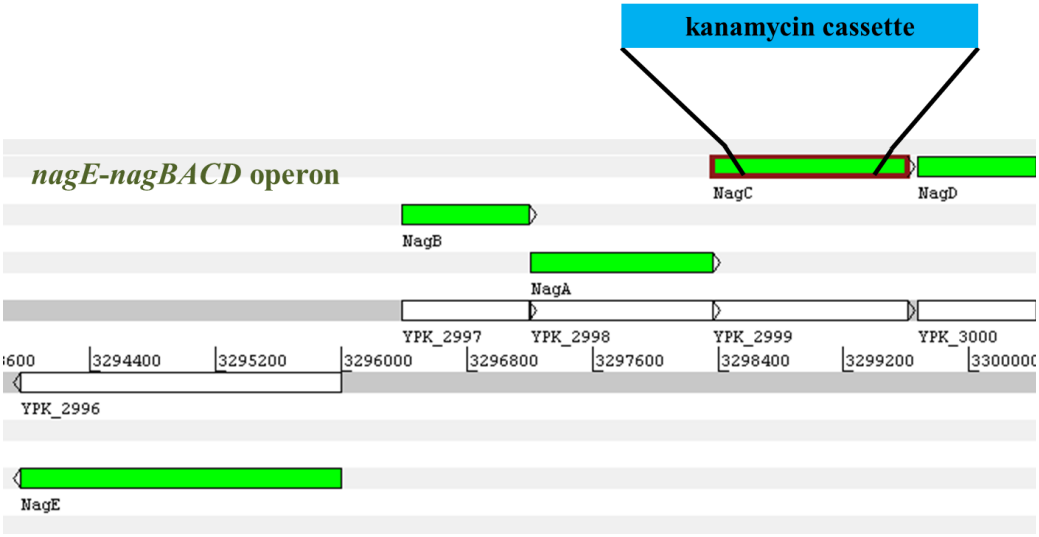


Figure 4.2: Schematic description of the mutation of *nagC* by substituting 1116 bp of *nagC* with a kanamycin cassette.

Y. pseudotuberculosis YpIII wild-type cells, containing the λ-red recombinase, allowing for recombination to occur. Mutants were screened by PCR using *nagC*-ko-check-F and *nagC*-ko-ch-R (Table 2.3), which resulted in a 1356 bp fragment (data not shown).

After confirming the mutation of Δ*nagC* by sequencing, Δ*nagC* was complemented by inserting a functional copy of *nagC* and its promoter region into the Tn*7* *glmS*/*pstS* site of *Y. pseudotuberculosis* YpIII as previously described (Choi *et al.*, 2005). To construct this, the promoter region (536 bp upstream of *nagC*) and ORF of *nagC* were amplified using primers *nagC*-comp-F and *nagC*-comp-R (Table 2.3) resulting in an 1817 bp product, which was cloned into pGEM-T-easy. An *Xho*I / *Bam*HI fragment of the *nagC* complementing construct was cloned into pUC18R6KT::Tc mini Tn*7*, resulting in pAW1. This construct, as well as the transposase harbouring vector pTNS2 were transformed into YpIII

Δ*nagC* and successful transformants were screened by PCR. Primers *lux* RV, Pst-up-2-F and GlmS-dn-2-R were used in different combinations to confirm that a functional copy of *nagC* had integrated downstream of *glmS*. Sequencing analysis confirmed the insertion of the construct 54 bases before the end of *glmS* in YpIII (data not shown).

4.2.2 Analysis of QS Regulation by NagC and *nagC* Regulation by QS

4.2.2.1 NagC is a Regulator of QS in *Y. pseudotuberculosis*

Bacterial *lux*-based promoter assays are a useful technique for investigating gene regulation in bacteria (Winson *et al.*, 1998). As demonstrated in several strains, such as QS and motility mutants of *Y. pseudotuberculosis*, by fusing QS gene promoters together with a *luxCDABE*-cassette in several strains, it is possible to detect alterations in gene expression by monitoring changes in bioluminescence levels (Atkinson *et al.*, 2008). To gain comparable results, the peak times and intensities of various promoter fusions in the different strains were observed.

To determine whether NagC is a regulator of QS genes, as predicted by the promoter pull-downs, QS gene promoter fusions (P_{ypsR}::*lux*, P_{ypsI}::*lux*, P_{ytbR}::*lux* and P_{ytbI}::*lux*) (Atkinson *et al.*, 2008) were incorporated into the *Y. pseudotuberculosis* YpIII wild-type, Δ*nagC* and complemented Δ*nagC* mutant by a single crossover. Growth and luminescence of the bacteria cells were measured at 22°C and 37°C in a Tecan® 96-well plate reader.

Figure 4.3 and Figure 4.4 show the bioluminescence of P_{ypsR}::*lux*, P_{ypsI}::*lux*, P_{ytbR}::*lux* and P_{ytbI}::*lux* fusions in Δ*nagC* compared with the YpIII wild-type and complemented mutant at 22°C and 37°C. At 22°C *ytbI* and *ypsI* (Figure 4.3 A and C) expression are ~16-fold or ~6-fold respectively higher in Δ*nagC* when compared with the wild-type and the complemented Δ*nagC* mutant after ~9 h of incubation. Expression of *ypsR* (Figure 4.3 D) is ~1.5 fold higher in Δ*nagC* and its expression peak is delayed for 1 h – 2 h compared to its expression in the wild-type and the complemented mutant. In contrast, expression of *ytbR* in Δ*nagC* is ~2.5 fold reduced compared to the wild-type and the complemented mutant after 10 h of incubation. This data suggest that under these conditions, NagC normally represses *ytbI*, *ypsI* and *ypsR* expression, whereas the expression of *ytbR* is activated at 22°C.

Similar results were obtained when performing the assay at 37°C (Figure 4.4) but the expression of all the QS genes peaked earlier compared with assaying at 22°C. At 37°C gene expression of *ytbI* and *ypsI* (Figure 4.4 A and C) is upregulated in Δ*nagC* to ~120-fold for *ytbI* and ~300-fold for *ypsI* compared to the wild-type and the Δ*nagC* complemented mutant. Expression of *ypsR* is delayed by 2 h in Δ*nagC* followed by a 1.3-fold activation (Figure 4.4 D) when compared to the wild-type and the Δ*nagC* complemented mutant. However, the expression of *ytbR* at 37°C remains unchanged compared to its expression in the wild-type (Figure 4.4 B).

These results suggest that NagC is a repressor of QS gene expression (with the exception of *ytbR*) at 37°C and it is an early activator of *ypsR* expression.

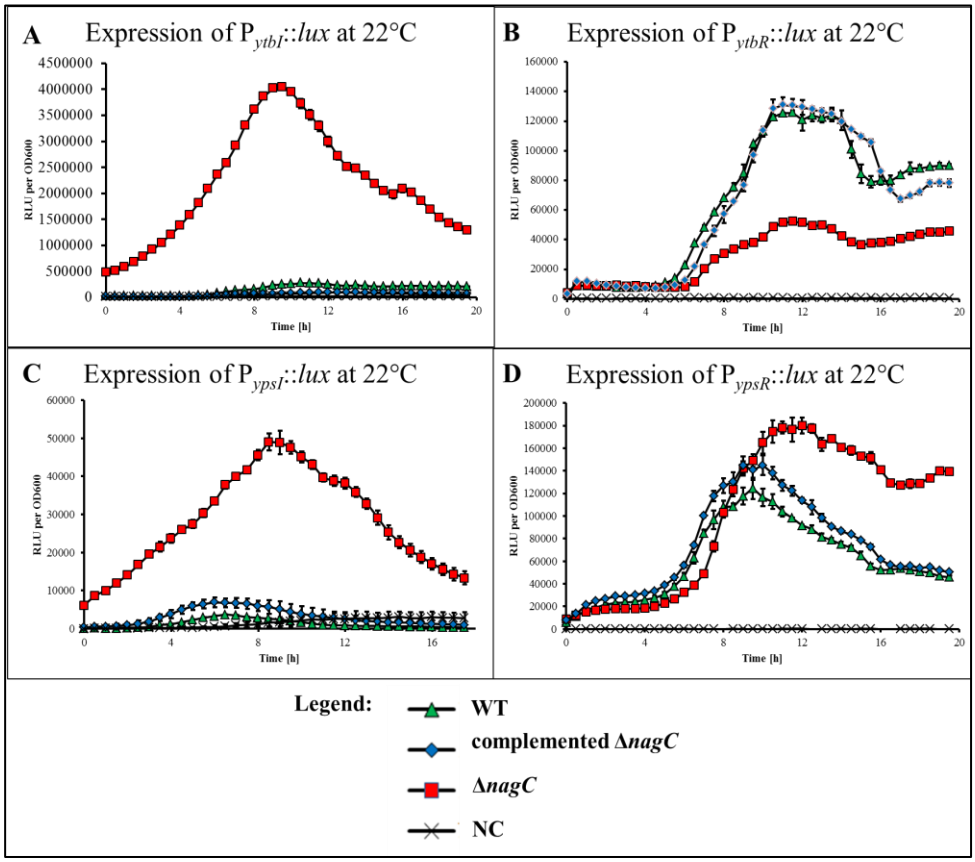


Figure 4.3: Expression of QS genes at 22°C. *ytbI* (A), *ypsI* (C) and *ypsR* (D) expression is activated in Δ*nagC* compared to their expression in the YpIII wild-type and Δ*nagC* complement, suggesting that NagC is a repressor of these QS genes. Expression of *ytbR* (B) is reduced in Δ*nagC*, leading to the assumption that NagC is an activator of this QS response regulator. Displayed is one representative out of three repeat assays.

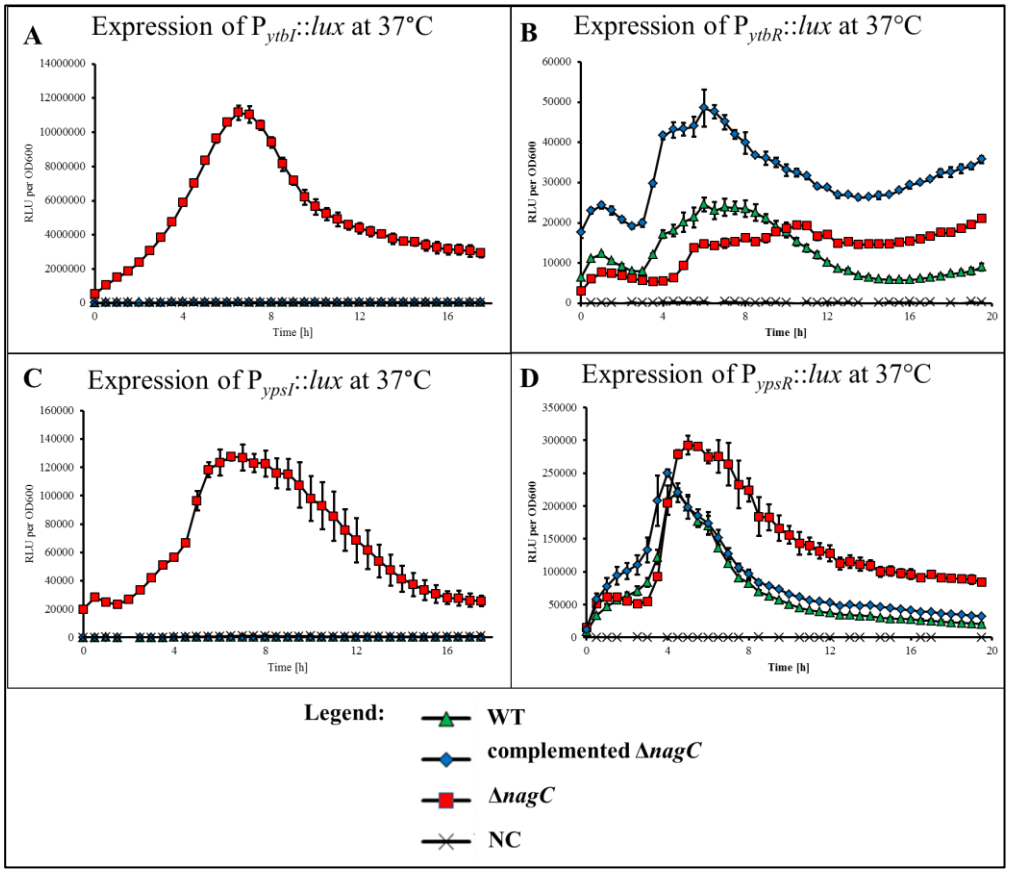


Figure 4.4: Expression of *Y. pseudotuberculosis* QS genes at 37°C. *ytbI* (A), *ypsI* (C) and *ypsR* (D) expression is activated in Δ*nagC* compared to their expression in the YpIII wild-type and complemented Δ*nagC*, suggesting that NagC is a repressor of these QS genes at 37°C. Expression of *ytbR* (B) in Δ*nagC* is similar to the wild-type. A representative dataset out of three repeat assays is shown.

4.2.2.2 QS Regulates Expression of *nagC*

After demonstrating that NagC acts as a regulator of QS, by being a repressor of the expression of the AHL synthase genes at both 22°C and 37°C and an activator of *ytbR* expression (4.2.2.1), a *nagC* promoter fusion was constructed to determine whether, QS reciprocally regulates the expression of *nagC*.

For assembly of a *nagC* promoter *lux* fusion, the 536 bp putative promoter region of *nagC* was amplified by using primers *nagC*-*lux*-F and *nagC*-*lux*-R (Table 2.3). The PCR product was ligated in front of the *luxCDABE* cassette (Winson *et al.*, 1998) in pGEM-T-easy. The construct was then subcloned into pDM4 or pDM4.2 and conjugated into Δ*ypsI*, Δ*ypsR*, Δ*ytbI*, Δ*ytbR*, Δ*ypsI*/Δ*ytbI*, Δ*ypsR*/Δ*ytbR* and Δ*nagC* and integrated into the chromosome by a single crossover event.

For all QS mutants *nagC* expression peaked at ~10 h when grown at 22°C and 4 h when grown at 37°C. Figure 4.5 A reveals that the expression of *nagC* at 22°C in Δ*ytbI*, Δ*ypsI*/Δ*ytbI* (ΔdI) and Δ*ypsR*/Δ*ytbR* (ΔdR) is 2-fold higher compared with the wild-type, whereas in Δ*ypsR* and Δ*ypsI* expression is reduced ~2-fold. Δ*ytbR* has little effect on *nagC* expression at 22°C suggesting that QS regulates the expression of *nagC*, in which YtbI and all AHL synthases/response regulators have a repressing effect on *nagC* expression; whereas YpsR and YpsI have activating effects on *nagC* expression at 22°C. The *nagC* promoter activity in Δ*nagC* is 8-fold higher compared to the wild-type and the complemented Δ*nagC* mutant (Figure 4.5 A). This shows that NagC negatively auto-regulates itself at 22°C.

Figure 4.5 B indicates that the expression of *nagC* in the wild-type at 37°C is approx. 4-fold higher than at 22ºC. Results of this assay further show that *nagC* expression is 2-fold downregulated in Δ*ytbR* compared to the wild-type and 3-fold downregulated in Δ*ypsR* and Δ*ypsI*. There is no activation or repression of *nagC* in the other QS mutant backgrounds at 37°C suggesting that under wild-type conditions YtbR, YpsR and YpsI negatively affect *nagC* expression, whereas YtbI and the combination of both AHL synthases/response (Δ*ypsI*/Δ*ytbI*, Δ*ypsR*/Δ*ytbR*) regulators do not affect *nagC*. The expression of *nagC* in Δ*nagC* is 16-fold higher compared to the wild-type and the complemented Δ*nagC* mutant (Figure 4.5 B), demonstrating that at 37°C NagC is also negatively autoregulated.

All of these results suggest that *nagC* expression is temperature dependent and that *nagC* is regulated by QS.

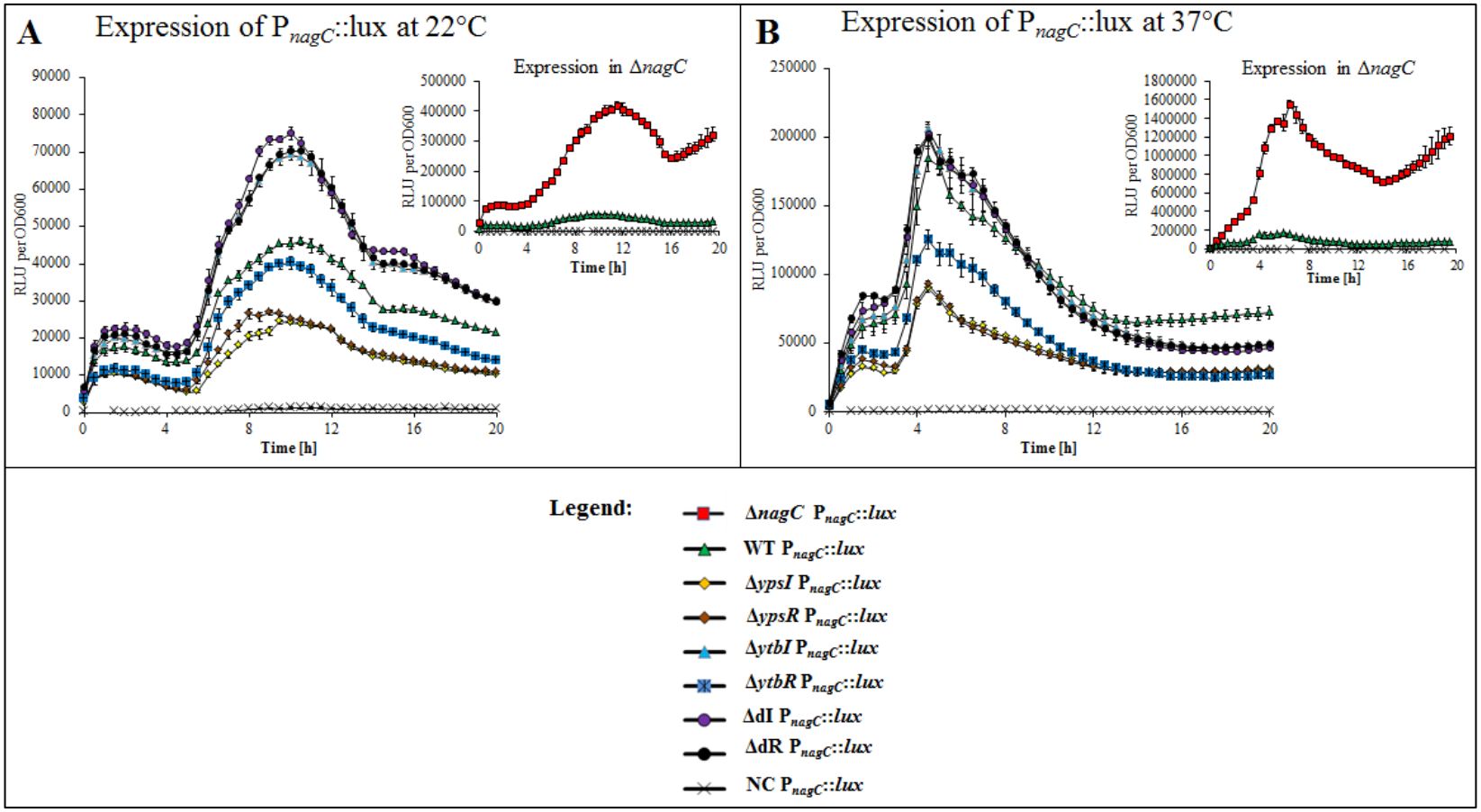


Figure 4.5: Expression of *nagC* in QS mutants and Δ*nagC* at 22°C (A) and 37°C (B). At 22°C expression of *nagC* is downregulated in ΔdI (Δ*ypsI*/Δ*ytbI*), ΔdR (Δ*ypsR*/Δ*ytbR*) and Δ*ytbI* and upregulated by Δ*ypsR*, Δ*ypsI* and Δ*ytbR*. At 37°C Δ*ypsR*, Δ*ypsI* and Δ*ytbR* also activate expression of *nagC*; whereas ΔdI, ΔdR and Δ*ytbI* have no effect on *nagC* expression compared to the wild-type. At both temperatures *nagC* expression is upregulated in Δ*nagC*. A representative dataset out of three repeat assays is shown.

4.2.2.3 Summary

Sections 4.2.2.1 and 4.2.2.2 show that NagC acts as a regulator of QS and that expression of *nagC* is in turn regulated by QS.

Figure 4.6 summarises the relationship between NagC and QS, demonstrating that NagC acts as a repressor for *ytbI*, *ypsI* and early *ypsR* expression at 22°C / 37°C and as an activator for *ytbR* expression at 22°C. Conversely, YpsI and YpsR activate the expression of *nagC* at 22°C and 37°C whereas YtbI represses *nagC* expression at 22°C. The expression of *nagC* in the wild-type is 4-fold higher at 37ºC compared to 22ºC. Furthermore, *nagC* expression in Δ*ytbI*, Δ*ypsI*/Δ*ytbI* and Δ*ypsR*/Δ*ytbR* at 22°C is repressed and at 22°C / 37°C NagC is negatively autoregulated.

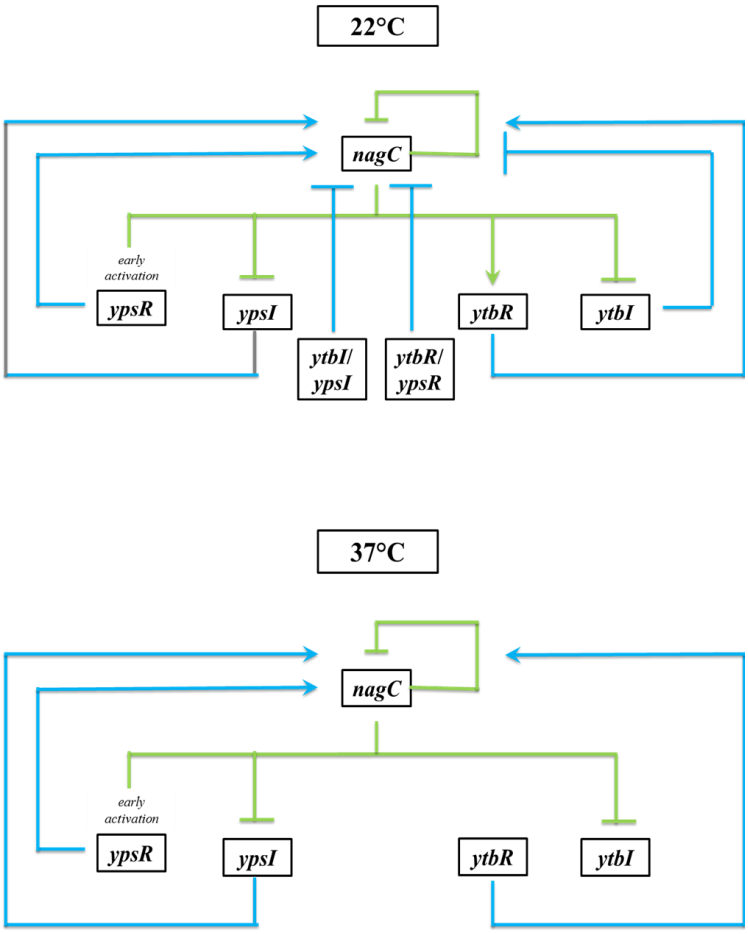


Figure 4.6: Illustration of the regulation of QS gene expression by NagC (green) and the regulation of *nagC* expression by QS (blue) at 22°C and 37°C. At 22°C and 37°C NagC represses expression of *ypsI* and *ytbI*. NagC is responsible for early activation of *ypsR* expression at 22 and 37°C. The expression of *ytbR* is activated by NagC at 22°C. The expression of *nagC* is activated by *ypsR*, *ypsI* and *ytbR* at 22°C and 37°C. Δ*ytbI*, Δ*ypsI*/Δ*ytbI* and Δ*ypsR*/Δ*ytbR* are repressing *nagC* expression at 22°C. NagC negatively regulates its own expression at both temperatures.

4.2.3 Impact of GlcNAc on QS and *nagC* Expression

Section 4.2.2 shows that there is a reciprocal regulatory relationship between NagC and QS, while NagC is repressing the expression of *nagC*. NagC is the regulator of the expression of the *nagE-nagBACD* operon in *E. coli* and *Yersinia* (Plumbridge, 1991, Deng *et al.*, 2002), which is known to be derepressed in the presence of GlcNAc (Plumbridge, 1990). Given the relationship between NagC and QS revealed in the previous section, there may also be a link between GlcNAc, NagC and QS in *Y pseudotuberculosis*. To test this 0.3 mM, 1.0 mM or 5 mM GlcNAc was added to the LB medium in *lux* promoter fusion assays.

Figure 4.7 reveals that at 22°C in the wild-type background *ytbI* and *ytbR* expression show a dose-dependent response to GlcNAc, with a comparative 1.5-fold reduction in their expression after the addition of 1.0 mM and 5.0 mM GlcNAc (Figure 4.7 A and C). However, addition of 5.0 mM GlcNAc leads to a 0.5-fold increase in *ypsR* expression (Figure 4.7 D). GlcNAc does not show any detectable effects on *ypsI* expression under these conditions (Figure 4.7 B). Figure 4.8 reveals an enhanced reduction of expression of *ytbI* (A) and *ytbR* (C) in the presence of GlcNA at 37°C compared to 22°C. A strong reduction in expression is detected as the concentration of GlcNAc increases. The peak of expression of both genes is earlier compared to 22°C. However, at 37°C GlcNAc has little or no effect on *ypsI* and *ypsR* expression (Figure 4.8 B and D).

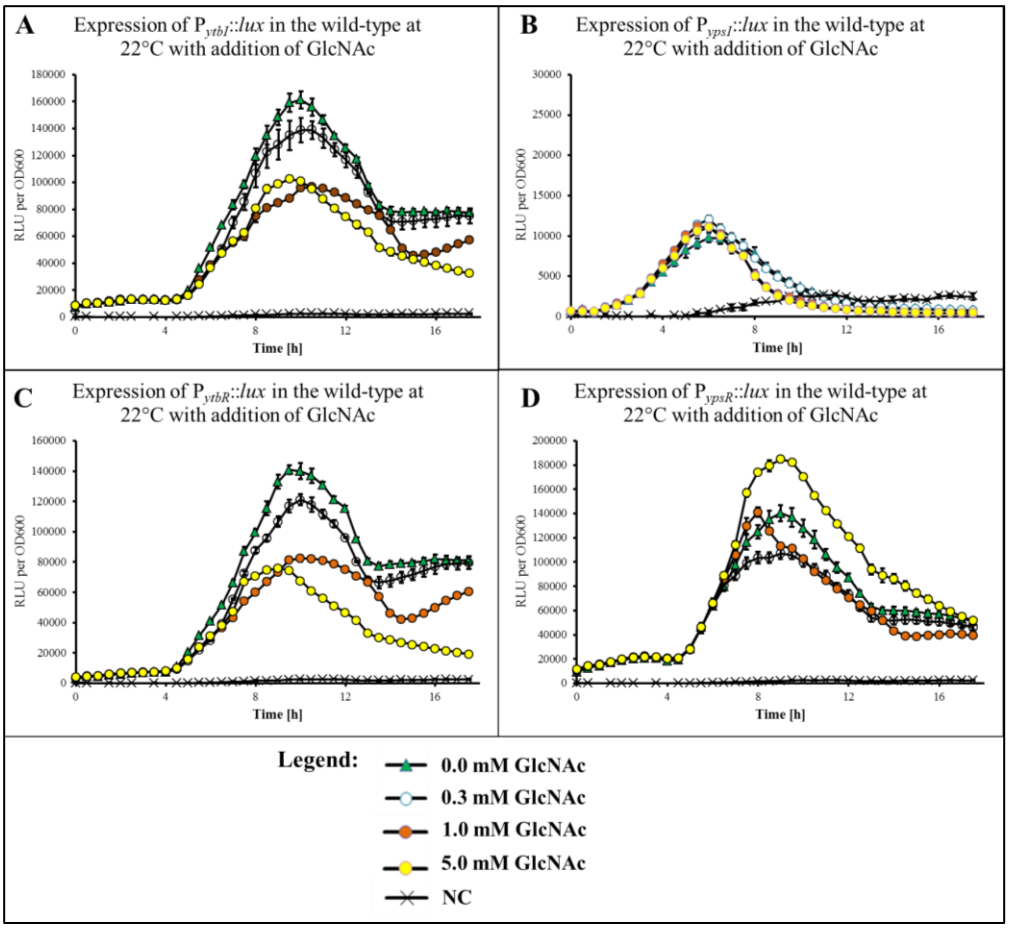


Figure 4.7: Expression of QS genes at 22°C in the wild-type after addition of GlcNAc. Addition of GlcNAc leads to a 1.5-fold reduction of *ytbI* (A) and *ytbR* (C) expression, after addition of 1.0 mM and 5.0 mM of GlcNAc. Addition of 5.0 mM GlcNAc increases the expression of *ypsR* 0.5-fold (D). Addition of GlcNAc does not affect gene expression of *ytbI* (B) A representative dataset out of three repeat assays is shown.

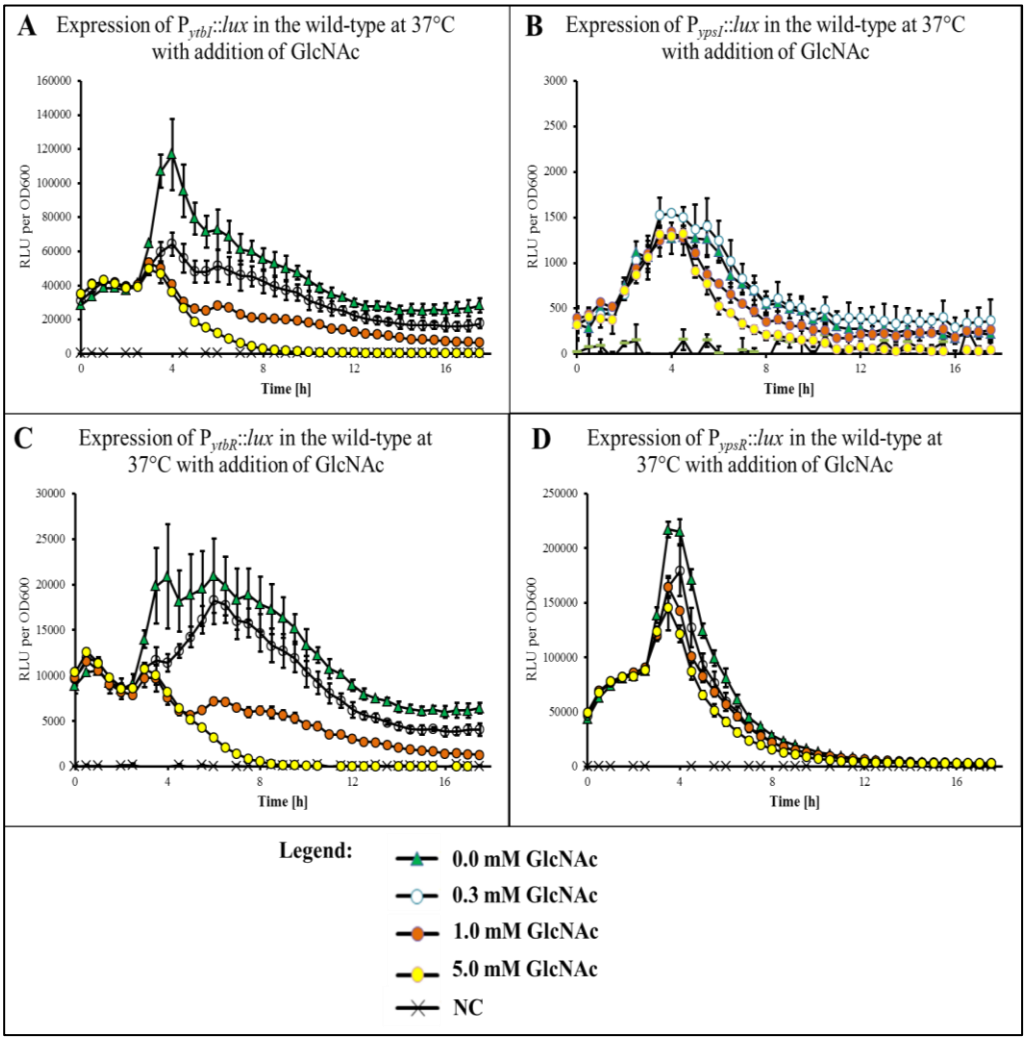


Figure 4.8: Expression of QS genes at 37°C in the wild-type after addition of GlcNAc. Addition of varying concentrations of GlcNAc leads to a dose dependent response with a 3-fold decrease of *ytbI* expression (A) and 2-fold decrease of *ytbR* expression (C). Addition of GlcNAc does not affect expression of *ypsI* (B) and slightly affects expression of *ypsR* (D). A representative dataset out of three repeat assays is shown.

This data suggests that GlcNAc has an impact on the expression of QS genes in the wild-type, by increasing *ypsR* expression at 22°C and by decreasing *ytbI* and *ytbR* expression at 22°C and 37°C.

After demonstrating that GlcNAc impacts on QS gene expression, the effect of GlcNAc on QS in the Δ*nagC* mutant was investigated to determine if there is a link between GlcNAc and NagC for QS gene expression or if GlcNAc impacts on QS independent of *nagC* regulation.

Figure 4.9 shows that the addition of GlcNAc to QS promoter *lux* fusions, in the Δ*nagC* background, at 22°C leads to a 2-fold increase in *ypsI* expression (Figure 4.9 B). Addition of 1.0 mM and 5.0 mM GlcNAc leads to 2-fold decrease in *ytbR* expression. However an addition of 5.0 mM GlcNAc further decreases *ytbR* expression after 8 h (Figure 4.9 C). GlcNAc has no effect on *ypsR* and *ytbI* expression at this temperature (Figure 4.9 A and D). Performing the assay at 37°C leads to a 2-fold decrease in *ypsI* expression with an early peak after 8 h compared to a peak in expression after 12 h when no GlcNAc was added (Figure 4.10 B). Expression of *ytbR* and *ypsR* responds to GlcNAc addition by a 4-fold and 2-fold decrease, respectively (Figure 4.10 C and D). At 37°C GlcNAc has little effect on *ytbI* expression (Figure 4.10 A). This data suggests that GlcNAc affects QS gene expression in Δ*nagC* through activation of *ypsI* expression and repression of *ytbR* at 22°C and a decrease of *ypsI*, *ytbR* and *ypsR* expression at 37°C.

The results obtained for QS fusions in the wild-type and in Δ*nagC* after addition of GlcNAc are slightly different. This suggests that the impact of GlcNAc on QS gene expression is partly independent of NagC. GlcNAc would not have shown an

effect on QS gene expression in Δ*nagC* if this compound would only impact on the release of NagC from QS promoters

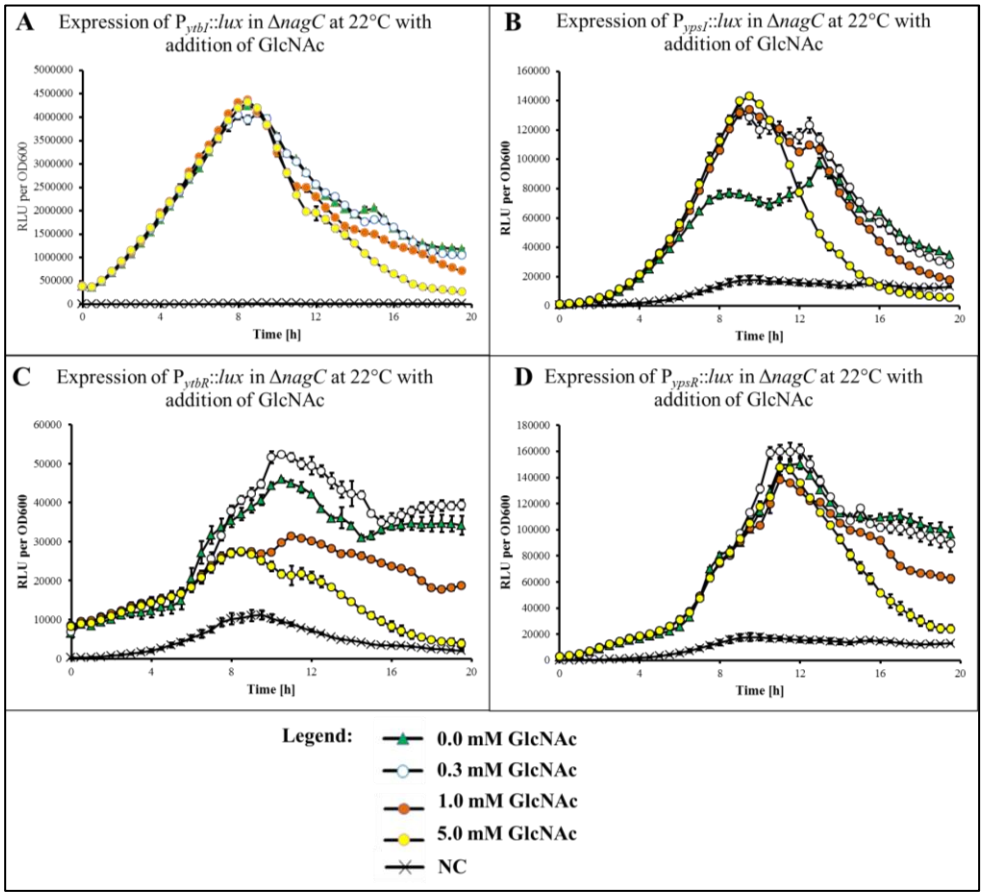


Figure 4.9: Expression of QS genes in Δ*nagC* at 22°C after addition of GlcNAc. Addition of GlcNAc leads to a 2-fold increase of *ypsI* expression (B). *ytbR* expression (C) reacts dose dependently to the addition of GlcNAc by a 2-fold decrease. GlcNAc does not affect expression of *ytbI* (A) and *ypsR* (D). A representative dataset out of three repeat assays is shown.

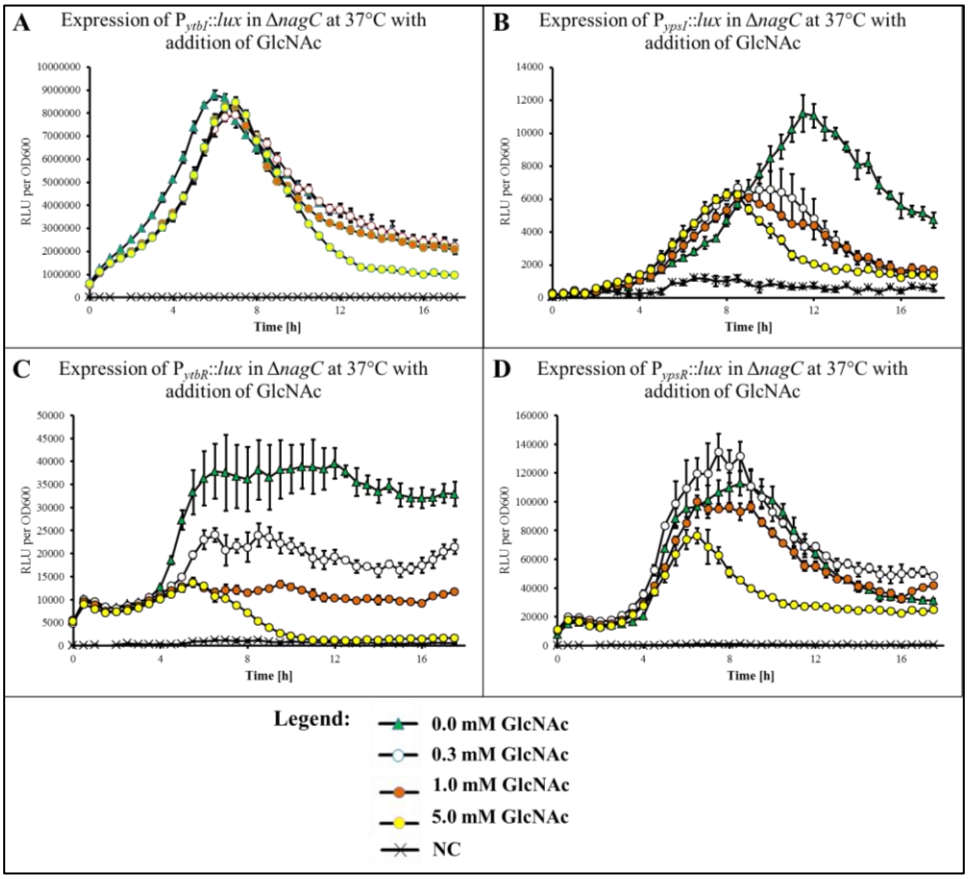


Figure 4.10: Expression of QS genes in Δ*nagC* at 37°C after addition of GlcNAc. Addition of GlcNAc leads to a 2-fold decrease of *ypsI* expression (B), to a 4-fold decrease of *ytbR* (C) and to a 2-fold decrease in *ypsR* expression (D). Addition of GlcNAc does not affect expression of *ytbI* in Δ*nagC* (A). A representative dataset out of three repeat assays is shown.

To confirm this finding, it was important to investigate whether *nagC* expression itself was influenced by GlcNAc, as in *E coli*, where the presence of GlcNAc derepresses the *nagE-nagBACD* operon including *nagC* (Plumbridge, 1990).

To evaluate this, a *nagC*-promoter *lux* fusion in the wild-type and the Δ*nagC* background was grown in the presence of 0.3 mM, 1.0 mM or 5.0 mM GlcNAc. The addition of GlcNAc to the wild-type leads to a dose-dependent response with a 5-fold increase in *nagC* expression at 22°C and with a less striking, but similar, trend at 37°C (Figure 4.11 A and B). This suggests that GlcNAc is taken up and the subsequent presence of intracellular GlcNAc-6P leads to a derepression of *nagC* which causes an increased *nagC* expression. In contrast, GlcNAc shows the opposite effect on the regulation of *nagC* expression in the Δ*nagC* background at 22ºC (Figure 4.11 C). In this mutant GlcNAc leads to a dose-dependent response reducing the expression of *nagC* approx. 2-fold. However, there is little effect on *nagC* expression in Δ*nagC* at 37°C (data not shown).

These results confirm the assumption that GlcNAc, as in *E coli* (Plumbridge, 1990), activates expression of *nagC* in *Y. pseudotuberculosis* by derepressing NagC in wild-type. For Δ*nagC* it was assumed that independent of GlcNAc the *nag*-operon would be continuously switched on in absence of its repressor NagC. However, GlcNAc leads to a decrease in *nagC* expression at 22°C showing that GlcNAc must affect other genes, which then cause the decrease of *nagC* expression.

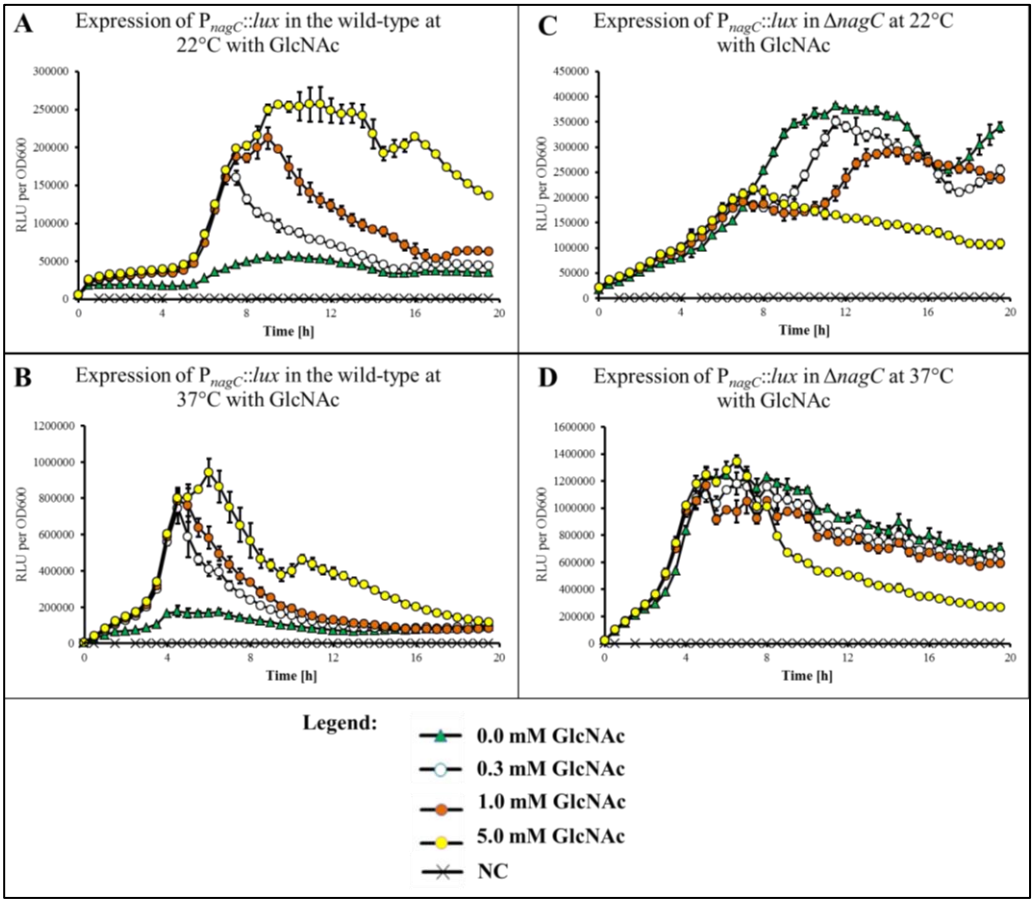


Figure 4.11: Expression of *nagC* in the wild-type and Δ*nagC* after addition of GlcNAc at 22°C and 37°C. *nagC* expression shows a dose dependend response to GlcNAc by increasing 5-fold in the wild-type at 22°C (A) and 37°C (B). In Δ*nagC* *nagC* expression is 2-fold decreased at 22°C (C) but does not respond to GlcNAc at 37ºC. A representative dataset out of three repeat assays is shown.

4.2.3.1 Summary

Figure 4.12 summarises how the amino sugar GlcNAc regulates the expression of QS genes and *nagC* in the YpIII wild-type and Δ*nagC* at 22°C and 37°C.

- At 22°C and 37°C GlcNAc decreases *ytbR* and *ytbI* expression in the wild-type.
- At 22°C GlcNAc activates *ypsR* expression in the wild-type.
- *ypsI* expression is not influenced by GlcNAc in the wild-type.
- At 22°C expression of *ypsI* is activated and expression of *ytbR* is repressed by GlcNAc in Δ*nagC*.
- At 37°C *ypsR, ypsI* and *ytbR* expression is decreased by addition of GlcNAc in Δ*nagC.*
- *ytbI* expression is not influenced by GlcNAc in Δ*nagC.*
- *nagC* expression is upregulated after addition of GlcNAc to the wild-type at 22°C and 37°C but is decreased in Δ*nagC* at 22°C.

These results show that GlcNAc is activating mechanisms that mainly lead to repression of QS gene expression in the wild-type (except *ypsR*) and Δ*nagC* (except *ypsI*). However, *nagC* expression is upregulated in the wild-type at 22°C and 37°C and downregulated in Δ*nagC* at 22°C.

Taken together these results show a link between QS, NagC and GlcNAc. GlcNAc influences QS gene expression independent of *nagC*, indicating that there are other regulatory mechanisms that control QS after addition of GlcNAc. The effect of GlcNAc on *nagC* expression in the absence of NagC supports this hypothesis.

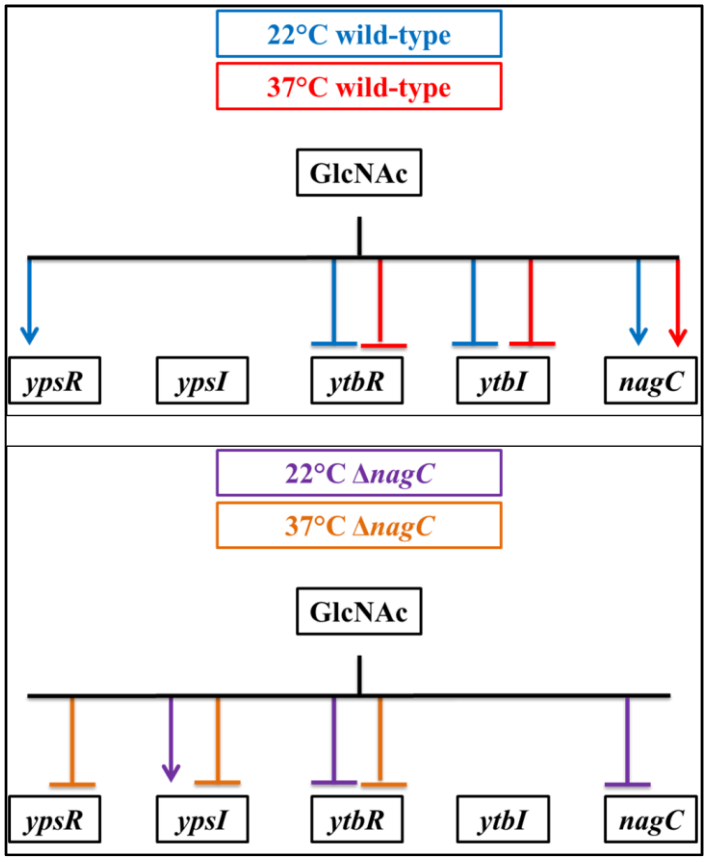


Figure 4.12: : Illustration of the regulation of QS gene expression in the wild-type at 22°C (blue lines and arrows) and 37°C (red lines and arrows) and Δ*nagC* at 22°C (purple lines and arrows) and 37°C (orange lines and arrows) after addition of GlcNAc.

Addition of GlcNAc to the wild-type decreases *ytbR* and *ytbI* expression at 22°C and 37°C and *ypsR* expression is activated at 22°C. Addition of GlcNAc to the *nagC* in Δ*nagC* leads to a reduction *ytbR* expression at 22°C and 37°C and to a decrease of *ypsR* expression at 37°C. *ypsI* expression is increased at 22°C and decreased at 37°C. Expression of *nagC* is increased by GlcNAc in the wild-type at 22°C and 37°C and decreased in Δ*nagC* at 22°C.

4.2.4 MurNAc Addition Affects QS Gene and *nagC* Expression

MurNAc and GlcNAc form the peptidoglycan layer of the bacterial cell wall [reviewed in (Madigan, 2012)]. In contrast to MurNAc, polyGlcNAc is also a main component of the extra cellular matrix of *Yersinia* biofilms, the formation of which are known to be QS controlled (Atkinson *et al.*, 2011, Joshua *et al.*, 2003). Since GlcNAc was found to have effects on *nagC* and QS gene expression, experiments were carried out, to determine whether MurNAc also has an effect on QS gene and *nagC* expression in the YpIII wild-type. These results will help to understand whether the influence of GlcNAc on QS is due to it being a major constituent of the ECM of *Yersinia* biofilms or through being a part of the peptidoglycan layer (Bobrov *et al.*, 2008). Since there is currently no published evidence for MurNAc being a component of biofilms, a change in QS gene expression by addition of MurNAc will show, if *Yersinia* can sense and react to cell debris or other bacteria by coming into contact with this part of the bacterial cell wall.

Figure 4.13 reveals that at 22°C there is a slight reduction of *ypsI* expression in the presence of 5.0 mM MurNAc, whereas for *ytbI*, *ypsR* and *ytbR* expression MurNAc has no effect (data not shown).

At 37°C expression of *ytbI*, *ypsI*, *ytbR* and *ypsR* all show a dose dependent response to increasing MurNAc concentrations with an ~3-fold decrease of *ytbI* (Figure 4.14 A), 7-fold decrease of *ypsI* (Figure 4.14 B), 2.5-fold decrease of *ytbR* (Figure 4.14 C) and 2-fold decrease of *ypsR* (Figure 4.14 D) at the maximum concentration of 5.0 mM MurNAc.

Addition of MurNAc to the *nagC* promoter fusion in the wild-type and Δ*nagC* has no effect (data not shown).

These findings suggest that *Y. pseudotuberculosis* is able to respond to MurNAc *via* QS.

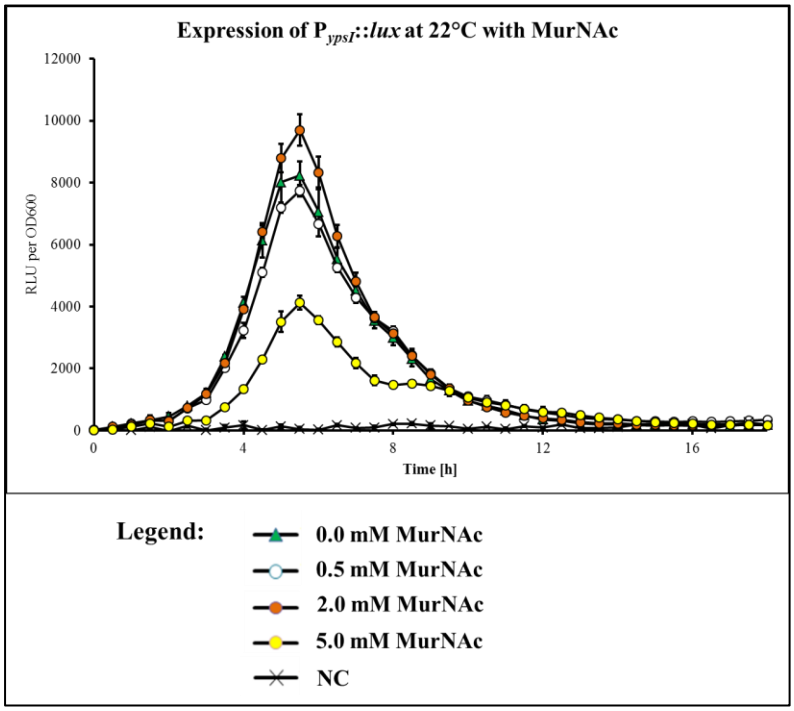


Figure 4.13: Expression of *ypsI* in the wild-type after addition of MurNAc at 22°C. The response to MurNAc is a 5-fold decrease in expression of *ypsI*. A representative dataset out of three repeat assays is shown.

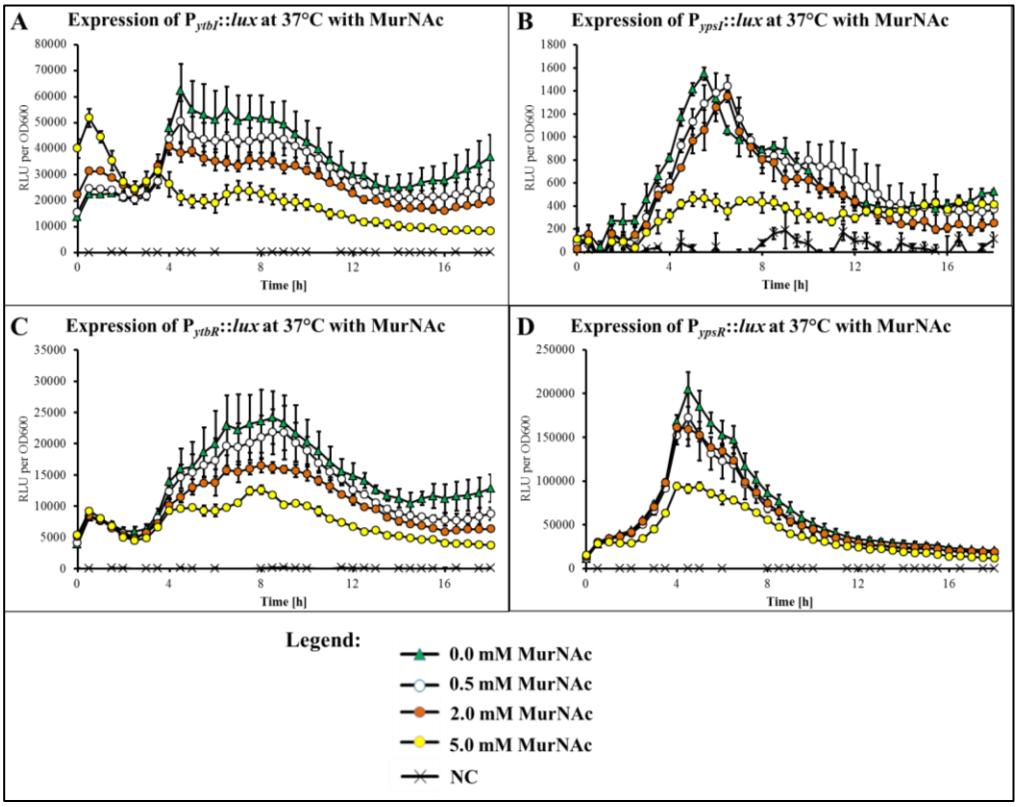


Figure 4.14: Expression of QS genes in the wild-type after addition of MurNAc at 37°C. Expressions of QS genes show dose-dependent changes in expresion to MurNAc with a 2-fold to 7-fold fall in expression after addition of 5.0 mM MurNAc. A representative dataset out of three repeat assays is shown.

4.2.4.1 Summary

Figure 4.15 shows, that MurNAc in general acts as a QS repressor for *ypsI* expression at 22°C and for *ytbI*, *ypsI*, *ytbR* and *ypsR* expression at 37°C. 5.0 mM MurNAc leads to a major decrease in QS gene expression showing that *Yersinia* may sense cell wall components by reacting to MurNAc.

Furthermore, MurNAc acts as an activator for *nagC* expression independent of availability of NagC, suggesting that NagC has no regulatory effect on MurNAc metabolism.

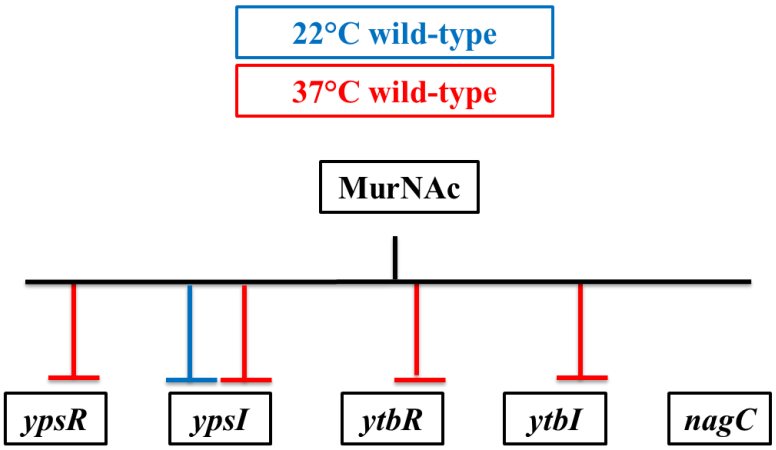


Figure 4.15: Illustration of the regulation of QS gene expression in the wild-type at 22°C and 37°C, after addition of MurNAc. The blue line demonstrates the influence of MurNAc on *ypsI* expression at 22°C and red lines show the influence of MurNAc on QS at 37°C. MurNAc represses *ypsI* expression at 22°C. At 37°C, expression of all four QS genes is repressed after addition of MurNAc. MurNAc has no effect on *nagC* expression.

4.3 Discussion

QS of *Y. pseudotuberculosis* regulates several phenotypes to facilitate the population's survival after changes in population density and environmental conditions. By regulating motility, QS can activate and repress the flagellar activity and cause bacterial populations to relocate to a suitable alternative environment for colonisation (Atkinson *et al.*, 2008, Blair, 1995, Guttenplan and Kearns, 2013). By promoting biofilm formation QS may help to facilitate bacterial settlement and aggregation on surfaces and the production of the matrix of the biofilm (Atkinson *et al.*, 2011). Bacteria then 'know' when they should settle on the surface, when to produce the appropriate biofilm component and when it is optimal for single cells to migrate [reviewed in (Atkinson *et al.*, 2007)]. The formation of biofilms was shown to be negatively affected by parts of the T3SS (Atkinson *et al.*, 2011). By regulating the T3SS, QS enables *Y. pseudotuberculosis* to produce the injectisome and inject the effector Yops at the appropriate population level and in line with the appropriate temperature (Perry and Brubaker, 1987, Atkinson *et al.*, 2011). Recently it was reported that QS regulated phenotypes are following hierarchical order. By using promoter fusion assays it was shown that QS regulates a number of interconnected phenotypes including biofilm formation and motility, which are also interlinked with genes of the histidine metabolism (Figure 4.16) (Joshua *et al.*, 2014, Atkinson *et al.*, 2008). However, although QS regulates these phenotypes little is known about the regulation of QS outside of the QS system itself (Atkinson *et al.*, 2008).

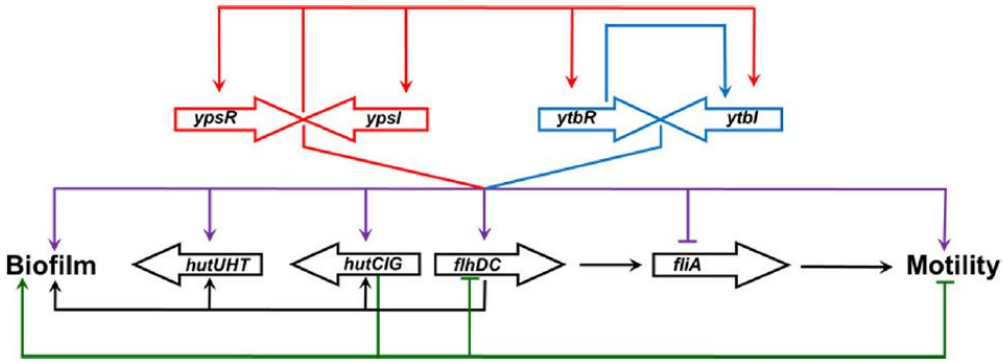


Figure 4.16: Regulation of the *Y. pseudotuberculosis* QS system and its impact on the *hutUHT* and *hutCIG* operons and *flhDC* and *fliA* for regulation of biofilm formation and motility. Colours indicate the activation/repression of genes/phenotypes by proteins of the same colour. This figure was taken from (Joshua *et al.*, 2014).

Due to NagC being a regulatory protein (Titgemeyer *et al.*, 1994) and since it was identified through promotor pulldowns as potentially involved in QS regulation (Goldstone, unpublished data), *lux*-based promoter fusion assays were used to determine the extent of NagC regulation of the QS system. The results obtained using a Δ*nagC* revealed that by deleting this regulatory protein, QS gene expression is altered at both 22°C and 37°C. NagC strongly represses *ypsI* and *ytbI* expression, by either direct binding to QS gene promoters or by affecting other genes whose products then bind to QS promoters. However, expression of *ypsR* is delayed and activated after mutation of *nagC* showing that NagC also represses this QS response regulator. Interestingly, the expression of *ytbR* is reduced in Δ*nagC* at 22°C and is not influenced by NagC at 37°C. This shows that NagC is a temperature dependent regulator of QS. YtbR was shown to positively regulate the expression of *ypsR*, *ypsI* and *ytbI* (Atkinson *et al.*, 2008). By activating *ytbR* NagC may indirectly activate expression of the QS genes, which might buffer the

negative impact that NagC has on QS gene expression. Further experiments such as promoter pull-down assays of the QS genes will give information as to whether NagC is directly binding to the promoters or if NagC is affecting other genes that impact on QS gene expression.

NagC is also known as an activator and repressor of the *glmUS* operon, important for GlcNAc synthesis (Plumbridge, 1995). This operon is present in *Yersinia* (Thomson et al., 2006, Parkhill et al., 2001) and it would therefore be interesting to determine whether NagC also controls the *glm*-operon in YpIII as in *E. coli*. Furthermore, since NagC influences QS gene expression in *Y. pseudotuberculosis*, it would be worth investigating whether the well characterised NagC of *E. coli* also impacts on the QS response regulator SdiA (Kanamaru *et al.*, 2000).

In *P. aeruginosa* Vfr, the cAMP receptor regulatory protein activates expression of *rhlR* (Albus *et al.*, 1997) and small RNAs such as CsrA regulate the entire QS system of *V. cholerae* (Lenz et al., 2005). Furthermore, post-transcriptional regulation also affects QS in *P. aeruginosa*, in which the expression of *lasI* is repressed by RsmA (Pessi *et al.*, 2001). So far, no previous studies with *Y. pseudotuberculosis* have revealed mechanisms for QS regulation.

The findings presented from this study do not explain how NagC represses some QS genes while activating others. However, there may be several other mechanisms involved which cause the effects NagC has on QS, as shown for *P. aeruginosa*, in which regulators affect the QS system which leads to a cascade of gene regulation. For instance Vfr activates *lasR*, which in turn activates

components of both the PQS and the *rhl* QS pathways while the *rhl* QS system represses the PQS system [reviewed in(Venturi, 2006)].

After identification of NagC as a QS regulator of *Y. pseudotuberculosis* it was important to understand whether QS also regulates *nagC* and if there is a feedback mechanism. Section 4.2.2.2 suggests that QS influences the expression of *nagC,* since YpsR, YpsI and YtbI all affect upregulation and YtbR affects downregulation of *nagC* expression at 22°C. YpsR, YpsI and YtbR promote activation of *nagC* at 37°C. These results show QS can influence *nagC* either positively or negatively.

However, since it is not known, whether NagC directly influences QS gene expression and if QS is directly influencing *nagC* expression, it is possible that there are other regulators within this cascade. Further work using promoter *lux*-fusions could investigate whether NagC is involved in controlling motility or the histidine metabolism. Investigations with a *nagE* mutant, the phosphotransferase of the *nagE-nagBACD* operon showed no clear evidence for any effect of NagE on QS gene expression, although *nagC* was downregulated in Δ*nagE* (data not shown). This suggests that the *nagE-nagBACD* operon may be upregulated by NagE. Section 4.2.2.2 shows that NagC represses its own expression and is therefore likely a repressor of the entire *nag*-operon. Further investigation on promoter *lux*-fusions on the *nag*-operon could confirm this and may show that GlcNAc metabolism in YpIII is similar to that in *E. coli* (Plumbridge, 1991).

This study has revealed that NagC is a regulator of QS and it was further proposed that addition of GlcNAc could affect QS, since addition of GlcNAc derepresses NagC from binding to the *nagE-nagBACD* of *E. coli* (Plumbridge, 1990). The results obtained in section 4.2.2.1 revealed that NagC generally represses QS gene expression in YpIII. Hence GlcNAc may derepress NagC binding behaviour on QS gene promoters, if NagC directly binds to QS promoters and if GlcNAc causes derepression of NagC.

The results obtained for GlcNAc on the wild-type fusions show that GlcNAc influences the expression of QS genes by repressing *ytbR* and *ytbI* expression at 22°C / 37°C and by activating *ypsR* expression at 22°C. As anticipated, addition of GlcNAc causes an increase in *nagC* expression which may be caused by derepression of NagC from the *nag*-operon as in *E. coli* (Plumbridge, 1990). However, since GlcNAc did not cause an increase of *ytbR, ypsR* and *ytbI*, it is unlikely that addition of GlcNAc causes derepression of NagC affecting these gene promoters, but it might cause derepression of *ypsI*.

When added to the QS promoter fusions in Δ*nagC*, GlcNAc altered their expression by activating *ypsI* and repressing *ytbR* expression at 22°C and by repressing *ypsR, ypsI* and *ytbR* at 37°C. Interestingly, expression of *nagC* was also reduced at 37°C in contrast to the increase of expression seen in the wild-type. If GlcNAc-6P was just derepressing NagC, addition of GlcNAc to Δ*nagC* should not affect QS gene expression. However, results reveal that GlcNAc may repress and derepress other regulators of QS gene expression.

In *E. coli* phosphorylated intracellular GlcNAc is the activator of the expression of the gene encoding for the GlcNAc-6P-deaminase NagB, showing that GlcNAc affects parts of the *nag*-operon directly (Alvarez-Anorve *et al.*, 2009). The data presented in this study reveals that GlcNAc can affect QS gene expression partly independent of NagC. Since addition of GlcNAc changed the expression of *ytbR*, *ypsR* and *ypsI* in a *nagC*-inactive mutant, it can be assumed that GlcNAc can also influence other QS regulators apart from NagC. It might also indicate that extra- or intracellular GlcNAc directly activates expression of the *nag*-operon independent of NagC since *nagC* expression is downregulated in Δ*nagC*. Furthermore, GlcNAc is important for bacterial and fungal cell-signalling, inhibiting formation of type-I-fimbriae important for *E. coli* virulence and also triggering the development of budding yeast into long filamentous hyphal cells of *C. albicans* (Sohanpal et al., 2004, Castilla et al., 1998). It is therefore likely that GlcNAc may also affect QS independently of NagC. Transcriptome analysis could give more information as to which genes of *Y. pseudotuberculosis* are generally activated by GlcNAc and which might be related to QS gene expression.

After showing that GlcNAc affects QS gene expression independent of NagC, it was important to find out why GlcNAc is influencing QS. For *P. aeruginosa* GlcNAc incorporated in peptidoglycan affects QS leading to increased pyocyanin production, showing that *P. aeruginosa* reacts to the presence of other bacteria (Korgaonkar and Whiteley, 2011). Therefore this study investigated, whether peptidoglycan may influence QS gene expression. To test this, MurNAc, the other

major sugar component of peptidoglycan was added exogenously to *Y. pseudotuberculosis* QS lux-promoter fusions to determine whether it stimulated changes in QS gene expression, like GlcNAc. Results show that MurNAc affects QS mainly by repressing QS genes at 37°C. However, the results obtained after MurNAc addition differ to those of GlcNAc. This shows that by being able to sense GlcNAc and MurNAc of the peptidoglycan, *Y. pseudotuberculosis* may respond to the presence of other bacteria through sensing shed peptidoglycan components. Evidence for this was obtained from a study of *E. coli*, in which GlcNAc-6P derived from peptidoglycan was shown to be important in allosteric regulation of *nagB* (Alvarez-Anorve et al., 2009). Since GlcNAc affects QS gene expression in a different manner than MurNAc it shows that other GlcNAc sources, like biofilms that contain polyGlcNAc, may have an impact on QS gene expression independent of the presence of peptidoglycan.

4.3.1 Summary

This chapter describes the relationship between NagC, GlcNAc, MurNAc and QS of *Y. pseudotuberculosis* and reveals:

- NagC regulates QS,
- QS regulates NagC,
- GlcNAc influences QS expression in the presence and absence of NagC,
- MurNAc influences QS.

5 The Influence of NagC and GlcNAc on QS-Dependent Phenotypes of *Y. pseudotuberculosis*

5.1 Introduction

The QS system of *Y. pseudotuberculosis* was shown to influence several phenotypes including motility, cell-cell aggregation, type-III-secretion and formation of biofilms on the head of *C. elegans* (Atkinson *et al.*, 1999, Atkinson *et al.*, 2008). The influence on swimming motility was discovered by deletion of the QS genes *ypsI* and *ypsR* which caused hypermotility of the bacteria on swim agar plates. Promoter-*lux*-gene fusions to the *Yersinia* flagellar master regulon *flhDC* and the flagellar specific sigma factor *fliA* indicated that the QS system generally upregulates the flagellar master regulator *flhDC* but downregulates the major motility sigma factor *fliA* at 22ºC. Cell-cell aggregation in *Y. pseudotuberculosis* at 37°C was firstly shown by (Skurnik *et al.*, 1984) and found to be related to QS due to an increased aggregation phenotype observed after mutating the QS response regulator *ypsR* (Atkinson et al., 1999). In this thesis it has been demonstrated that both QS response regulators and synthases are important for aggregation but it could not be shown that mutations in single QS genes had any effect. Furthermore, Tn*5* transposon mutagenesis and mutation of the injectisome parts YscF and YscV revealed that the injectisome is the aggregation factor of *Y. pseudotuberculosis* (Chapter 3).

C. elegans, a 1 mm – 2 mm long nematode, is an important model organism used in genetics e.g. evolutionary studies due to its short life cycle and limited genetic variation. This nematode can be found worldwide and its natural habitat is linked to the presence of a high quantity of microorganisms such as those found in human-made composts. *C. elegans* can also be distributed in soil by various

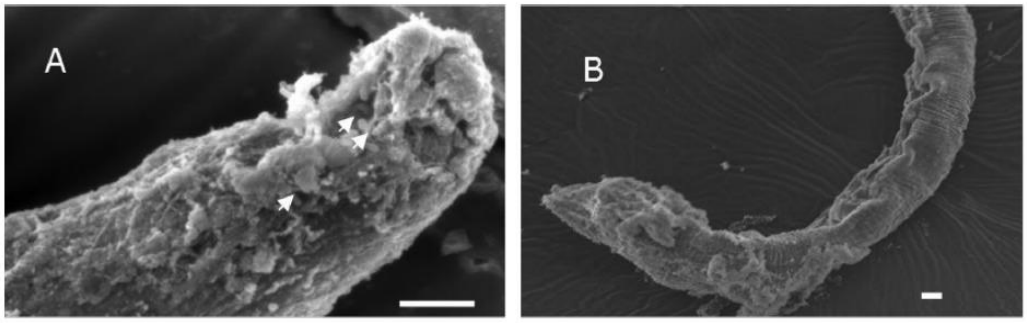


Figure 5.1: Scanning electron microscope (SEM) picture of *C. elegans* infected with *Y. pseudotuberculosis* (bars ≙ 10 µm) after 24 h of incubation [adapted from (Joshua *et al.*, 2014, Atkinson *et al.*, 2011)]. Figure A shows the head of an infected nematode including biofilm and attached bacteria cells (white arrows). Figure B shows an infected worm with biofilm along its body.

vectors including slugs, snails and isopods, in whose digestive system the nematode can survive whilst in the larval dauer-state [reviewed in (Félix and Braendle, 2010)].

Y. pseudotuberculosis YpIII was shown to form biofilms at the anterior end of *C. elegans* (Joshua et al., 2003)(Figure 5.1). Deletion of *ypsI*/*ytbI* and *ypsR*/*ytbR* causes a reduction and delay in biofilm formation on the nematode, which demonstrates that QS impacts on this phenotype (Atkinson *et al.*, 2011).

Biofilms of *Y. pseudotuberculosis* consist of several components, such as proteins, eDNA and polysaccharides. The biofilm matrix forms around the bacteria and protects the cells from environmental challenge, facilitates bacterial cell aggregation and maintains the population density which is important for QS. Furthermore, the growth and turnover of the biofilm through release of bacterial cells allows bacteria to colonise new habitats [reviewed in (Flemming and Wingender, 2010)]. One exopolysaccharide component of the extracellular matrix of *Yersinia* biofilms is polyGlcNAc, production of which is regulated by genes of

the *hms* locus (Bobrov *et al.*, 2008). In addition the *hms* locus encodes genes for the hemin or Congo Red storage also known as the pigmentation phenotype in virulent *Yersinia* species (Perry *et al.*, 1990). In *E. coli* the synthesis of GlcNAc is regulated by the *glmUS* operon and the degradation of extracellular GlcNAc by the *nag*-operon. The regulator of the GlcNAc metabolism of *E. coli* is NagC, which acts as an activator and repressor of the *glmUS* operon and as repressor of the *nagE-nagBACD* operon in *E. coli* (Plumbridge, 1995, Titgemeyer *et al.*, 1994). Considering that *nagC* of *Yersinia* shares 78 % sequence homology to that of *E. coli* , it is assumed that they share a common function (Deng et al., 2002).

Biofilm formation on abiotic surfaces are likely to be induced by the release of polysaccharides into the environment (Nesper *et al.*, 2001), whereas biofilms on *C. elegans* are induced by the release of host and bacterial recognition molecules before settling of the bacteria on the surface of the nematode head while the worm is feeding from a lawn of *Y. pseudotuberculosis*. This was shown by mutating several genes (genes encoding surface antigens, C-lectin and fatty acid desaturases) of *C. elegans* that enable the nematode to become resistant to biofilm formation of *Y. pseudotuberculosis* (Joshua *et al.*, 2003, Joshua *et al.*, 2014). In addition, several genes of *Y. pseudotuberculosis* have been recently shown to be important for biofilm formation and include those for histidine utilisation and motility since feeding the nematode with a *hutC* mutant and motility mutants *flhDC, fliA* and *flhA* caused reduction of biofilms on *C. elegans* (Atkinson et al., 2011, Joshua et al., 2014). Interestingly, it was further shown that these genes are linked or regulated by quorum sensing, since *hut*-operons (*hutCIG* and *hutUHT*)

and the motility regulator *flhDC* are activated by QS and the flagellar sigma factor *fliA* is repressed by QS (Atkinson *et al.*, 2008, Joshua *et al.*, 2014).

However, QS directly affects biofilm formation on *C. elegans*. This was shown by the mutation of AHL synthases *ypsI*/*ytbI* or QS response regulators *ypsR*/*ytbR* which reduced biofilms by more than 50 % compared to the *Y. pseudotuberculosis* wild-type after 24 h of incubation. An extended incubation time of 96 h led to the formation of a severe biofilm on the nematode showing that biofilm formation is delayed in these QS double mutants (Atkinson *et al.*, 2011). Furthermore, the pYV encoded injectisome of the T3SS, which is located in the cell membrane was shown to be important for production of biofilms because a triple mutant of the *ypsI*/*ytbI* and the injectisome component YscJ restored biofilms to wild-type levels. Growing bacteria under injectisome forming conditions inhibited biofilm formation, whereas curing pYV of the mutants restored biofilm. These results indicate that T3S blocks biofilm formation in the wild-type (Atkinson *et al.*, 2011).

Usually the T3SS of *Yersinia* exports Yops *in vitro* in absence of Ca^{2+} at 37°C (Yother and Goguen, 1985) but following the mutation of *flhDC, ypsR, ytbR, ytbI, ypsI/ytbI* and *ypsR*/*ytbR Y. pseudotuberculosis* exports Yops under non-permissive conditions at 30°C. This shows that a deletion of *ypsI/ytbI* results in injectisome formation at temperatures lower than 37°C, which gives evidence for the reduction of *C. elegans* biofilms when fed with this YpIII mutant (Atkinson *et al.*, 2011).

However, the injectisome needle was shown to be the aggregation factor (Chapter 3) so that the T3SS may be the intermediate linking biofilm formation and aggregation.

5.1.1 Aim of this Study

Chapter 4 described the effects of mutation of *nagC*, revealing that NagC regulates the expression of QS genes and QS influences the expression of *nagC*. Furthermore, since QS regulates biofilm formation and *flhDC*, *fliA*, *flhA*, which in turn influence biofilm formation and regulate motility (Atkinson *et al.*, 2008, Atkinson *et al.*, 2011, Joshua *et al.*, 2014), it is possible the mutation of *nagC* may influence these QS related phenotypes. Considering that Chapter 3 revealed that QS influences the T3SS promoted aggregation and since Atkinson *et al.*, 2011 showed that QS mutants (except *ypsI*), *flhDC*, *fliA* and *flhA* express Yops at low temperatures it might be that NagC may also affect the T3SS and the aggregation phenotype.

NagC regulates GlcNAc metabolism by activating/repressing GlcNAc synthesis and repressing GlcNAc degradation (Plumbridge, 1995, Titgemeyer *et al.*, 1994). Furthermore, polyGlcNAc is an important component of the extracellular matrix of *Yersinia* biofilms (Bobrov *et al.*, 2008). In this study the effects of NagC and the availability of GlcNAc were investigated, assuming that after *nagC* mutation, there might be no repression of GlcNAc degradation and no synthesis of GlcNAc which might lead to an absence of biofilm on *C. elegans*.

To clarify all questions raised, the Δ*nagC* mutant was used in several phenotypic assays to investigate whether:

- NagC regulates motility,
- NagC affects aggregation,
- Biofilms on *C. elegans* are attenuated in Δ*nagC*,
- Addition of GlcNAc affects biofilms,
- NagC regulates Yop secretion.

5.2 Results

5.2.1 Swimming Motility of *Y. pseudotuberculosis* Δ*nagC*

It was shown that the YpsR/I locus represses motility by controlling expression of *flhDC* and *fliA* and that the YtbR/I locus activates flagellar expression (Atkinson et al., 2008). Since these results together with further findings demonstrated that NagC is a regulator of QS in *Yersinia* (Chapter 4), motility assays were carried out to determine if NagC has a visible effect on flagellar mediated motility.

Firstly, to determine whether Δ*nagC* has an altered motility phenotype compared to the wild-type, 20 µl of overnight cultures grown at 22°C were observed under a phase-contrast microscope as described by Atkinson *et al*. (1999). Strains tested were the YpIII wild-type, Δ*nagC*, the Δ*nagC* complemented mutant and the hypermotile Δ*ypsR* control. None of the strains were hypermotile (data not

shown). Additionally, swimming motility in swim agar of the YpIII wild-type, Δ*nagC*, the Δ*nagC* complemented mutant and a positive control Δ*ypsR* (Atkinson *et al.*, 1999) was examined. Figure 5.2 shows that after 48 h of incubation Δ*nagC* is not motile on swim agar, like the wild-type and the Δ*nagC* when compared to the hypermotile Δ*ypsR*. This result suggests that, unlike YpsR/I, NagC is not acting as a repressor of flagellar-mediated motility either directly or *via* its control of QS. However, since the YpIII wild-type strain is not motile, this assay cannot clarify, whether NagC regulates flagellar gene expression in *Y. pseudotuberculosis*.

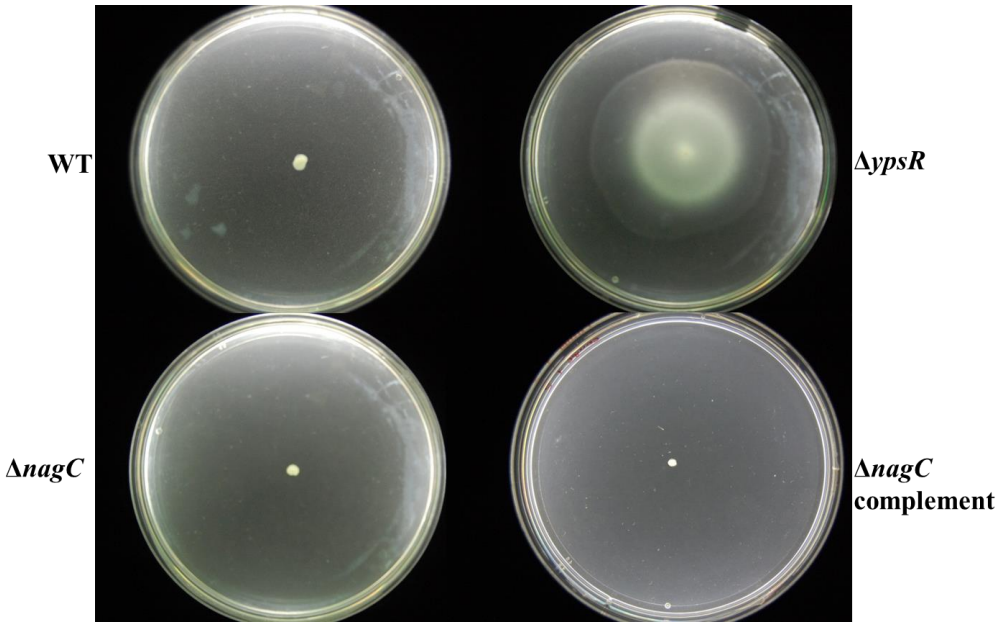


Figure 5.2: Flagellar swimming assay of *Y. pseudotuberculosis* Δ*nagC* in comparison to the wild-type, the hypermotile Δ*ypsR* and the Δ*nagC* complement. Pictures of the swimming plates were taken after 48 h of static incubation time at room temperature. Results show that like the YpIII wild-type, Δ*nagC* and its complemented control are not swimming, whereas the positive control (Δ*ypsR*) does. Pictures of plates show one representative of three individually conducted assays.

5.2.2 Aggregation of Δ*nagC*

The aggregation phenotype of *Y. pseudotuberculosis* was shown to be promoted at 37°C by the T3S injectisome encoded on the virulence plasmid; hence strains that lack pYV do not aggregate. However, it was also revealed that a deletion of *ypsI/ytbI* or *ypsR/ytbR* increases aggregation which confirms the contribution of QS to this phenotype (Chapter 3). Since NagC regulates the expression of QS genes at 37°C by downregulation of *ypsI*, *ytbI* and early activation of *ypsR* expression (Chapter 4), it was also important to determine whether the influence of NagC on QS also affects aggregation.

To examine this, the YpIII wild-type, Δ*ypsI*/Δ*ytbI*, Δ*nagC* and the Δ*nagC* complement with or without pYV were grown overnight at 37°C and were transferred into cuvettes. Figure 5.3 shows cultures left to settle in cuvettes over 40 min and microscope images with a 4,000-fold magnification were taken from the same cuvette. All pYV^+ strains aggregated whereas cells that were cured of the plasmid remained planktonic. However, the aggregation intensity is enhanced in a pYV^+ Δ*ypsI*/Δ*ytbI* and in Δ*nagC*, which reveals a role for AHL synthases and NagC towards aggregation in a pYV-dependent manner.

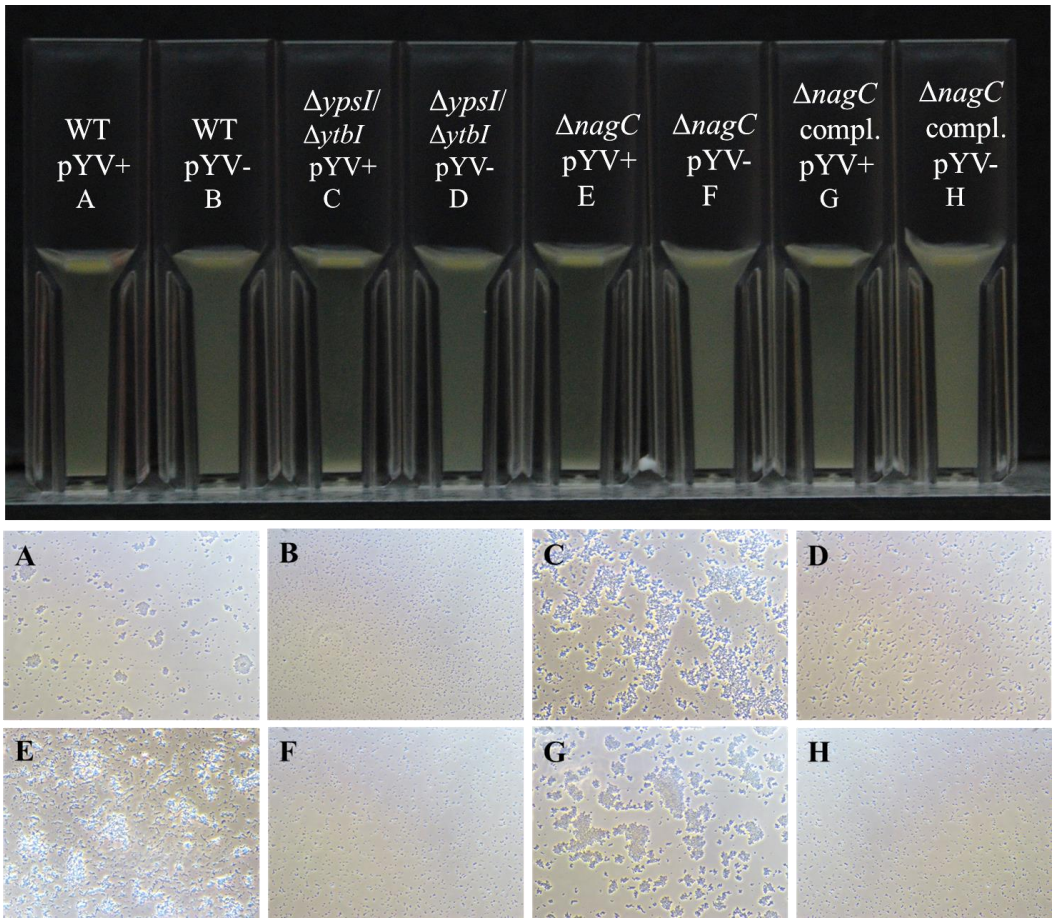


Figure 5.3: Cell aggregation assay of *Y. pseudotuberculosis* Δ*nagC* (E+F) and its complemented mutant (G+H) compared with the wild-type (A+B) and the Δ*ypsI*/Δ*ytbI* (C+D) with or without pYV. Cuvette pictures were taken after 40 min of incubation at 22°C. All *Yersinia* strains aggregate when containing pYV (Cuvettes A, C, E, F) whereas the Δ*ypsI*/Δ*ytbI* and Δ*nagC* show enhanced aggregation.

5.2.3 Biofilms on *C. elegans*

5.2.3.1 NagC is Important for Biofilm Formation on *C. elegans*

Several strains of *Y. pseudotuberculosis* form biofilms on the head and body of *C. elegans* (Joshua *et al.*, 2003). Furthermore, it is known that QS is involved in the formation of *C. elegans* biofilms, since mutation of both AHL synthases reduces the biofilm when compared to the wild-type strain (Atkinson *et al.*, 2011). In the same study it was revealed that biofilm formation is dependent on T3S, since biofilms are increased when *C. elegans* were fed with *Y. pseudotuberculosis* cured of pYV or by a mutation of *yscJ*, a T3S structural gene connecting the inner and outer membrane parts of the injectisome. Also by mutating both AHL synthases, T3S effectors were excreted in non-permissive conditions of low temperatures and in the presence of Ca^{2+} (Atkinson *et al.*, 2011). Considering that NagC is the regulator of the GlcNAc metabolism in *E. coli* and *Yersinia* and that polyGlcNAc is a major component of the exopolysaccharide present in the ECM of *Yersinia* biofilms (Titgemeyer *et al.*, 1994, Deng *et al.*, 2002, Bobrov *et al.*, 2008), it was important to determine, whether deletion of *nagC* caused changes in the biofilm phenotype of *Y. pseudotuberculosis* on *C. elegans*. Since it is known that NagC also impacts on QS expression and that the AHL deletion mutant Δ*ypsI*/Δ*ytbI* is reduced in its ability to form biofilms on *C. elegans* (Atkinson et al., 2011), it was proposed that NagC could also affect biofilms in that manner.

To investigate biofilms on *C. elegans*, the *Y. pseudotuberculosis* YpIII wild-type, Δ*nagC* and the Δ*nagC* complemented mutant were transformed with the GFP encoding p*xylA*p (Table 2.2). *C. elegans* were transferred onto plates containing the wild-type, Δ*nagC* or the Δ*nagC* complemented mutant and biofilm was examined after 24 h. Figure 5.4 shows confocal microscopy pictures (1,000-fold magnification) of *C. elegans* fed with the *Y. pseudotuberculosis* wild-type (A), Δ*nagC* (B) and the Δ*nagC* complemented mutant (C) and stained with rhodamine labelled wheat-germ-agglutinin (WGA) which binds to the polyGlcNAc in the biofilm ECM. When fed with the wild-type and the Δ*nagC* complemented mutant, red fluorescent biofilm can be detected on the head of *C. elegans* in which green fluorescent bacteria are embedded. A layer of biofilm can also be visualised on the cuticule of the worm fed with the wild-type. However, when fed with Δ*nagC* no red fluorescence can be detected and green fluorescing bacteria are distributed along the digestive tract of the nematode showing that the worm can feed on Δ*nagC* without blockage of its mouth region. This blockage would prevent the nematode from swallowing the bacteria, which may have occurred by feeding *C. elegans* with the wild-type and the Δ*nagC* complemented mutant since no fluorescent bacteria can be seen inside the gut.

A reason for the differences in biofilm expression of Δ*nagC* and the wild-type/ Δ*nagC* complement may be due to inhibition of biofilm formation due to a change in the GlcNAc metabolism by relief of repression of the *nag*-operon in the absence of NagC. This would lead to unregulated metabolism of GlcNAc which is needed

for building the ECM of the biofilm and lack of biofilm will inhibit bacterial accumulation at the mouth region of the nematode.

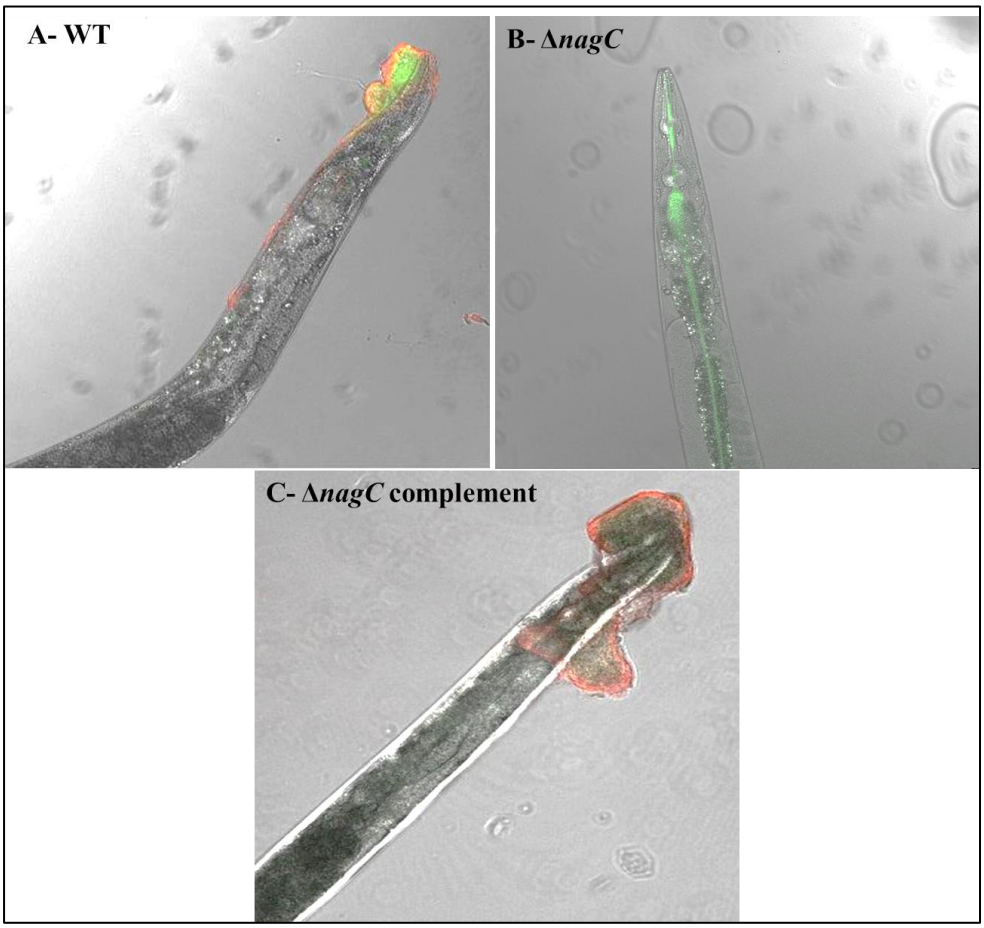


Figure 5.4: Biofilm formation on the head of *C. elegans* caused by *Y. pseudotuberculosis* after incubation for 24 h at 22°C. The YpIII wild-type and the Δ*nagC* complemented mutant form biofilms on the head of the nematode (A and C), whereas feeding the worm with Δ*nagC* does not result in biofilm formation (B).

5.2.3.2 Addition of GlcNAc Restores Biofilm Formation on *C. elegans*

Since a deletion of *nagC* leads to attenuated biofilm formation on *C. elegans,* it was necessary to investigate what additional regulatory changes are occurring in this mutant. The first step taken was to explore the importance of GlcNAc for biofilm formation by Δ*nagC* since a deletion of *nagC* may result in enhanced degradation of GlcNAc and it is assumed that with a decreased GlcNAc concentration, *Yersinia* is not able to form the biofilm ECM. In order to confirm that biofilm is a GlcNAc-mediated process, GlcNAc was added to NGM plates.

Confocal microscopy (Figure 5.5 and Figure 5.6) revealed that after addition of 1.0 mM GlcNAc there is no change in biofilm formation by Δ*nagC* (Figure 5.5 B) since red-fluorescence could not be detected and green fluorescently labelled bacteria can be seen in the gut of the nematode. These results are similar compared to those of the previous section in which GlcNAc was not added to the NGM plates (5.2.3.1, Figure 5.4).

In contrast, addition of 5.0 mM GlcNAc to the NGM restores biofilm formation by Δ*nagC* (Figure 5.6 B), since red-fluorescence of the biofilm ECM can be detected surrounding the head and the vulva region of the nematode.

Biofilm severity index (BSI) was calculated and Figure 5.7 shows the BSIs for the nematodes fed with either the *Y. pseudotuberculosis* wild-type, Δ*nagC* or the Δ*nagC* complemented mutant on plates without GlcNAc supplement (A), 1.0 mM GlcNAc (B) and 5.0 mM GlcNAc (C) and reveals that the YpIII wild-type causes ~70 % biofilm on the worm, whereas feeding the nematode with Δ*nagC* does not lead to any biofilm formation. Complementing Δ*nagC* with a functional copy

restores the biofilm to ~80 %. Similar results are obtained in the presence of 1.0 mM GlcNAc to the NGM plates (Figure 5.7 B). The addition of 5.0 mM GlcNAc leads to restoration of biofilm on *C. elegans* by Δ*nagC* to ~40 % of that of the wild-type and Δ*nagC*.

These results suggest that NagC is an important regulator for the metabolism of GlcNAc and after deletion; degradation of GlcNAc by the *nag*-operon gene products will be enhanced, leading to a non-biofilm phenotype. However, addition of 5.0 mM GlcNAc increases the amount of GlcNAc to a level high enough for the bacteria to re-develop a biofilm and suggests that free exogenous GlcNAc is important for biofilm formation on the surface of *C. elegans*.

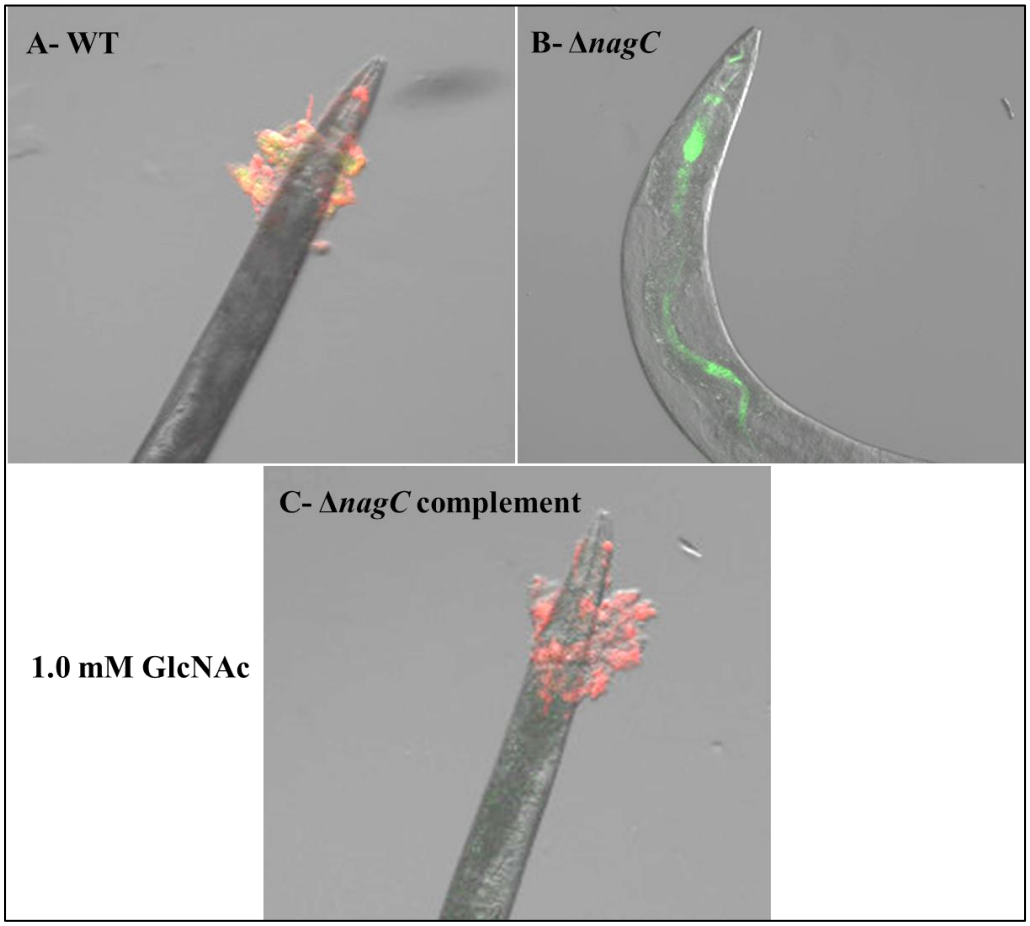


Figure 5.5: Biofilm formation on the head of *C. elegans* by *Y. pseudotuberculosis* after incubation for 24 h at 22°C on NGM plates containing 1.0 mM GlcNAc. The YpIII wild-type and the Δ*nagC* complemented mutant form biofilms at the head of the nematode (A and C), whereas feeding the worm with Δ*nagC* does not show biofilm formation (B).

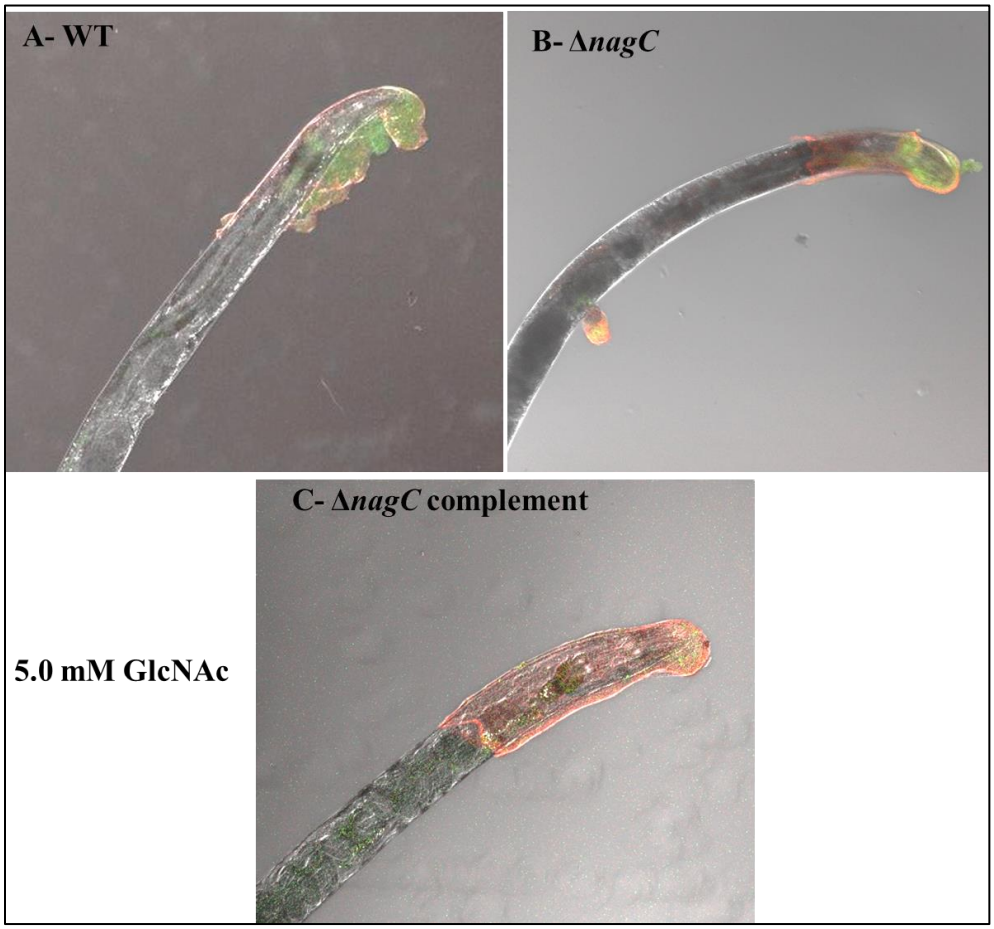


Figure 5.6: Biofilm formation on the head of *C. elegans* by *Y. pseudotuberculosis* after incubation for 24 h at 22°C on NGM plates containing 5.0 mM GlcNAc. The YpIII wild-type, Δ*nagC* and the Δ*nagC* complemented mutant form biofilms at the head of the nematode (A, B and C).

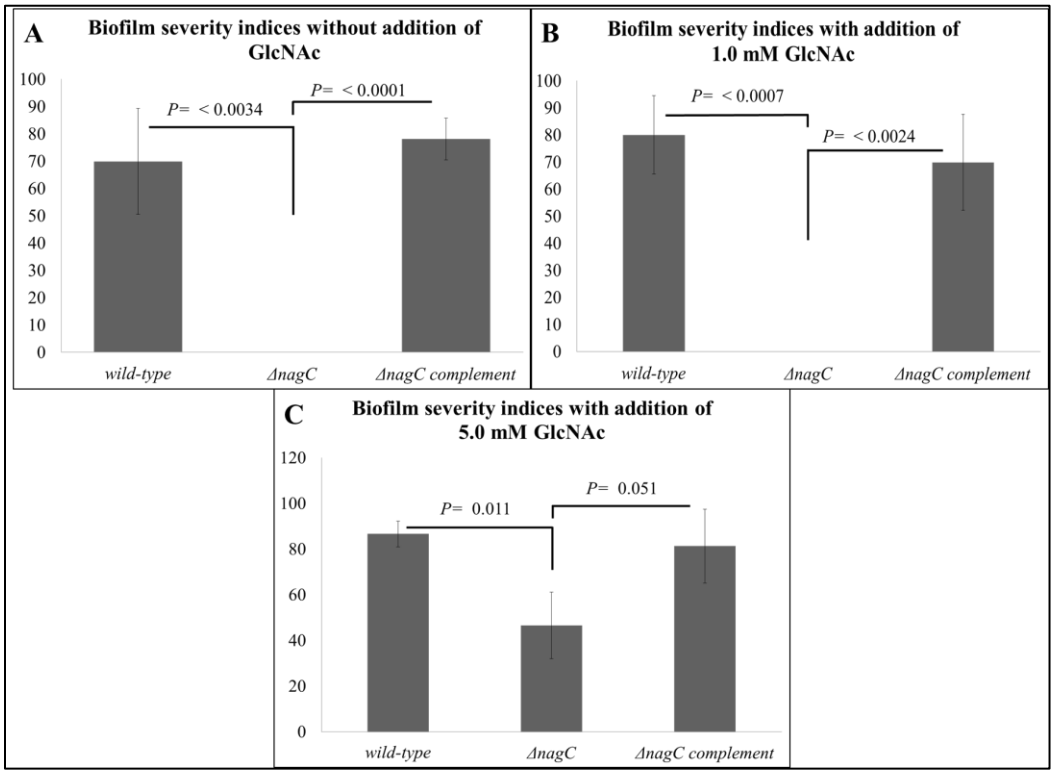


Figure 5.7: Biofilm severity indices (BFIs) of *C. elegans* fed with the YpIII wild-type, Δ*nagC* and the Δ*nagC* complemented mutant after 24 h incubation at 22°C with or without addition of GlcNAc. Figure A and B show the BSIs for nematode on NGM medium without and with addition of 1.0 mM GlcNAc. The wild-type and the Δ*nagC* complement show severe biofilm formation but no biofilm was formed by Δ*nagC*. Biofilm formation of Δ*nagC* is restored when 5.0 mM GlcNAc was added (C). The sample size for each was n=3. *P*-values were calculated using a t-test with two tailed distribution and two sample equal variance standard deviation.

5.2.4 Mutation of *nagC* has No Effect on Yop Secretion

In vitro the expression of the T3SS is linked with a temperature requirement of 37°C and Yops are only released in absence of Ca^{2+} (Perry and Brubaker, 1987). However, several QS mutants were shown to have lost this regulation and secreted Yops at 30ºC in presence of Ca^{2+} revealing that QS is regulating T3S (Atkinson *et al.*, 2011). After mutating *nagC* and confirming its role as a regulator of QS gene expression, it is also important to investigate whether NagC also takes part in the regulation of T3S.

Yop secretion assays were carried out at 37ºC in the presence of the Ca^{2+}-chelator Magnesium oxalate (MgC_2O_4) to compare the patterns of Yop secretion by Δ*nagC* with the YpIII wild-type and the Δ*nagC* complemented mutant. Figure 5.8 shows

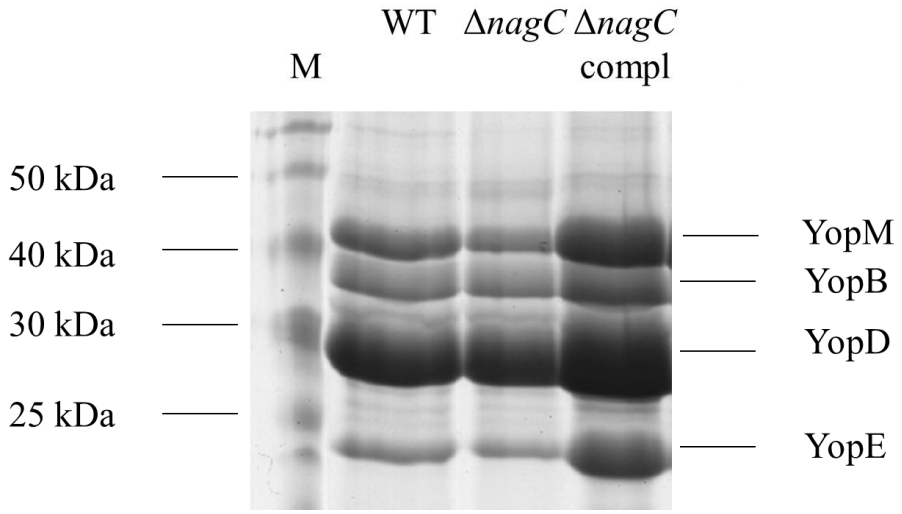


Figure 5.8: Yop secretion assay of *Y. pseudotuberculosis* wild-type, Δ*nagC* and the Δ*nagC* complemented mutant grown without Ca^{2+} at 37°C. Δ*nagC* shows the same Yop profile as the wild-type and the complemented strain. The ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa), was used as marker.

Yops extracted from Δ*nagC* exhibit the same profile as the Yops extracted from the wild-type and Δ*nagC* complemented mutant when grown under Yop inducing conditions, which suggests that NagC does not have an effect on the T3SS under these conditions.

5.2.5 Summary

The results in this chapter have led to the identification of several phenotypes linked to NagC. Mutation of *nagC* enhances aggregation of *Y. pseudotuberculosis* to levels reported in Δ*ypsI/* Δ*ytbI*, showing that NagC, like QS normally represses aggregation factors. Furthermore, mutation of *nagC* attenuated biofilm formation on *C. elegans* which could be restored by addition of 5.0 mM GlcNAc, revealing that a deletion of *nagC* causes a continuous degradation of GlcNAc by the *nag*-operon, which functions as an essential part of the biofilm. However, a role for NagC in motility or T3S could not be observed despite NagC regulating QS and QS being known to affect those phenotypes.

5.3 Discussion

Chapter 4 described the reciprocal regulatory mechanism between NagC and QS and therefore this chapter set out to investigate the effects NagC has on QS related phenotypes of *Y. pseudotuberculosis* YpIII.

After investigating the motility of Δ*nagC* by microscopy and on swimming agar, it was found that Δ*nagC* behaves like the wild-type when grown at 22ºC (Figure 5.2).

Since Chapter 4 revealed that Δ*nagC* upregulates *ypsI*, *ytbI* and *yspR* and *ytbR* gene expression, the behavioural similarity to the wild type may be due to this regulation. The increase in *ypsI* and *ypsR* may cause further repression of motility, since those were shown to repress motility under normal conditions. In addition, expression of *ytbR*, which activates motility, is downregulated in this mutant. In contrast, *ytbI* expression is activated in Δ*nagC* which may cause an activation of

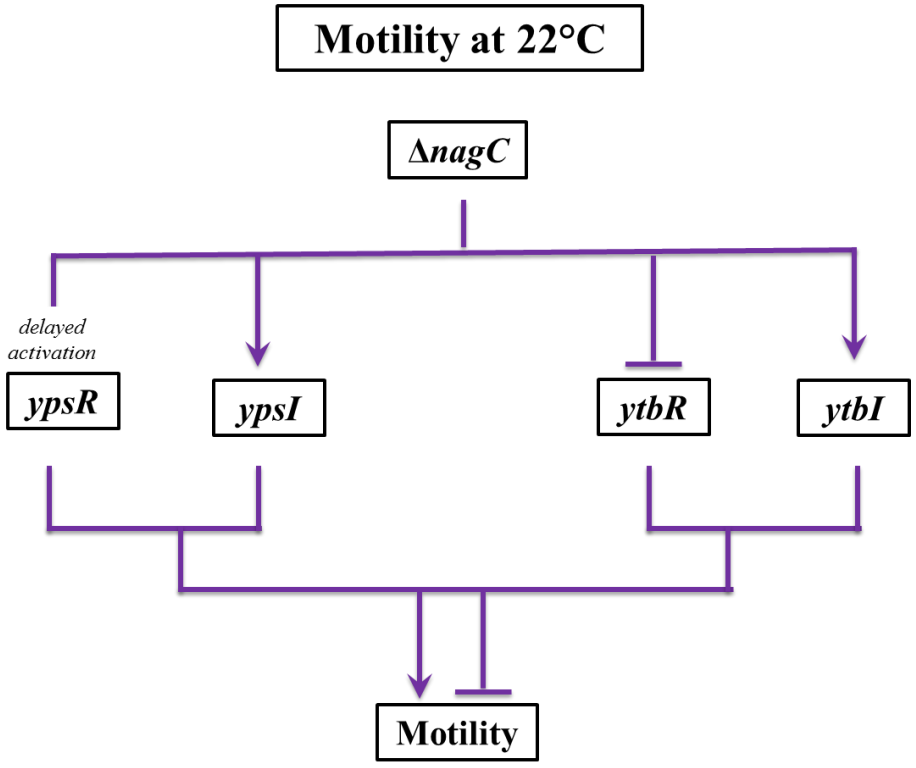


Figure 5.9: Illustration of how a mutation in NagC may effect motility. YpsI/YpsR have been shown to downregulate motility, whereas YtbI/YtbR are known to activate motility in the wild type (Atkinson *et al.*, 2008). Deletion of *nagC* leads to an activation of *ypsI* and *ypsR*, a reduction of *ytbR* and an increase of *ytbI* which may balance positive and negative effects of QS on motility.

motility genes and therefore balance the repression of motility caused by the activation/downregulation of the other QS genes (Figure 5.9). This then results in neither an increase, nor a decrease of motility in Δ*nagC*.

To determine whether NagC downregulates motility genes, promoter *lux* fusions of the flagellar master regulon *flhDC* and the flagellar specific sigma factor *fliA* could be inserted into Δ*nagC*. These constructs could be investigated together with promoter fusions of these two genes in double mutants of QS genes and *nagC* to analyse their expression patterns in comparison to the wild-type.

Chapter 3 presented data to suggest that QS plays a role in cell-cell aggregation since mutation of both AHL synthases and both response regulators increased the aggregation of cells at 37ºC (Figure 3.4). Since Chapter 4 presented work to support a role for NagC in controlling QS, it was investigated whether NagC also plays a role in aggregation.

When investigating the aggregation phenotype, deletion of *nagC* causes the cells to aggregate more densely than the YpIII wild-type and the Δ*nagC* complemented mutant and shows aggregation intensity similar to the Δ*ypsI*/Δ*ytbI* (Chapter 3). However, since *ypsI* and *ytbI* are upregulated in Δ*nagC* (Chapter 4), it would be assumed that if the direct regulation of Δ*nagC* towards QS is regulating aggregation, the aggregation phenotype would not result in denser aggregation. Since this is not the case, it may be assumed that NagC affects aggregation independently of QS. By conducting transposon mutagenesis on Δ*nagC* and investigating the resulting mutants, factors linking NagC and aggregation could be identified.

Another phenotype which was shown to be influenced by NagC is biofilm formation on *C. elegans*. Biofilm formation on *C. elegans* by *Y. pseudotuberculosis* was firstly described by Darby *et al.* (2002) and was shown to be regulated by QS (Atkinson *et al.*, 2011) due to the reduction of biofilm observed when the worms were infected with the Δ*ypsI*/Δ*ytbI* mutant. A major exopolysaccharide component of the ECM of the *Y. pseudotuberculosis* biofilm is polyGlcNAc, which is produced by the *hms*-locus (Bobrov *et al.*, 2008). In *E. coli* GlcNAc is produced *via* the *glmUS*-operon which is positively and negatively controlled by NagC (Plumbridge, 1995). Since this operon also exists in *Y. pseudotuberculosis* with a high similarity, it may perform a similar or identical function to the *glmUS*-operon in *E. coli* (identity of *glmU/glmS* compared to *E. coli*: 82 % / 85 %). This study demonstrated that a deletion of *nagC* leads to a biofilm negative phenotype of *Y. pseudotuberculosis* in comparison with the wild-type and the Δ*nagC* complemented mutant. Staining the biofilm on the worms with rhodamine-labelled WGA showed that there is no extracellular GlcNAc, hence no ECM produced by Δ*nagC* (Figure 5.4). However, addition of 5.0 mM GlcNAc to the agar plates restored the biofilm to about 50 % of the YpIII wild-type and the complemented mutant (Figure 5.6, Figure 5.7). These data suggest that bacteria unable to express *nagC* possess an enhanced metabolism of GlcNAc, due to derepressed expression of the *nag*-operon. This may cause a reduction of the biofilm ECM due to a lack of the main component, polyGlcNAc. The derepression of the *nag*-operon is illustrated in Figure 5.10. PolyGlcNAc in the biofilm is not essential for the bacteria's survival, however some GlcNAc

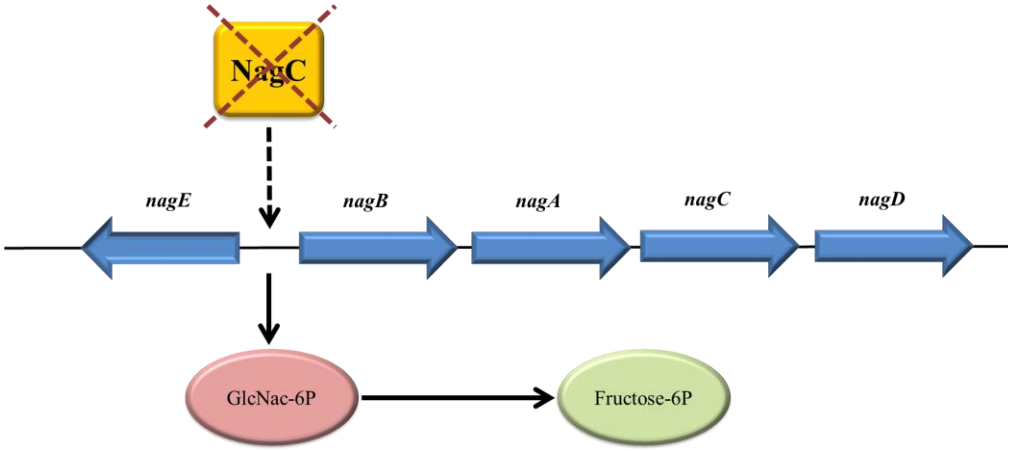


Figure 5.10: Possible effects of the mutation of *nagC* on the expression of the *nagE-nagBACD* operon. Absence of NagC might lead to derepression of the *nag*-operon, which may cause enhanced metabolism of GlcNAc to fructose-6P.

needs to be kept inside the cell for the synthesis of peptidoglycan (Madigan, 2012). Hence when GlcNAc is in short supply, the cells might divert it away from polyGlcNAc synthesis. However, addition of 5 mM of GlcNAc restored the biofilm, showing that this amount is sufficient to restore the production of polyGlcNAc.

It was previously suggested that bacteria release ECM material and will be passively taken off the lawn by the mouth of *C. elegans* while the nematode is moving (Tan and Darby, 2004). A more recent study suggests that bacteria attach to the worm and then secrete ECM material or that bacteria already attached to the worm become in contact with biofilm material from the lawn (Joshua *et al.*, 2014). This study supports the first observation, since when visualising the GFP expressing Δ*nagC* there are no bacteria seen attached to the surface of the worms (Figure 5.4).

Interestingly, while conducting this study; worms were often observed to have separate biofilms on their tail or vulva region as demonstrated in Figure 5.11.

Worms infected with *Y. pseudotuberculosis* usually show exaggerated body-bends, which are likely to be regulated by a decrease in intracellular signalling genes (*gls*) as a reaction to the attachment of the bacteria and after exposure to potential bacterial toxins (Atkinson *et al.*, 2011, Joshua *et al.*, 2014). It was further shown that the deletion of both AHL synthases (YpsI/YtbI) reduced the biofilm on the worm by more than 50 % but still showed this body-bending phenotype (Atkinson *et al.*, 2011). Interestingly, when fed with Δ*nagC*, these exaggerated movements of *C. elegans* are not seen. This suggests that mutation of *nagC*

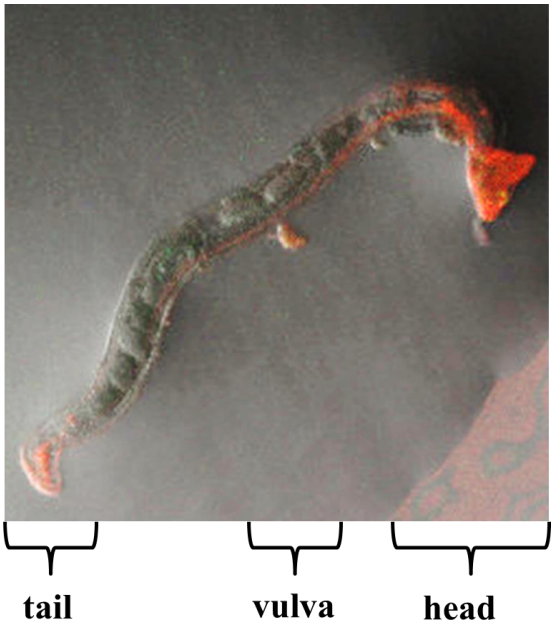


Figure 5.11: Confocal microscopy of *C. elegans* fed with the Δ*nagC* complemented mutant for 24 h. The nematode shows separate biofilms on its head, its vulva and its tail. Magnification = 5000 -fold.

prevents the interactions which trigger the exaggerated body-bend phenotype in *C. elegans*. Since it is known that *C. elegans* changes the expression of several genes, such as C-lectin and fatty acid desaturase genes as a reaction to the *Y. pseudotuberculosis* wild-type (Joshua *et al.*, 2014), it would be interesting to perform a genome-wide gene expression study of the nematode after feeding with Δ*nagC*.

5.3.1 Summary

After studying the NagC and GlcNAc related phenotypes of *Y. pseudotuberculosis*, it can be proposed that

- Δ*nagC* shows the same motility phenotype as the wild-type,
- NagC is involved in reducing cell-cell aggregation,
- mutation in *nagC* leads to attenuation of biofilm on *C. elegans*, probably due to enhanced degradation of GlcNAc,
- addition of 5.0 mM GlcNAc restores the biofilm of Δ*nagC*.

6 Conclusions and Future Directions

6.1 Conclusions

Studies of the QS mechanisms of *Y. pseudotuberculosis* have been ongoing for almost 20 years and a lot of knowledge about QS regulated phenotypes has been accumulated. However, there are still many gaps to fill to understand all the mechanisms that are ongoing in the bacteria under different environmental conditions. This study set out to better understand the role of QS on bacterial behaviour and to gain new insights into the regulation of QS.

The QS regulated phenotypes of *Y. pseudotuberculosis* which already have been described are flagellar mediated motility, biofilm formation on *C. elegans*, cell-cell aggregation and T3S (Atkinson *et al.*, 1999, Atkinson *et al.*, 2008, Atkinson *et al.*, 2011). It was shown that flagellar mediated motility is activated at 22ºC since the flagellar master regulon *flhDC* is activated and the sigma factor *fliA* is downregulated by QS (Atkinson *et al.*, 2008). Furthermore, biofilm formation on *C. elegans* is in turn activated by FlhDC and T3S was shown to be downregulated by both AHL synthases and response regulators and by motility genes at 30ºC and 37ºC which demonstrated that QS is an important factor for the repression of T3S under non-host conditions (Atkinson *et al.*, 2011). In addition, it was demonstrated that biofilm formation was delayed in an AHL negative mutant (Δ*ypsI*/Δ*ytbI*). Further investigations revealed that genes for a functioning T3S injectisome need to be available in the Δ*ypsI*/Δ*ytbI* for a decrease in biofilm formation. This shows that the T3SS and biofilm formation are linked by QS (Atkinson *et al.*, 2011).

Taken together, it is apparent that the QS related phenotypes are linked into a complex inter-dependent regulatory network.

This study aimed to expand this network by further investigating how QS impacts on aggregation. Aggregation was first detected in *Y. pseudotuberculosis* in the 1980s by growth of bacteria in liquid medium at 37ºC (Skurnik *et al.*, 1984). Aggregation was linked to QS by Atkinson *et al.*(1999) and it was thought that the virulence plasmid encoded adhesion YadA is the aggregation factor (Roggenkamp *et al.*, 1995, El Tahir and Skurnik, 2001). A more recent study showed that also supernatant factors may be involved in aggregation and that a mutant of the injectisome component *yscJ* abolished aggregation (Goldstone, 2011). To confirm these findings and to get a better understanding of this phenotype, it was important to test how QS regulates cell-cell aggregation. Furthermore, additional investigations were performed to identify the aggregation factor.

Results of this study showed that *Y. pseudotuberculosis* aggregates when grown at 37ºC in liquid culture and that aggregates settle to the bottom of a 1 ml cuvette within 45 min – 50 min. Deletion of the AHL synthases (Δ*ypsI*/Δ*ytbI*) and response regulators (Δ*ypsR*/Δ*ytbR*) caused increased aggregation, showing that QS represses aggregation. Incubating cultures with a protease revealed that surface protein(s) are involved in promoting aggregation. Furthermore, curing the bacteria of the virulence plasmid pYV lead to a loss of aggregation, suggesting that the aggregation factor is pYV encoded. Interestingly, several pYV genes inactivated by transposon insertion resulted in the loss of bacterial aggregation. Those genes all code for parts of the T3SS injectisome, such as YscV, YscU, YscT and YscR

of the export apparatus and YscN of the ATPase. A separate mutation of the *yscV* and the needle component *yscF* in the wild-type and Δ*ypsI*/Δ*ytbI* also abolished the aggregation of *Y. pseudotuberculosis* cultures, suggesting that the injectisome or a component of the injectisome is the aggregation factor.

This result is in contrast to the work demonstrating that YadA is the aggregation factor of *Y. pseudotuberculosis* in rich medium (Skurnik *et al.*, 1984, El Tahir and Skurnik, 2001). Recent investigations revealed that YadA is not related to cell-cell aggregation when grown in complex medium since a mutation in *yadA* did not abolish this phenotype (Joanne Purves, personal communication), which supports the findings of this study .

Taking these results together with findings of Atkinson *et al.* (2011), which showed that QS represses the T3SS in wild-type conditions, it can be assumed that a deletion of Δ*ypsI*/Δ*ytbI* might lead to an increase in injectisome production at 37ºC. Assuming that the injectisome is the aggregation factor and that the Δ*ypsI*/Δ*ytbI* might produce more needles, may explain why this mutant shows enhanced aggregation. This would indicate that there is a link between QS, aggregation and T3S. To check for enhanced expression levels in the Δ*ypsI*/Δ*ytbI*, promoter *lux*-fusions of T3SS genes, such as *yscV* and *yscF*, could be integrated into Δ*ypsI*/Δ*ytbI* and can be compared to the wild-type. Furthermore antibody staining or fluorescent tags on YscF together with microscopy could verify if more injectisomes are present on the surface of the Δ*ypsI*/Δ*ytbI*.

Previous studies of *S.* Typhimurium and *P. aeruginosa* also showed that the T3S is important for cell-cell aggregation but also showed that the aggregates were

surrounded by biofilm (Jennings *et al.*, 2012, Tran *et al.*, 2014). Furthermore, small aggregates with fewer than 10^5 *P. aeruginosa* cells surrounded by biofilms were found to be physiological heterogenous (Wessel *et al.*, 2014). In contrast, this study on *Y. pseudotuberculosis* could not detect biofilms enclosing aggregated cells but detected eDNA. Degradation of eDNA by DNAse-I did not abolish aggregation, which implies existence of a surrounding substance that is not important for aggregation but might be released by the cells. Similar findings were described in a study of *P. aeruginosa* which showed that cells aggregated when grown in liquid culture and that they also release eDNA (Schleheck *et al.*, 2009). In general it is assumed that aggregation helps bacteria to survive in the environment outside the host and inside the host in the early stages of infection, which also helps them to invade the host more effectively (Jennings *et al.*, 2012, Tran *et al.*, 2014). *Y. pseudotuberculosis* aggregation at 37ºC may be important for the early stages of infection and confers on the invading bacteria a degree of protection from host defences. However, aggregation of cells and T3S might work against each other when effectors need to be injected into the neighbouring cell which suggests that there must be another regulating factor. These could be regulatory mechanisms that enable only the single cells attached to host cells to release Yops. Hence it may be that single cells are used to inject Yop effectors into host cells and aggregates protect against phagocytosis and may even act as a repository for single cells that can be released when necessary.

After revealing that the QS regulated aggregation phenotype is caused by interaction of cells using their injectisomes, the next step of the project was aimed to identify regulators of T3S and the other QS phenotypes. Since a previous project (Robert Goldstone, unpublished) revealed NagC as a potential QS regulator, this study set out to investigate the role of NagC. This transcription factor is a known regulator of GlcNAc-6P synthesis and GlcNAc metabolism (Deng *et al.*, 2002, Plumbridge, 1991). In addition, GlcNAc has been associated with virulence, host cell colonisation and biofilm formation in *E. coli*, *P. aeruginosa* and *V. cholerae* (Chang et al., 2004, Korgaonkar et al., 2013, Ghosh et al., 2011).

The results obtained revealed that NagC regulates QS by reducing *ypsI* and *ytbI* expression and by early activation of *ypsR*. However, the expression of *ytbR* is activated by NagC at 22ºC. Interestingly, NagC not only regulates QS but there is also reciprocal regulation.

By investigation of the effects of NagC on QS phenotypes by mutation of *nagC*, it was shown that cell-cell aggregation is enhanced to levels similar to the Δ*ypsI*/Δ*ytbI* genotype. It is assumed that, since NagC downregulates *ytbI* and *ypsI* gene expression and a mutation in *ypsI*/*ytbI* leads to enhanced aggregation phenotype due to higher production of injectisomes, Δ*nagC* possesses an enhanced aggregation phenotype.

Biofilm formation on *C. elegans* was shown to be promoted by NagC. Furthermore, addition of 5.0 mM GlcNAc to Δ*nagC* could restore the biofilm on the nematode. This suggests that the effects observed for NagC on biofilm

formation may occur as a consequence of NagC acting as a repressor of the *nag*-operon (Plumbridge, 1991) and a mutation of *nagC* leads to an enhanced degradation of GlcNAc, which is needed for production of the biofilm matrix (Bobrov *et al.*, 2008). However, it is unlikely that NagC is indirectly regulating biofilm formation *via* QS, since NagC was shown to repress AHL synthases and a mutation of *nagC* would therefore lead to an increase in biofilm formation.

Furthermore, addition of MurNAc to QS promoter *lux*-fusions in the wild-type and Δ*nagC* lead to changes in QS gene expression. Since MurNAc together with GlcNAc form the major part of the peptidoglycan layer of the bacterial cell wall (Madigan, 2012), this finding suggests that *Y. pseudotuberculosis* may be able to sense other bacteria and react to them.

In summary, by repressing expression of both AHL synthases at 37ºC, NagC indirectly represses the T3SS, hence deletion mutants lacking both synthases cannot be repressed by NagC and the number of injectisomes may be increased. By repression of the AHL synthases and activation of the response regulators, NagC shows the same motility phenotype as the wild-type at 22ºC. However, the biofilm phenotype may be regulated independently by NagC and QS (Figure 6.1).

Since unpublished studies revealed that the T3S regulators YmoA and VirF also regulate QS and affect aggregation (Amy Slater, personal communication), it can be assumed that there are many more factors that need to be taken in account when studying the network between QS regulators, QS and QS regulated phenotypes.

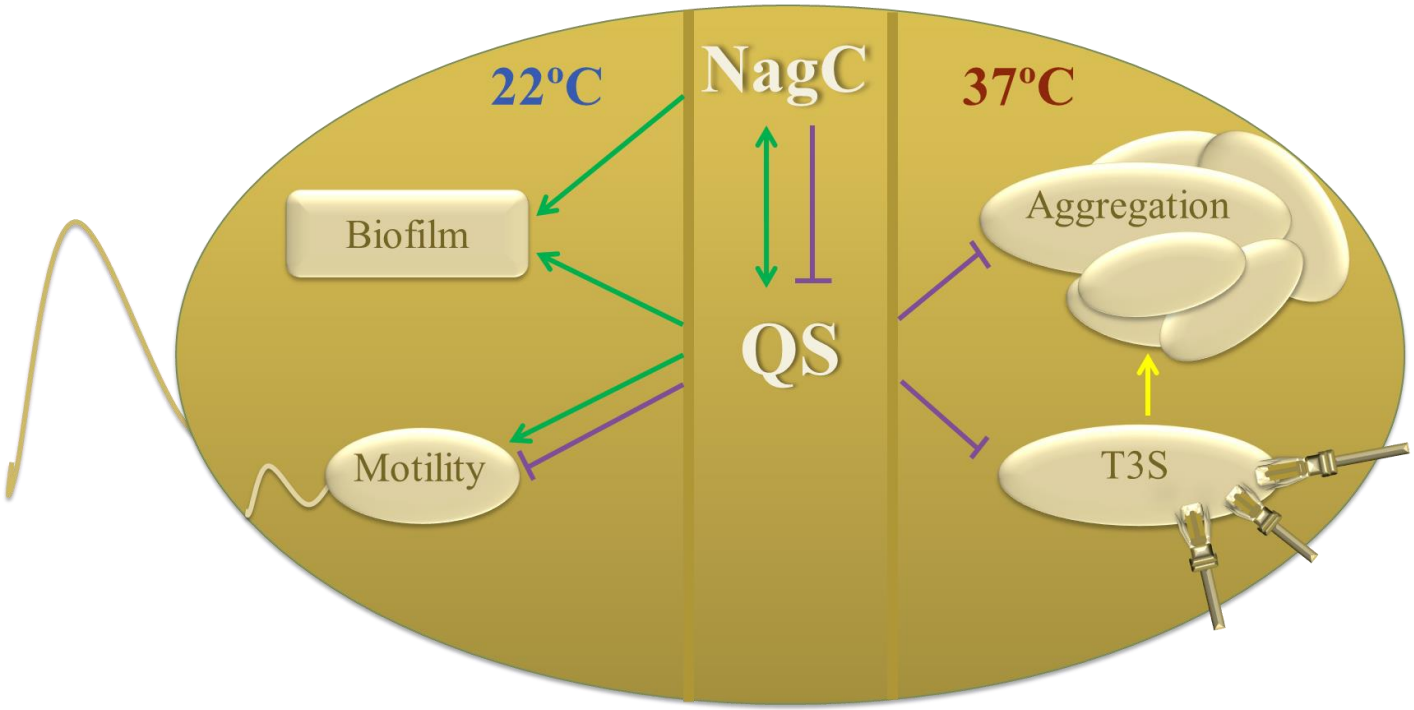


Figure 6.1: Regulation of phenotypes by NagC and QS at 22ºC and 37ºC. NagC represses aggregation and T3S at 37ºC by downregulating QS gene expression. NagC positively and negatively affects motility by regulation of QS genes at 22ºC. The biofilm phenotype is affected by NagC independently of QS independent of QS gene regulation.

6.2 Future Directions

After gaining more insights into QS and QS phenotype regulation by NagC, particular the aggregation phenotype linked to the T3S injectisome, further questions arise requiring further work.

Firstly, it would be good to demonstrate, how TS3 needles induce formation of *Y. pseudotuberculosis* aggregates. It is possible that injectisome needles are interacting with each other leading to aggregation, or perhaps, cells have receptors that the needles bind to. It is necessary to identify the parts of the needle that are responsible for aggregation, since it is possible that a single surface protein component of the injectisome promotes aggregation. It could also be possible that hydrophobic parts of the injectisome interact with the negatively charged cell membrane of other cells. Next to YscF, this could also be the translocon, consisting of YopB and YopD, as identified recently for *P. aeruginosa* (Tran et al., 2014). Deletions of *yopB* and *yopD* will give more information. Furthermore, fluorescent tags in combination with electron-microscopy or cryo-electron tomography might be able to image injectisomes bridging between aggregated cells (Kudryashev *et al.*, 2015) and clarify how the injectisomes aggregate or if receptors are involved. This would also confirm whether the Δ*ypsI*/Δ*ytbI* aggregates more because it produces more injectisomes. Another experiment that needs to be carried out is to identify, whether bacteria aggregate in a host cell environment or if it is an *in vitro* artefact. For this, bacterial aggregates and single cells should be incubated with human cell lines and observed under a microscope.

In addition, promoter *lux*-fusions of several needle components in the wild-type and Δ*ypsI*/Δ*ytbI* would also show, if injectisome parts are more highly expressed in the AHL deletion mutant. Extraction of injectisome components from the bacterial surface will further clarify, if the Δ*ypsI*/Δ*ytbI* possesses more needles. In addition, inserting the injectisome promoter *lux*-fusions into Δ*nagC* could further identify, if NagC is directly regulating the T3SS or indirectly by controlling QS.

After revealing that NagC affects QS phenotypes, it is important to find out more about the QS regulatory network of *Y. pseudotuberculosis*. A recent study in this laboratory showed that the T3S regulators LcrF and YmoA negatively regulate QS and that QS activates *ymoA* expression. Cultures with mutations in *ymoA* and *lcrF* do not aggregate, which underpins the link between the T3SS and the aggregation phenotype (Amy Slater- personal communication). However, it needs to be investigated how these findings link to NagC. It is assumed that NagC affects T3S since it represses *ypsI* and *ytbI* and that this leads to the enhanced aggregation phenotype of Δ*nagC*. YmoA and LcrF might activate aggregation either directly or *via* repression of QS. Taken together this suggests that there might be a network between NagC, LcrF, YmoA and QS to regulate aggregation of the wild-type. This could be tested by performing promoter *lux*-fusion studies of all their gene promoters and by promoter pull-down assays.

Furthermore, there are several other regulators which might influence QS, such as HutC which may be involved in motility and QS regulation (Joshua *et al.*, 2014) and needs to be fitted into this regulatory network.

In addition, GlcNAc was shown to be important for virulence in several bacterial species [reviewed in (Naseem and Konopka, 2015)], hence it would be important to study *Yersinia* infection using the wild-type and Δ*nagC in vivo* to detect changes in virulence behaviour.

Conducting all the assays to answer all these questions will be a new, interesting and insightful future project.

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