Investigating the role of the *V. cholerae* integron/gene cassette system in biofilm formation and resistance to protozoa

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CERTIFICATE OF ORIGINAL AUTHORSHIP

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This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Abstract

Vibrio cholerae is a marine aquatic bacterium and the causal agent of the devastating diarrhoeal cholera disease that sickens millions of people each year. Persistence in the environment of this pathogen is an important area of research as this is where is resides in between periodical disease outbreaks. In the environment, *V. cholerae* deals with numerous stresses including temperature fluctuations, lack of nutrients, salinity and bacteriophage. Additionally, predatory action by free-living heterotrophic protists called protozoa is a major problem for *V. cholerae* growth and survival in the environment. Several mechanisms are employed by *V. cholerae* in environmental persistence, of which biofilm formation is considered a key mechanism. *V. cholerae* preferably forms biofilm on chitin, a long chain polymer present in the exoskeletons of crustaceans in the aquatic environment which apart from being a nutritive source, induces natural competency that allows free uptake of DNA through a mechanism of lateral gene transfer (LGT) called transformation. LGT is when DNA is transferred between bacterial cells and is considered an important evolutionary mechanism in *V. cholerae* through acquisition of novel genetic traits usually on mobile genetic elements such as genomic islands, phage or transposons. Chromosome 2 of *V. cholerae* carries one such mobile genetic element called the integron that facilitates the insertion of mobile genes called gene cassettes and contains more than 150 gene cassettes of which 80-90 % are of unknown or uncharacterised function. Integration, deletion or rearrangement of gene cassettes is dependent on a recombinase called the integron-integrase (encoded by *intIA*) that recognises *attC* sites associated with gene cassettes and facilitates their insertion within the *attI* site of the integron and to a lesser extent, *attC* sites of gene cassettes in the cassette array. *intIA* is induced by the SOS response a regulatory cascade requiring RecA that is activated by the presence of single-stranded DNA (ssDNA) due to stalled DNA replication from damage (e.g. fluoroquinolones, β lactams, UV exposure) or from acquisition of ssDNA from LGT processes.

In order to study chitin induced transformation and *intIA* transcription in *V. cholerae*, this thesis described the construction of circular and linear gene cassettes and investigated their transfer into the *V. cholerae* chromosomal integron of chitin-competent cells and demonstrating that the integron is a novel site for adding DNA for complementation of mutations in *V. cholerae*. Additionally, insertion of the artificial gene cassettes into *attI* and two different *attC* sites were shown to affect bacterial surface properties and biofilm formation most likely due to enhanced transcription of downstream gene cassettes due to the presence of internal promoters in the

artificial gene cassettes. Finally, one of the artificial gene cassettes was used to investigate cassette transfer dynamics in *V. cholerae* in the presence of two bacteriovorist protozoa, the ciliate *Tetrahymena pyriformis* and the amoeba *Acanthamoeba castellanii*. This thesis shows that following internalization and packaging of *V. cholerae* into the food vacuoles (also called phagosomes) of both protozoa, intracellular ROS induces the SOS response leading to enhanced integron-integrase expression and gene cassette recombination (up to 405-fold more cassette integration). ROS production is a key feature of killing within the phagosome causing DNA damage leading to mutagenesis that results in stalling of DNA replication and generating an excess of single ssDNA. In addition, this thesis shows that due to the indiscriminate feeding behaviour of protozoa, co-localisation of different species such as *V. cholerae* and *E. coli* in the same phagosome can facilitate LGT. It is shown that *V. cholerae* utilizes its T6SS to kill and release artificial gene cassette DNA from *E. coli* to make it accessible for uptake and subsequent integron-integrase mediated integration.

Taken together, this thesis, through the creation of artificial gene cassettes to study integron integration dynamics, highlights the importance of integron-associated gene cassettes in biofilm formation and shows the importance of protozoa in driving LGT-driven adaptation and evolution in *V. cholerae*.

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List of Abbreviations

AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ATCC	American type culture collection
ATR	Acid tolerance response
C°	Degrees celcius
cAMP	Cyclic adenosine monophosphate
CAI	Cholera autoinducer
c-di-GMP	Cyclic di-guanosine monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CT	Cholera toxin
DGC	Diguanylate cyclase
DR	Direct repeat
dsDNA	Double stranded deoxyribonucleic acid
eDNA	Extracellular DNA
EFV	Expelled food vacuole
x g	Gravitational force
GI	Genomic island
GFP	Green fluorescent protein
h	Hour
HOCl	Hypochlorous acid
ICE	Integrative conjugative element
IS	Insertion sequence
IR	Inverted repeat sequence
LB	Luria Bertani broth
LPS	Lipopolysaccharide
LCV	*Legionella*-containing vacuole
LGT	Lateral gene transfer
MGE	Mobile genetic element
min	Minute
ml	Millilitre

µl	Microlitres
mm	Millimetre
mM	Millimolar
µM	Micromolar
MSHA	Mannose-sensitive haemagglutinin
nM	Nanomolar
NSS	Nine salts solution
NaCl	Sodium chloride
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PYG	Proteose yeast extract
QS	Quorum sensing
rpm	Revolutions per minute
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RT	Room temperature
RER	Rough endoplasmic reticulum
ssDNA	Single stranded deoxyribonucleic acid
T6SS	Type VI secretion system
TCP	Toxin-coregulated pili
VPI-1	Vibrio pathogenicity island-1
VPI-2	Vibrio pathogenicity island-2
VPS	Vibrio polysaccharide
WHO	World Health Organization
WT	Wild type

Chapter 1 : General introduction and literature review

1.1 Introduction

Among the versatile microorganisms on Earth, bacteria are the most successful. They persist and adapt rapidly to a continually changing environment dealing with stresses such as temperature fluctuations, starvation, salinity and bacteriophage attack (Lutz et al. 2013). Additionally, a substantial proportion of bacteria are consumed by free-living heterotrophic protists called protozoa (Mallory et al. 1983) and digested within food vacuoles constraining their growth and survival (Jürgens & Matz 2002; Lutz et al. 2013). Despite bacteria being exposed to multiple stresses within the protozoal food vacuole (i.e. acidity, proteolytic enzymes, antimicrobial peptides and reactive oxygen/nitrogen species), some are able to survive and grow intracellularly (Al-Khodor et al. 2008) before escaping into the extracellular environment (Barker & Brown 1994).

To persist and adapt to a changing environment, bacteria must employ a wide range of adaptive strategies. Transient physiological responses are the main strategies whereby genes contained within regulons are transcribed or repressed in response to surrounding environmental conditions (Barker et al. 2001; Hammer & Bassler 2003; Silva & Benitez 2004) leading to phenotypic changes such as a biofilm formation on abiotic surfaces or biotic surfaces (Hall-Stoodley, Costerton & Stoodley 2004). In other instances, bacteria rely on mechanisms that bring permanent genetic changes such as gene mutation, re-arrangement of genes, or lateral gene transfer (LGT) (Walker 1996). Lateral gene transfer is one of the most important mechanisms driving bacterial evolution potentially providing a quick and drastic evolutionary "jump" in ecological and pathogenic fitness through acquisition of novel genetic traits (Ochman, Lawrence & Groisman 2000).

This PhD thesis investigated an adaptive genetic element called the integron in the cholera-causing pathogen *Vibrio cholerae* and explored its role in biofilm formation and adapting to bacteriovorist protozoa. Therefore, this introductory chapter will focus on *V. cholerae* and the importance of biofilm formation and, LGT in its evolution. Additionally, the known bacterial defence mechanisms against bacteriovorist protozoa and, the integron will be reviewed in detail.

1.2 *Vibrio cholerae*

1.2.1 General description

Vibrio cholerae is a gram-negative, motile, curved rod-shaped bacteria with a size ranging between 1.4-2.6 μm in length and belongs to the *Gammaproteobacteria* class of the Vibrionaceae family (Thompson, Iida & Swings 2004). *Vibrio* species are facultative anaerobes that are metabolically versatile and exist as natural inhabitants of aquatic and estuarine environments (Reen et al. 2006; Thompson et al. 2004). The pathogenic strains of *V. cholerae* are responsible for approximately 3-5 million cholera cases per annum, leading to more than 100,000 deaths around the world (Ali et al. 2012). Ingestion of contaminated food and drinking water is the primary route of transmission of the disease, which is prevalent in regions that lack adequate sanitation and access to pure drinking water (Charles & Ryan 2011). Cholera is characterized by an enormous excretion of fluid from the body that can cause hypotensive shock and death within few hours of infection if not treated immediately (Charles & Ryan 2011; Kaper, Morris & Levine 1995).

V. cholerae contains diverse serogroups (>200 serogroups)*,* classified based on the lipopolysaccharide containing O-antigen structure. Only two, O1 and O139, are linked with epidemic cholera and produce cholera toxin (CT) with O1 dominant and responsible for pandemics (Kaper, Morris & Levine 1995). The O1 serotype of *V. cholerae* is further divided into biotypes called classical and El Tor on the basis of a variety of phenotypic differences including susceptibility to bacteriophage and the antibiotic, polymyxin B (Faruque, Albert & Mekalanos 1998; Kaper, Morris & Levine 1995). A hybrid biotype that contains characteristics of both classical and El Tor emerged in the early 2000's (Nair et al. 2002).

1.2.2 Epidemiology and evolution of *V. cholerae*

Since the first *V. cholerae* pandemic in 1817, at least seven pandemic waves have occurred globally. Generally, new strains of *V. cholerae* replace pre-existing strains from pandemic to pandemic (Figure 1.1). The identity of the *V. cholerae* strains causing the first five pandemics are unknown, as they occurred pre-1884, the year when the causal agent of cholera was first cultured (Pollitzer 1959). The sixth pandemic (1899-1923) was caused by *V. cholerae* O1 of the classical biotype and is believed to be the causal agent for the preceding pandemics (Scrascia et al. 2009). In support of this, DNA sequencing of a preserved intestine from a 2nd

pandemic cholera victim showed the *V. cholerae* DNA sequence to be most related to the O1 classical biotype of *V. cholerae* (Devault et al. 2014). The classical biotype was responsible for sporadic disease occurrences in the period between the sixth and seventh pandemics (Pollitzer 1959).

The seventh pandemic began in Indonesia in 1961 and is caused by the El Tor biotype replacing the classical biotype and witnesses the most severe and fatal of the cholera pandemics (Hu et al. 2016). During the seventh pandemic in late 1992, a new serogroup of *V. cholerae* called O139 appeared in the delta regions of Bangladesh and India causing epidemic outbreaks but not moving beyond these regions (Albert et al. 1993; Faruque, Albert & Mekalanos 1998; Faruque et al. 2003). This serogroup arose from the O1 E1 Tor lineage due to changes in the lipopolysaccharide region of the genome (Weintraub et al. 1994) probably from LGT with a non-O1/O139 strain (Blokesch & Schoolnik 2007b). After 2005, cholera disease by this serogroup has been sporadic and sometimes rare (Chowdhury et al. 2015) with the E1 Tor biotype acting as the main agent of the current seventh pandemic.

The emergence of new strains of O1 *V. cholerae* has been observed in many Asian and African countries such as the Bangladeshi Matlab variants and variants from Mozambique and Haiti (Grim et al. 2010). These new variants are a result of recombination between the two biotypes (classical and El Tor) resulting in the emergence of 'hybrid' or 'atypical' El Tor strains (Safa, Nair & Kong 2010). The aforementioned observations that *V. cholerae* changed from the classical to El Tor biotype, and, from the O1 to O139 serogroup and more recently, the emergence of hybrid El Tor strains in the early 1990's shows *V. cholerae* to be capable of generating substantial genetic diversity for which the lateral exchange of DNA is regarded as a main driver (Pang et al. 2007).
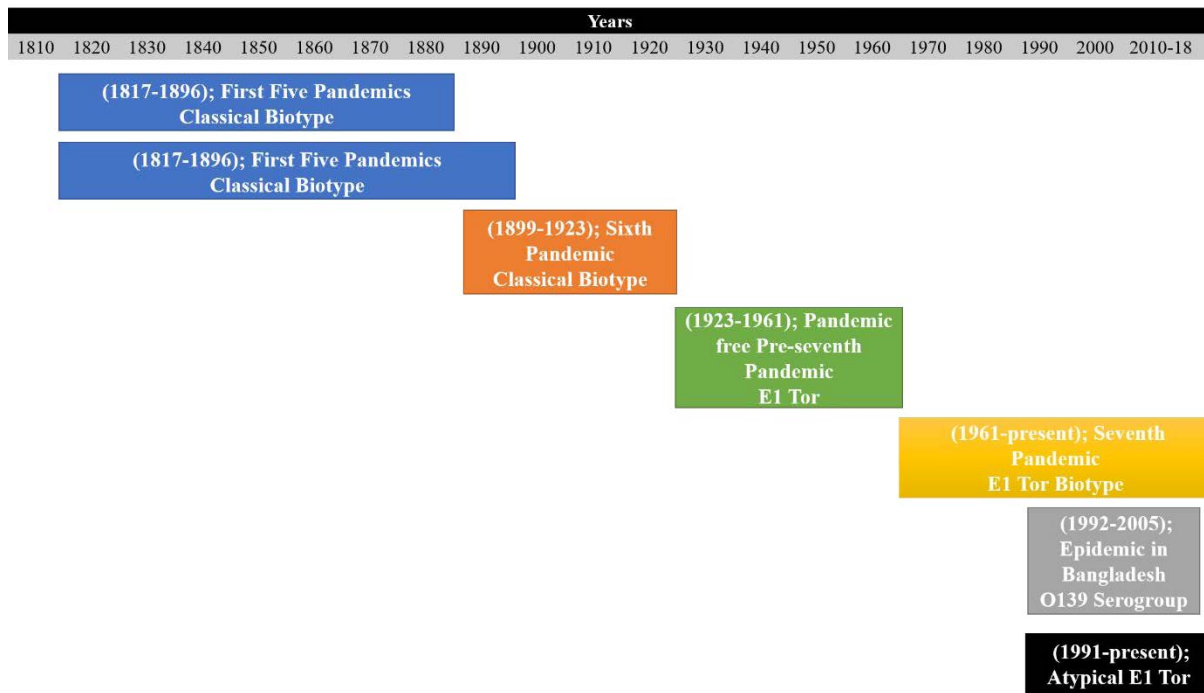
Figure 1.1: Timeline for cholera pandemics through the emergence of new strains since 1817. Timeline of each cholera pandemic with their causative agents. Acquisition of novel genes has driven the emergence and progression of *V. cholerae* pathogens over their evolutionary periods. This figure has been recreated using from a figure in Rahman et al. 2015 (Rahaman et al. 2015).

1.2.3 Pathogenicity of *V. cholerae*

The pathogenicity of cholera causing *V. cholerae* depends on the existence of genes encoding cholera toxin (CT) and toxin-coregulated pilus (TCP) widely present within O1 and O139 serogroup strains. CT is a heat-stable toxin responsible for the profuse and highly watery diarrhoea with a characteristic foul odour in infected patients. However, non-toxigenic (i.e. non-CT) environmental strains of *V. cholerae* (non-O1/O139) can create similar symptoms due to the action of another heat stable toxin (Morris Jr 1994; Rodrigue, Popovic & Wachsmuth 1994; Saha et al. 1996). CT is encoded by two genes (*ctxAB*), which are part of the CTX lysogenic bacteriophage (Waldor & Mekalanos 1996). The TCP is an intestinal adhesin that is essential for intestinal colonization and interestingly, is the receptor for the CTX phage. TCP's production and assembly is mediated by proteins encoded by the *tcp* and *acf* genes that are situated on a genomic island called Vibrio Pathogenicity Island I (VPI-1). VPI-1 is 39.5-kilobases and also encodes regulatory proteins (i.e. ToxR and ToxT) that control the expression of both CT and TCP (Karaolis et al. 1998).

1.2.4 The lifecycles of *V. cholerae*

V. cholerae does not exclusively circulate in human hosts. It naturally exists in the coastal aquatic environment (environmental lifecycle), which acts as the principal reservoir for this bacterium (Colwell, Kaper & Joseph 1977; Silva & Benitez 2016). How this bacterium survives and adapts in the environment and identifying the underlying factors which allows it to periodically causes cholera outbreaks are intriguing questions in infectious disease ecology (Colwell 1996).

1.2.4.1 Pathogenic lifecycle

In the human host, the infection begins (Figure 1.2) with the consumption of contaminated food and water containing pathogenic strains of *V. cholerae* however, *V. cholerae* must overcome a number of obstacles in the human body for successful infection to proceed. For example, most of the consumed bacterial cells are destroyed by the action of the bactericidal effects of the acidic environment in the stomach and, bile acids and antimicrobial peptides in the small intestine (Almagro-Moreno, Pruss & Taylor 2015; Harris et al. 2012; Shikuma et al. 2013). Therefore, the dose required to cause infection is important and depending on the condition of the inocula, the dose may range between 10^6-10^{11} cells (Cash et al. 1974; Levine et al. 1981). *V. cholerae* can also enter into the human host as metabolically quiescent cells (VBNC; viable but non-culturable state) (Alam et al. 2007; Colwell et al. 1985) or as microcolonies that are considered hyperinfectious (Tamayo, Patimalla & Camilli 2010), by-passing the low pH stress in the gastric compartments through its acid tolerance response (ATR) (Colwell et al. 1996; Wong & Wang 2004). Once the bacteria enter into the intestinal lumen, the structural properties of this bacterium such as outer membrane porins, lipopolysaccharide and capsule play a role in inhibiting the entry of bile acids and antimicrobial peptides into the cell (Mathur & Waldor 2004; Provenzano et al. 2000). Subsequently, only the surviving bacteria can adhere and colonize to the human intestinal epithelial cells.

The toxin coregulated pilus (TCP) is the principal factor mediating *V. cholerae* colonization to the human intestine (Herrington et al. 1988; Taylor et al. 1987). Following adhesion, *V. cholerae* grows and forms a biofilm where subsequently cholera toxin is secreted binding to the GM1 ganglioside receptor on the intestinal epithelial cells (King & Van Heyningen 1973; Pierce 1973) and catalyzing ADP ribosylation. ADP ribosylation turns on the activity of adenylate cyclase leading to an increase in intracellular cAMP levels that prevents sodium chloride (NaCl) absorption and decreases chloride and bicarbonate ion secretion causing

massive watery diarrhoea (Kaper, Morris & Levine 1995). It is assumed that approximately 10-100 million *V. cholerae* cells per milliliter of watery stool are released back into the environment from cholera victims (Morris Jr 2011).
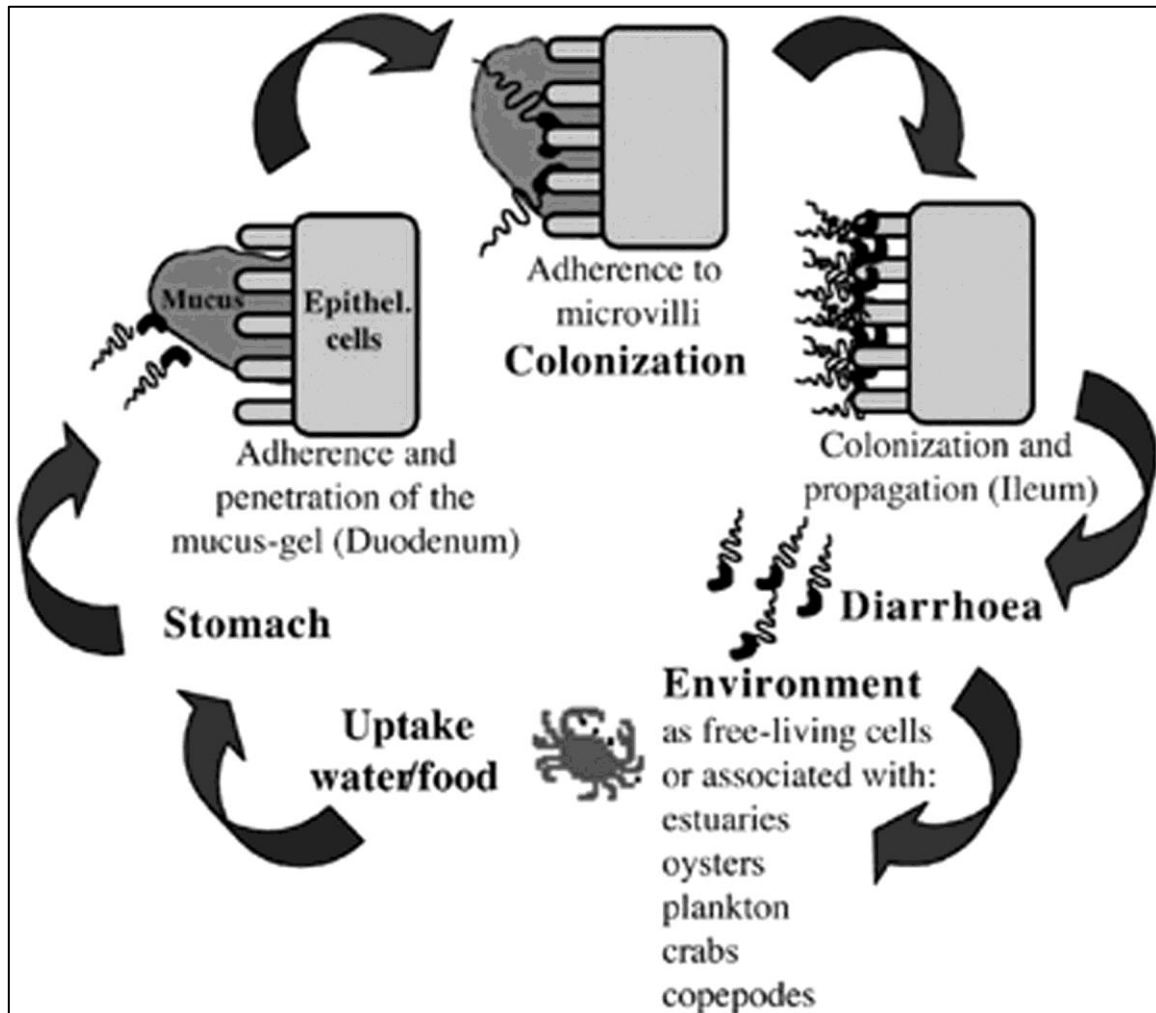
Figure 1.2: A typical infection cycle of *V. cholerae*. After ingestion of pathogenic *V. cholerae* through contaminated food or water, cells must by-pass the acidic stomach environment before they can penetrate the mucosal wall of the duodenum and colonize the epithelia of the ileum of the small intestine. Colonization and multiplication results in cholera disease through the secretion and action of CT. Subsequently, multiplied cells are flushed out and disseminate into the environment and interact with numerous organisms such as oysters, plankton, crabs, copepods, etc., which act as a reservoir for the pathogen in between outbreaks. This picture has been reproduced from J. Reidl, K.E. Klose, 2002. (Reidl & Klose 2002) and used with permission from the publisher.

1.2.4.2 **Environmental lifecycle**

In diverse environmental niches, *V. cholerae* is faced with temperature fluctuations, lack of nutrients, chemicals, bacteriophage attack and protozoan predation (Faruque & Nair 2002; Høiby et al. 2010; Matz et al. 2005; Sultana et al. 2012) and employs a range of adaptive mechanisms such as transitioning to the VBNC (viable but non-culturable) state (Islam et al.

1994), growth arrest and increasing number of persister cells (Silva-Valenzuela et al. 2017) and, biofilm formation (Teschler et al. 2015; Yildiz & Schoolnik 1999).

Biofilm formation is an ancient and a key mechanism that drives survival and persistence in the diverse environmental niches (Hall-Stoodley, Costerton & Stoodley 2004). *V. cholerae* forms biofilms on various biotic and abiotic surfaces, but numerous studies have reported that *V. cholerae* favourably forms biofilms on chitinous surfaces such as on zooplankton (Rawlings, Ruiz & Colwell 2007; Tamplin et al. 1990). Surface attachment and biofilm formation provides numerous selective advantages such as increased stress resistance, increased nutrient access and subsequent dispersal for colonizing new niches (Hall-Stoodley, Costerton & Stoodley 2004). Given the importance of biofilm formation by *V. cholerae*, its developmental process and regulation are reviewed in detail below.

Phases of biofilm development	Initial attachment	Formation of microcolony	Mature biofilm	Dispersal
Structures involved	Polar flagellum, MSHA pili	MSHA pili	Vibrio polysaccharide (VPS), matrix proteins (RmbA, RmbC, Bap 1), extracellular DNA (eDNA)	Extracellular nucleases (Dns and Xds), Polysaccharide lyase
Regulators	Cyclic dimeric guanosine monophosphate (C-di-GMP)		C-di-GMP, Quorum sensing (QS)	C-di-GMP, Quorum sensing (QS)

Figure 1.3. Environmental persistence of *V. cholerae* as a biofilm state. *V. cholerae* uses chemotaxis to locate preferable surfaces to attach. Attachment is enhanced by surface associated protein such as MSHA pili. Consequently, formation of microcolonies and synthesis of extracellular matrix components such as VPS, matrix associated proteins and small amounts of extracellular DNA (eDNA) are necessary for continued biofilm formation and maturation. The regulation of *V. cholerae* biofilm formation is complex. The small nucleotide signalling molecules, c-di-GMP is primarily involved in the transition from the motile form of *V. cholerae* to a sessile form. Furthermore, c-di-GMP and quorum sensing enhance the maturation of biofilm and its subsequent dispersal (see text for details).

1.2.4.2.1 Biofilm formation by *V. cholerae*

There are several steps (Figure 1.3) in the development of *V. cholerae* biofilms. This process includes (i) surface attachment and microcolony formation, (ii) biofilm maturation and (iii) biofilm dispersal (Utada et al. 2014; Watnick & Kolter 1999).

1.2.4.2.1.1 Surface attachment and microcolony formation

At first, *V. cholerae* scans the surface utilizing a roaming or orbiting movement that is facilitated by the polar flagellum and mannose-sensitive hemagglutinin (MSHA) pili (Utada et al. 2014; Watnick & Kolter 1999). MSHA is a type IV pilus (TFP) that facilitates irreversible binding of *V. cholerae* to the surface and formation of microcolonies through TFP driven motility (Utada et al. 2014). Secretion of vibrio polysaccharides (VPS) and extracellular DNA helps direct movement of cells into microcolony structures (Gloag et al. 2013; Zhao et al. 2013).

1.2.4.2.1.2 Biofilm maturation

After surface attachment and microcolony formation, *V. cholerae* synthesizes various extracellular matrix components, which are necessary to attain mature biofilms with a three-dimensional architecture. The extracellular matrix principally consists of vibrio polysaccharides (VPS), matrix proteins (RbmA, RbmC, and Bap1) and extracellular DNA (eDNA) (Reichhardt, Fong, et al. 2015; Seper et al. 2011; Yildiz & Schoolnik 1999). VPS is the key element that makes up half of the total biofilm matrix mass and plays the principal role in maturation (Fong et al. 2010; Yildiz et al. 2014; Yildiz & Schoolnik 1999). In addition to biofilm formation, VPS is also essential for transitioning *V. cholerae* from a smooth (lower quantity of VPS) colony morphological state to a rugose (higher quantity of VPS) colony morphological state (Figure 1.4). This rugose morphological state provides an extra level of adaptive ability in response to diverse stresses such as chlorine (Yildiz & Schoolnik 1999), lower pH (Zhu & Mekalanos 2003), oxidative radicals and osmotic pressure (Wai et al. 1998) and bacteriovorist protists (Sun, Kjelleberg & McDougald 2013).

V. cholerae rugose morphology   *V. cholerae* smooth morphology
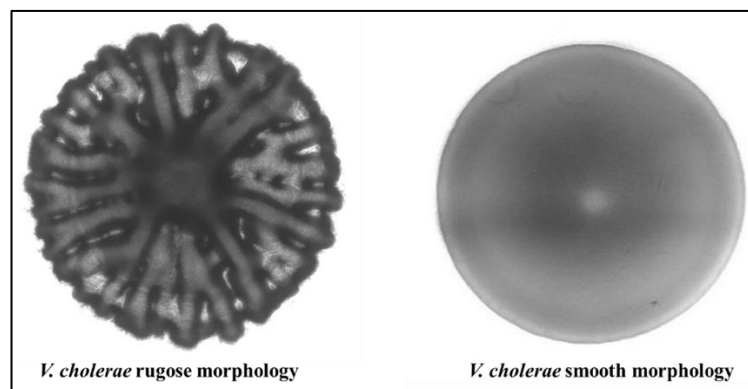
Figure 1.4: Colony morphological variation of *V. cholerae*. *V. cholerae* produces corrugated-rough colonies called rugose (left) due to higher production of VPS and exhibits increased levels of resistance to stress. Lower production of VPS results in opaque, translucent colonies called smooth (right). This image is derived from this PhD research.

The matrix-associated proteins (RbmA, RbmC and Bap1) have exclusive functions within the biofilm. Specifically, RmbA, which is produced in the early stages of biofilm formation and acts as a cohesive material for cell-to-cell adhesion (Berk et al. 2012; Fong & Yildiz 2007). RmbC and Bap1 appear in the later stages of biofilm formation and together with VPS, encases clusters of cells. Additionally, Bap1 found at the cell surface interface prevents cell dispersal by maintaining mechanical strength of the biofilm structure (Hollenbeck et al. 2014).

1.2.4.2.1.3 Biofilm dispersal

Following biofilm maturation, cells are capable of detaching and colonizing new niches. The underlying mechanism of this process in *V. cholerae* is still not completely understood. As extracellular DNA (eDNA) is an important element of the biofilm matrix, degradation of eDNA by the extracellular nucleases Dna and Xds is capable of decreasing biofilm formation and facilitating the detachment of biofilm cells (Seper et al. 2011). Moreover, *rbmB* encodes a VPS degrading enzyme, termed polysaccharide lyase, that has been proposed to have an role in detachment of cells (Fong & Yildiz 2007)

1.2.4.2.2 Core regulation of *V. cholerae* biofilm formation

As VPS and matrix-associated proteins are the main drivers of *V. cholerae* biofilm maturation, this portion of the review will focus on regulation of the VPS structural genes. These genes and subsequent biofilm formation are regulated by a complex integrated regulatory network including transcriptional activators (VpsR, VpsT and AphA); transcriptional repressors (HapR and H-NS); alternative sigma factors; a set of small regulatory RNAs and small nucleotide signalling molecules (Figure 1.5) (Teschler et al. 2015).

VpsR is the master positive regulator of *V. cholerae* biofilm formation and is a two-component signal transduction response regulator family member. Deletion of *vpsR* results in a reduction of VPS and matrix protein synthesis and, abolishes biofilm formation (Zamorano-Sánchez et al. 2015). VpsR controls the expression of genes necessary for VPS and matrix protein synthesis (*rbm*) through binding in their respective promoter regions (Zamorano-Sánchez et al. 2015). VpsR also upregulates *aphA*, a master virulence regulator, suggesting that it has a role in pathogenesis. VpsR expression is regulated by VpsT (positive regulation) and the quorum sensing (QS) regulator HapR (negative regulation) (Beyhan & Yildiz 2007; Yildiz, Dolganov & Schoolnik 2001). Similar to *vpsR*, disruption of *vpsT* decreases VPS and matrix protein production and diminishes biofilm formation (Krasteva et al. 2010; Zamorano-Sánchez

et al. 2015). VpsT is also positively regulated by VpsR, AphA and the alternative sigma factor RpoS, but negatively regulated by HapR (Beyhan et al. 2007; Yang et al. 2010). A nucleoid modulating protein, termed histone-like protein (H-NS), is also a transcriptional regulator and represses *vpsT* resulting in down regulation of biofilm formation (Wang et al. 2012).

1.2.4.2.2.1 Quorum sensing regulation of biofilm formation

In *V. cholerae* biofilm formation, HapR is a master negative regulator as deletion of *hapR* enhances biofilm formation (Hammer & Bassler 2003; Zhu & Mekalanos 2003). Transcription of *hapR* is controlled by QS, which is a cell communication system that allows bacteria to switch on or repress genes in high cell density populations (Ng & Bassler 2009). In *V. cholerae*, QS is important for biofilm formation and subsequent cell dispersal (Liu, Stirling & Zhu 2007; Müller et al. 2007). Mutational studies have shown that QS mutants develop thicker biofilms and do not separate from each other as easily as compared to the wild type (Liu, Stirling & Zhu 2007; Zhu & Mekalanos 2003). *V. cholerae* quorum sensing consists of three systems: system 1 that consists of the autoinducer CAI-1 synthesised by CqsA and sensed by CqsS; system 2 consists of the autoinducer AI-2 synthesised by LuxS and sensed by LuxQP; and a less characterized system 3 (Waters & Bassler 2005).

At low cell densities, the concentrations of CAI-1 and AI-2 are low, resulting in phosphotransfer events in which CqsS and LuxQP donate their phosphate to the LuxU phosphotransfer protein which then transfers it to LuxO (Ng & Bassler 2009). Phospho-LuxO (LuxO-P) in association with an alternative sigma factor RpoN activates the transcription of a set of small RNAs genes Qrr1-4 (Lenz et al. 2004), which work with the sRNA chaperone Hfq to inhibit the translation of *hapR* mRNA, resulting in decreased HapR production and upregulation of biofilm formation (Miller et al. 2002; Ng & Bassler 2009; Zhu et al. 2002). In contrast, at high cell densities, the levels of CAI-1 and AI-2 increase resulting in LuxO dephosphorylation and increased HapR production causing repression of the *vps* genes and *aphA* and ultimately results in downregulation of biofilm formation (Ng & Bassler 2009).

Several additional regulators influence HapR production and affect *V. cholerae* biofilm formation. The two-component system, VarS-VarA responds to an unidentified environmental signal and represses biofilm formation by upregulating *hapR* transcription. This process is complex and comprises the regulatory sRNAs called CsrB, CsrC, and CsrD, which bind with CsrA (an RNA binding protein) and interfere with LuxO mediated activation of Qrr1-4

enhancing HapR production (Lenz et al. 2005; Tsou et al. 2011). On the other hand, Fis is a small protein which positively regulates the QS responsive Qrr1-4 sRNAs, resulting in enhanced HapR repression (Lenz & Bassler 2007). Additionally, a hybrid histidine kinase VpsS can enhance biofilm formation through the QS pathway by providing a phosphate group to LuxU promoting HapR repression (Shikuma et al. 2009).

1.2.4.2.2.2 Regulation of biofilm formation by small nucleotide signals

V. cholerae biofilm formation is influenced by at least three small nucleotide signalling molecules including c-di-GMP (cyclic dimeric guanosine monophosphate), cyclic AMP (cAMP) and (p)ppGpp (the combination of guanosine tetraphosphate and guanosine pentaphosphate). They regulate the expression and repression of major regulators of *V. cholerae* biofilm formation including HapR, VpsT and VpsR in response to appropriate environmental cues (e.g. temperature fluctuations, lack of nutrients, oxygen depletion) (Toyofuku et al. 2016). Cyclic dimeric guanosine monophosphate (c-di-GMP) is a key signalling molecule that controls *V. cholerae*'s transition from the planktonic to the sessile form and vice versa (Ha & O'Toole 2015; Römling, Galperin & Gomelsky 2013). c-di-GMP is produced by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs) (Yang et al. 2010). c-di-GMP inhibits transcription of flagellar genes at transcriptional level. FlrA is a master regulator of flagellar gene expression. c-di-GMP binds to FlrA and changes it activity, impairing FlrA's ability to activate the expression of the *flrBC* operon encoding a histidine/response regulator that regulates the transcription of FlrA (; Srivastava et al. 2013). Mutants that cannot produce the flagellar structural protein (FlaA) exhibit increased VPS synthesis and biofilm formation indicating that loss of flagella constitutes an unknown signal for upregulation of these phenotypes (Watnick et al. 2001). However, mutation in the *flaA* and *vpsR* that encode the sodium driven motor (*mot*) and response regulator, VpsR in non-flagellated strains respectively, reduces VPS synthesis and biofilm formation that suggests the sodium driven motor (*mot*) and *vpsR* are involved in enhanced biofilm formation in *V. cholerae* (Biswas et al. 2019; Lauriano et al. 2004). Additionally, higher levels of intracellular c-di-GMP enhances transcription of MSHA pilus encoding genes (Beyhan et al. 2006) resulting in surface attachment and biofilm formation. In addition to regulation of motility and surface attachment, elevated cellular c-di-GMP levels stimulate increased transcription of genes involved in *V. cholerae* biofilm formation. For example, c-di-GMP enhances the binding of VpsT to *vps* promoter areas (Krasteva et al. 2010) (Figure 1.5).

In contrast to c-di-GMP, cAMP (a second messenger) is synthesized by adenylyl cyclase (CyaA) due to various cellular responses such as glucose limitation. cAMP downregulates *V. cholerae* biofilm formation (Liang, Silva & Benitez 2007) through binding with its receptor protein, CRP. This cAMP-CRP complex promotes HapR production and represses VpsR, leading to down regulation of expression of the VPS and matrix protein genes (Fong & Yildiz 2008). On the other hand, Fis is a small protein which positively regulates the the QS responsive sRNAs, Qrr1-4, results in enhancing HapR repression (Lenz & Bassler 2007).

Finally, *V. cholerae* biofilm formation is enhanced by the stringent response, which is initiated by nutritional stress. This stress enhances the synthesis of (p)ppGpp through the activity of RelA, SpoT and RelV (He et al. 2012). These three synthases are required for the transcription of *vpsR*, but only RelA can induce the transcription of *vpsT* partially through an alternative sigma factor RpoS (He et al. 2012). RpoS also negatively regulates *V. cholerae* biofilm formation by promoting HapR production (He et al. 2012).
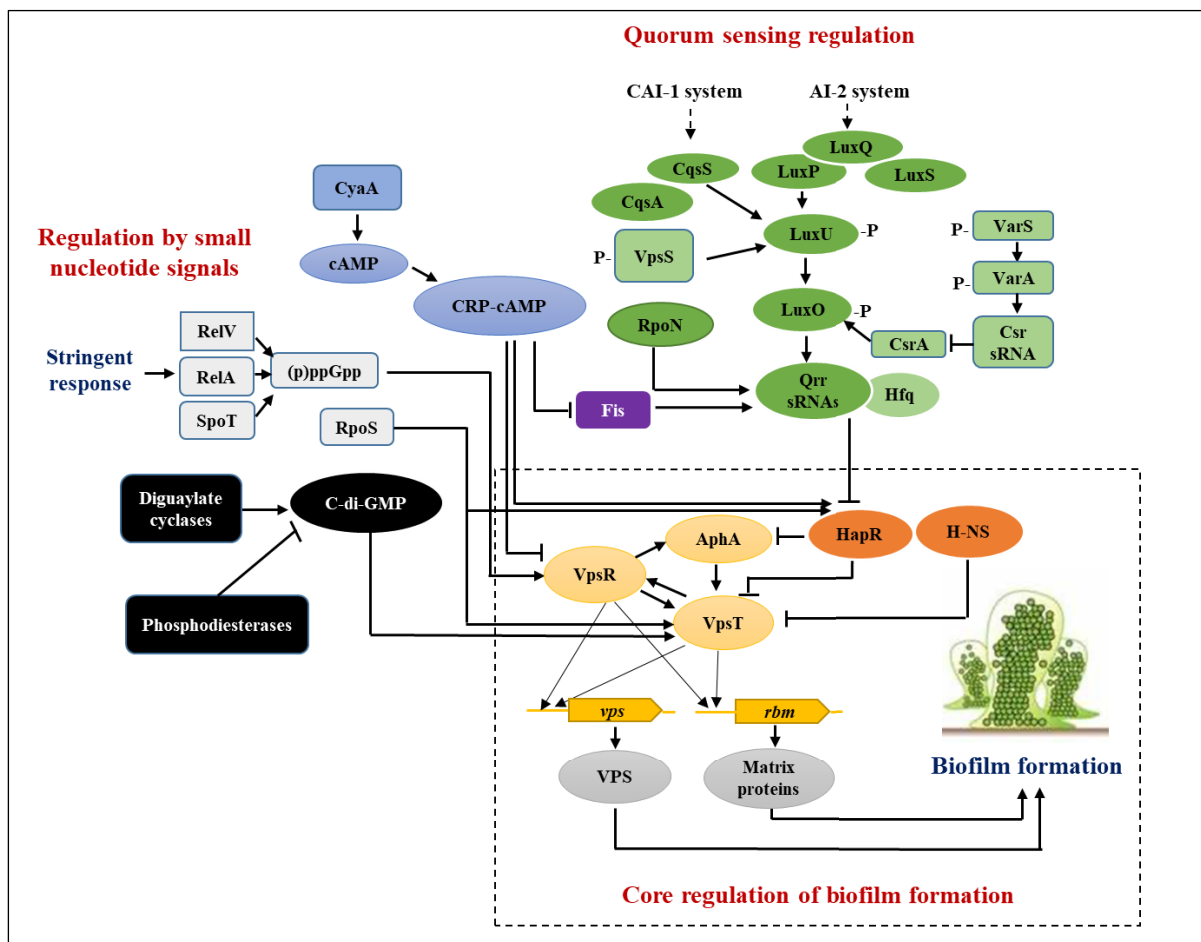
Figure 1.5: Regulatory network controlling formation of biofilm by *V. cholerae*. Briefly, the principal activators of *V. cholerae* biofilm formation are VpsR, VpsT and AphA, whereas HapR and H-NS are the main repressors. The regulation of biofilms formation is controlled by a complex regulatory network

that integrates at least 3 nucleotide secondary messengers including c-di-GMP, (p)ppGpp and cAMP and, quorum sensing (QS).

1.3 Protozoa

As a member of the natural bacterioplankton community, *V. cholerae* is an integral part of the pelagic microbial food web and is constrained in its growth and survival by the predatory action of free-living heterotrophic protists called protozoa (Jürgens & Matz 2002; Sherr & Sherr 2002). Protozoa are a diverse and highly specialised class of eukaryotic cells of which some consume bacteria for carbon nitrogen (Lutz et al. 2013). In general, the size of the protozoa can vary from 2 μm to 4.5 mm and based on their morphology, locomotion and feeding mode, are classified into three different groups: flagellates, amoeba and ciliates.

Flagellates contain one or more flagella used for movement and internalisation of prey (Chrzanowski & Šimek 1990; Parry 2004). Flagellates are considered to be one of the major predators of bacteria in aquatic environments with bacteriovory rates ranging from 2 – 300 bacteria per flagellate per hour (Bott 1995). According to their habitat, they can be grouped into freshwater or marine flagellate groups (Scheckenbach et al. 2006).

Amoebae have the ability to attach to biotic and abiotic surfaces (Rodríguez-Zaragoza 1994) and are well known for feeding on attached bacteria ranging from 1 – 1465 bacteria per amoeba per hour and do not feed efficiently on planktonic bacteria (Parry 2004). They generally utilize their cytoplasm filled projection called pseudopodia for moving on surfaces through extension and retraction (Parry 2004), which are mostly coordinated by actin microfilament activation (Tekle & Williams 2016). Generally, the size of the amoebae can vary, ranging between 20 μm to 2 mm and they inhabit a wide range of environments such as soil, fresh and marine water systems and, in places with extreme conditions of humidity and temperature (Rodríguez-Zaragoza 1994). They possess two different life forms: an active feeding form called "trophozoite" (vegetative form) and a highly resistant structure called a "cyst" (dormant form) (Bradley & Marciano-Cabral 1996), which is enclosed by a thick layer of polysaccharide and chitin that protects them from stressful conditions such as starvation, dehydration and the presence of biocides (Lambrecht et al. 2015). Generally, amoebae are closely associated with bacterial biofilms (Erken, Lutz & McDougald 2013; Lutz et al. 2013) and control their biomass by feeding on them. For example, *Acanthamoeba castellanii* is an amoeba that can be found as free living cells attached to biotic or abiotic surfaces in the aquatic environment where it grazes on complex biofilm communities composed of different microorganisms (Huws, McBain &

Gilbert 2005). Although this amoeba is mainly found in soil, it is also occasionally found in aquatic environments and has been widely used as a model to understand the interaction of amoeba with pathogenic and non-pathogenic bacteria (Guimaraes et al. 2016). The intracellular trafficking and digestion processes in *A. castellanii* are conserved and are similar to other phagocytic and mammalian cells (Guimaraes et al. 2016), a fact that makes *A. castellanii* an interesting model (including in this PhD thesis) for understanding the intracellular mechanisms of resistance to digestion displayed by many bacterial pathogens.

Ciliates possess a variable number of cilia near to mouth that provide it with enhanced prey capture by creating feeding currents which direct prey towards the cytostome (Hogg 2013; Laybourn-Parry 1984; Patterson & Hedley 1996). Their shapes vary and their size range is between 10 μm to 1 mm (Epstein, Burkovsky & Shiaris 1992). Based on their distribution in a wide range of habitats, they are considered to be cosmopolitan organisms and to date, it is estimated that more than 8,000 species of ciliates have been described. Ciliated protozoa possess specialised characteristics, for example, they can develop a complex oral groove used for the ingestion of numerous prey particles that are packaged into food vacuoles (Figure 1.6) (Parry 2004). Several reports have shown that ciliated protists have the ability to consume planktonic cells and efficiently graze on bacterial biofilms. Bacteriovory rates for ciliates is high and reach values of 200 - 5000 bacteria per ciliate per hour (Bott 1995; Parry 2004). Many ciliates have been used as models for the study of the interactions between bacteria and protozoa. One of the most widely used model ciliates, including this PhD thesis, is *Tetrahymena pyriformis* which is a freshwater ciliate that ranges in size between 50 to 60 μm and has a worldwide distribution.

1.3.1 Protozoal digestion

Following ingestion, bacteria are packaged into phagosomes (food vacuoles) (Figure 1.6) where intracellular trafficking and food vacuole maturation begins, followed by several coordinated reactions that end with food vacuole-lysosome fusion (Guerrier et al. 2017), a key process that is required for an efficient and complete digestion (Figure 1.6). Lysosomes pinch off from the rough endoplasmic reticulum (RER) and contain several destructive substances including acidic proteolytic enzymes, antimicrobial peptides (AMPs) and an acidic pH (Elliott & Clemmons 1966; Nusse 2011). In addition, food vacuoles receive DNA damaging agents such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) from the mitochondria that can turn into a toxic milieu within a few minutes (Nusse 2011). Degradation

of the contained food materials is initiated by the coordinated action of these destructive substances. When the digestion is complete, the end products are deposited in the cytoplasm, in massive quantities by means of blebs and/or by pinocytosis.
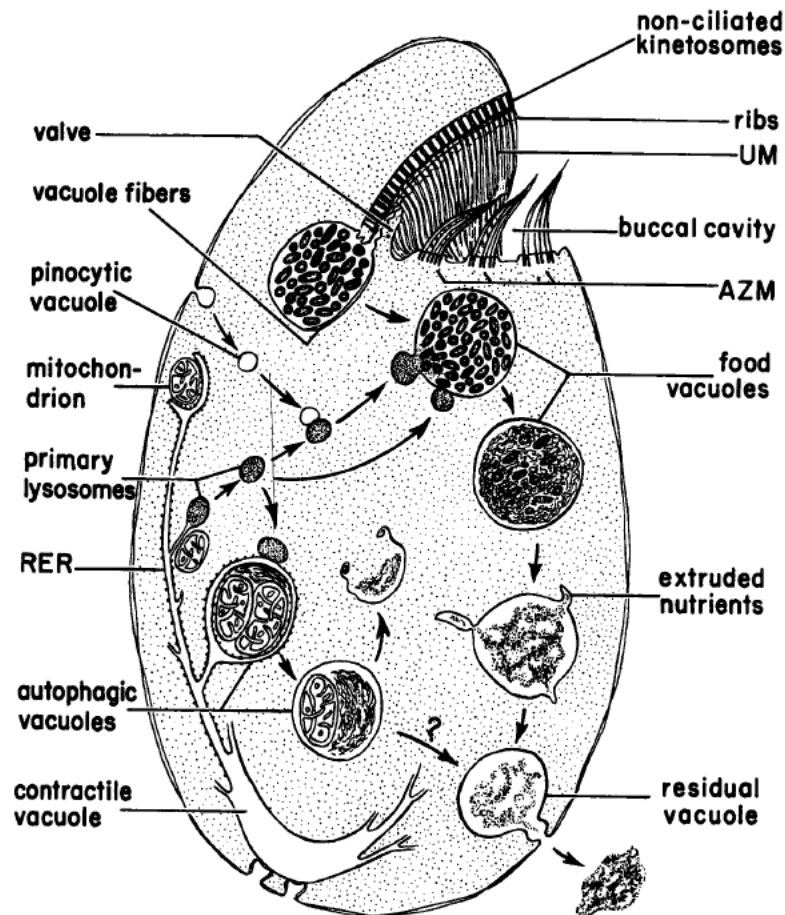
Figure 1.6: Schematic representation of pathways of intracellular digestion in *T. pyriformis*. This image represents the overall scenario of intracellular digestion process in protozoa. Bacterial cells are ingested through the buccal cavity into food vacuoles which then merge with lysosomes produced by the rough endoplasmic reticulum (RER) delivering harmful substances that kill the bacteria and facilitate digestion. Nutrients are extracted and fed into the cytoplasm and the residual vacuole is excreted. This image is reproduced and used from Elliot and Clemmons 1966 (Elliott & Clemmons 1966) with permission from the publisher.

1.3.2 Bacterial resistance mechanisms to predation

Bacteria have developed a variety of strategies that improves their fitness during interaction with protozoa (Matz & Kjelleberg 2005; Matz et al. 2005; Noorian et al. 2017). These include pre-ingestional and post-ingestional fitness (Matz & Kjelleberg 2005). Pre-ingestional fitness includes alteration of bacterial shape and size, increased motility, modification of cell surface, aggregation or biofilm formation and release of extracellular toxin(s) (Figure 1.7). Post-ingestional fitness includes digestion resistance, interruption of intracellular trafficking,

intracellular toxin release, and intracellular multiplication (Figure 1.7) (Sun, Noorian & McDougald 2018).
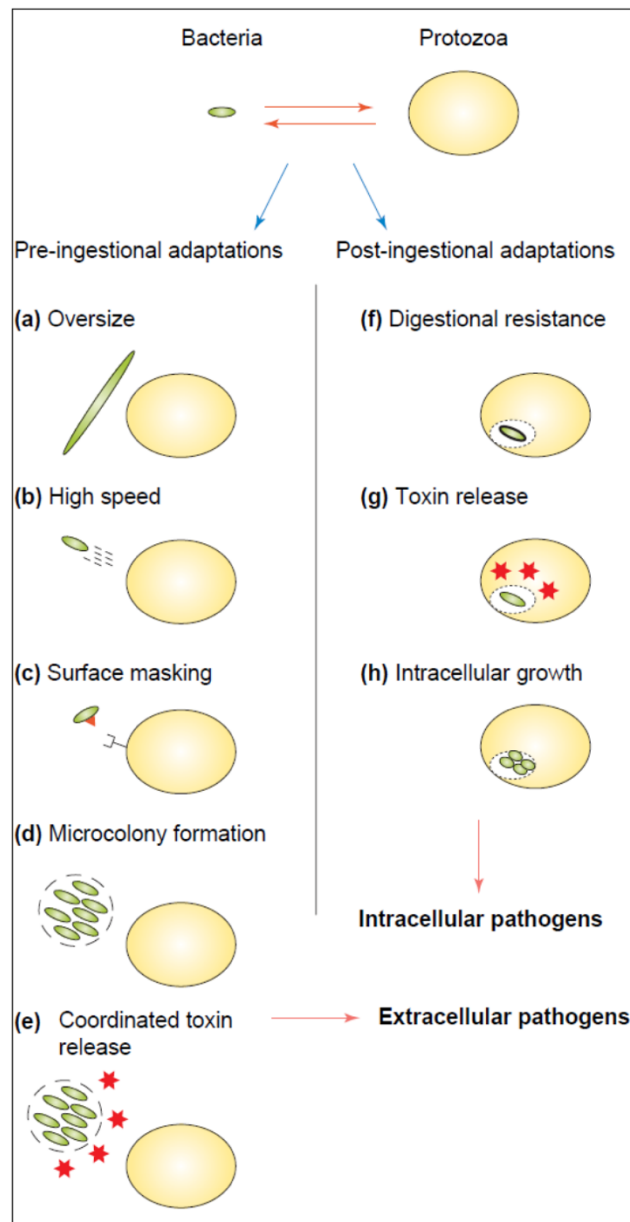
Figure 1.7: Schematic representation of potential pathways of bacterial resistance against predation by protozoa. Blue arrows indicate the two divergent strategies of pre-ingestional (a-e) and post-ingestional (f-h) adaptations emerging from bacteria-protozoa interactions (see details in the text). This image is reproduced and used from Matz and Kjelleberg 2005 (Matz & Kjelleberg 2005) with permission from the publisher.

1.3.2.1 **Pre-ingestion resistance mechanisms**

Changes in cell size and shape is an important strategy displayed by many bacteria that results in significant reduction of bacterial ingestion by bacterivorous protists (Wu, Boenigk & Hahn 2004). For example, grazing experiments between the flagellate *Ochromonas* sp. and the

bacterium *Comamonas acidovorans* showed that the bacteria undergo filamentation to evade predation (Hahn & Hofle 1998). This phenomenon was also observed in *Flectobacillus* sp. when exposed to the same grazer (Corno & Jurgens 2006; Hahn, Moore & Höfle 1999).

Increased bacterial motility is another strategy that is recognized as an important anti-grazing mechanism. During co-culture of bacteria and protozoa, increased bacterial swimming speed is observed resulting in a lower bacterial ingestion rate (Matz & Jurgens 2005). Although *Bdellovibrio bacteriovorous* is not a eukaryotic protist, it is a bacteriovorous bacterium and it was recently reported that *V. cholerae* increases its motility to resist predation from this predator (Duncan et al. 2018).

Since some bacterivorous protozoa must recognise specific bacterial cell surface proteins in order to internalise them, modification of the bacterial cell surface is an effective way to avoid predation. For example, some amoeba ingest bacteria through recognition of bacterial cell surface polysaccharide binding proteins (Brown, Bass & Coombs 1975). Pre-treatment with various carbohydrates such as galactose and mannose results in a significant reduction of ingestion (Medina et al. 2014).

Biofilm formation is a principal adaptive mechanism that helps protect bacteria from bacterivorous predation. When *V. cholerae* is in a biofilm, it is protected from the nanoflagellate *Rhynchomonas nasuta* and the amoeba *A. castellanii* (Sun, Kjelleberg & McDougald 2013) due to the generation of QS regulated toxic ammonia (Sun, Kjelleberg & McDougald 2013). Indeed, QS which can be achieved in high density biofilms seems to play an important role in *V. cholerae* resistance against protozoan predation with lower survival observed when the QS regulatory gene, *hapR,* was deleted (Matz et al. 2005). Similarly, a mucoid strain of *Pseudomonas aeruginosa* (PDO300) showed grazing resistance through formation of large microcolonies in response to surface feeder *R. nasuta* (Matz, Bergfeld, et al. 2004).

Additionally, toxins secreted by bacteria act as a powerful weapon to resist protozoan predation and can induce apoptosis in protozoa, block their feeding or cause cell death. For example, the Shiga toxin (AB5 toxin) by *E. coli* O157:H7 shows toxicity against the ciliate *Tetrahymena thermophile* (Lainhart, Stolfa & Koudelka 2009). Violacein, a toxic alkaloid produced by Chromobacterium *violaceum* and *Pseudoalteromonas tunicata* can cause apoptosis and feeding inhibition in nanoflagellates (Matz, Deines, et al. 2004; Matz et al. 2008). Biosurfactants by

Pseudomonas fluorescens and *Pantoea ananatis* have been shown to be toxic toward protozoa (Smith et al. 2016) and the extracellular protease, PrtV is involved in *V. cholerae* resistance against many predators such as *T. pyriformis* and *Cafeteria roenbergensis* (Vaitkevicius et al. 2006). The Type 6 secretion system (T6SS), a surface needle structure utilized by several Gram-negative bacteria to translocate toxins (also known as effector proteins) directly into adjacent target cells (Pukatzki et al. 2006) is known to be active against protozoa (Dong et al. 2013; Ma et al. 2009). For example, *V. cholerae* O1 strains containing T6SS are active towards the ameoba *Dictyostelium discoideum* (Pukatzki et al. 2007; Pukatzki et al. 2006) with VasX the effector protein responsible through its interaction with protozoal membrane lipids (Miyata et al. 2011).

1.3.2.2 **Post-ingestion resistance mechanisms**

Eukaryotic phagocytic or non-phagocytic cells digest bacteria for fulfilling their nutrients requirement, which is essential for maintaining their viability and reproduction (Hahn & Hofle 2001; Sibille et al. 1998). However, many bacteria have developed tactics that allow them to overcome protozoan intracellular digestion and in some instances, grow and multiply. Following ingestion by protozoa bacteria can, i) interrupt phagosomal intracellular trafficking, ii) survive within the phagosome and iii) escape to the cytosol to reproduce causing subsequent cell lysis of the host (Sun, Noorian & McDougald 2018). This section will focus on these processes and intracellular multiplication of bacteria and, their connection to the emergence of diversified pathogenic factors.

Some bacteria can interrupt the intracellular trafficking process to avoid phagosome-lysosome fusion. For example, *Legionella pneumophila* (Escoll et al. 2013; Richards et al. 2013) secretes a specialised ankyrin-like protein (AnkB) through the Doc/Icm Type IV secretion system that modulates host cell trafficking events to isolate the *Legionella*-containing vacuole (LCV) from the host endocytic pathway. This isolation allows the LCV to escape lysosomal fusion preventing bacterial death and allowing growth and multiplication (Escoll et al. 2013; Lomma et al. 2010; Richards et al. 2013). In the case of *Mycobacterium tuberculosis,* secretion of several proteins inside the phagosome of human and murine macrophages (which share similar phagocytic pathways to protozoa) leads to reduced acidification and reduced decoration of the phagosome with lysosomal markers preventing phagosome-lysosome fusion (Awuh & Flo 2017; Kolonko et al. 2014).

In addition to the interference of intracellular trafficking, intracellular resistant bacteria modify their gene expression to induce mechanisms that enhance survival and multiplication within the protozoa. *E. coli* induces its iron uptake gene, *irp,* to adapt to the low concentrations of divalent metal ions within the soil amoeba *D. discoideum* (Adiba et al. 2010). In addition, limited levels of magnesium inside the phagosome activates the PhoP/PhoQ system of *Salmonella enterica*, inducing genes that enhance resistance to AMPs and acidic conditions (Riquelme et al. 2016). Another antibacterial strategy used by protozoa is to introduce toxic metal ions such as copper into the phagosome. Deletions in *copA* in *E. coli*, a gene involved in copper resistance, led to a significant reduction in the number of *E. coli* recovered from within *D. discoideum* (Hao et al. 2016). In addition, the accumulation of zinc within phagosomes of *D. discoideum* has been shown (Buracco et al. 2017) suggesting that zinc resistance might also be important for survival of intracellular bacteria.

Another well-known micro-biocide encountered by bacterial pathogens inside phagosomes are ROS. ROS are short-lived, highly reactive, oxygen-containing molecules, which are commonly produced inside the phagosome (Nusse 2011). Two molecules of superoxide anion (O_2^-) react non-enzymatically to produce hydrogen peroxide (H_2O_2) (Hébrard et al. 2009), with concentrations reaching between 1 μM to 2 mM in the phagosome (Slauch 2011). Furthermore, hypochlorous acid (HOCl) can be generated from H_2O_2 and chloride that is catalyzed by the myeloperoxidase enzyme (Winterbourn et al. 2006). ROS production is considered a key feature of killing within the phagosome causing DNA damage and mutagenesis (Baharoglu & Mazel 2011; Colin et al. 2014; Srinivas et al. 2019). Mutagenesis causes stalling of DNA replication generating an excess of single stranded DNA (ssDNA) that initiates the SOS response through the DNA repair protein RecA which inactivates LexA that represses genes in the SOS regulon (Baharoglu & Mazel 2011; Taddei, Matic & Radman 1995). Bacteria have evolved numerous mechanisms for protecting themselves from ROS and induce these through dedicated regulatory pathways such as the the ArcAB two-component regulatory system of *S. enterica* (Pardo-Esté et al. 2018). Studies have shown that in response to oxidative stress, several enzymes including catalase-peroxidase (*katG*), alkyl hydroperoxide reductase (*ahpC*), glutathione oxidoreductase (*gorA*), superoxide dismutase (SOD) are induced in enteric bacteria to protect DNA and other cell constituents (Dhandayuthapani, Mudd & Deretic 1997; McCord & Fridovich 1969).

In the case of *V. cholerae*, survival and replication within protozoa have been reported. Different strains of *V. cholerae* including O1 and O139 strains can survive and multiply within the trophozoites/cysts of *A. castellanii* (Abd et al. 2007). Other reports have also shown that in addition to the multiplication within protozoa, *V. cholerae* can escape into the extracellular environment either as free or encased cells through access into the secretory system of *A. castellanii* (Noorian 2013; Van der Henst et al. 2016). As a result, *V. cholerae* ultimately returns to its aquatic habitat through lysis of *A. castellanii*, a process that depends on the production of VPS (Van der Henst et al. 2016). Similarly, *V. cholerae* can reproduce and survive inside the food vacuoles of *T. pyriformis* with food vacuoles being expelled and acting as a protective environment and as potential vector for disease transmission (Espinoza-Vergara et al. 2019). Production of these expelled food vacuoles (EFVs) and the ultimate escape of the pathogen from the EFVs into the environment is facilitated by the *V. cholerae* containing outer membrane protein, OmpU, which is positively regulated by ToxR (Espinoza-Vergara et al. 2019). Although several studies have tried to understand the mechanisms that *V. cholerae* uses to survive and grow inside protozoa, there is still more to be understood. Relevant to this thesis is that ROS are known to induce components of LGT (e.g. the integron-integrase, see section 1.4.5.6) through the SOS response. As a result, it is possible that protozoal grazing may have an important role in enhancing LGT-driven evolution.

1.4 Lateral gene transfer (LGT)

Studies into the genomes of *V. cholerae* strains shows that it evolves and attains a substantial amount of its genetic diversity by acquiring DNA from other cells including close and distantly related bacterial species *via* a process known as lateral gene transfer (LGT) (Ochman, Lawrence & Groisman 2000). LGT is an important process that allows for the transfer of DNA that often encodes important proteins with adaptive functions (Thomas et al. 2017). The amount of DNA attained by LGT may constitute up to 25 % of a bacterial genome (Ochman, Lawrence & Groisman 2000), with 20 % of *V. cholerae's* total genome content showing signatures of being acquired by LGT (Deshpande et al. 2011).

For LGT to drive adaptation and evolution, there are three requirements. Firstly, DNA must be exchanged between a donor and a recipient cell, secondly, the transferred DNA sequence must be integrated into the genome (or into a resident replicon such as plasmid) of the recipient cell and thirdly, the integrated DNA must be functionally transcribed and expressed (Lawrence 1999; Ochman, Lawrence & Groisman 2000). The first step of LGT proceeds through one of

three mechanisms: transformation, conjugation and transduction (Figure 1.8) (Ochman, Lawrence & Groisman 2000; Zaneveld, Nemergut & Knight 2008).

1.4.1 Transformation

Some bacteria are able to uptake free circular or linear DNA from their environment in a process called transformation (Figure 1.8) (Borgeaud et al. 2015; Frost et al. 2005). 'Competence' is a physiological state of the bacterial lifecycle that allows the bacterium to take up DNA by transformation and is driven by the expression of specialized proteins that function in DNA uptake and processing (Chen & Dubnau 2004; Meibom et al. 2005). Competence in *V. cholerae* is induced by growth on chitin, a long chain polymer present in the exoskeletons of crustaceans in the aquatic environment. This naturally induced competence is an important driver for the evolution of *V. cholerae* (Blokesch 2012a) and occurs when *V. cholerae* colonizes and forms thick biofilms on chitinous exoskeletons (Blokesch & Schoolnik 2007a; Meibom et al. 2005). Specifically, chitinase secretion by *V. cholerae* is the key enzyme required for chitin utilization that converts the insoluble polymer into soluble oligosaccharides that when taken up by the cell, act as signal to induce natural competency (Hayes, Dalia & Dalia 2017). Chitin also induces expression of the *V. cholerae* T6SS gene cluster that enables *V. cholerae* to kill neighbouring cells releasing free DNA that can be taken up by transformation (Borgeaud et al. 2015; Chourashi et al. 2018; Thomas et al. 2017). Indeed, *V. cholerae* is likely to be in mixed species biofilms providing ample opportunity for unique LGT events. Perhaps the most remarkable example showing the significance of chitin-induced natural transformation in the evolution of *V. cholerae* strains is the serogroup switch from the O1 to O139 *via* the acquisition of the genes from a non-O1/O139 strain (Blokesch & Schoolnik 2007a).
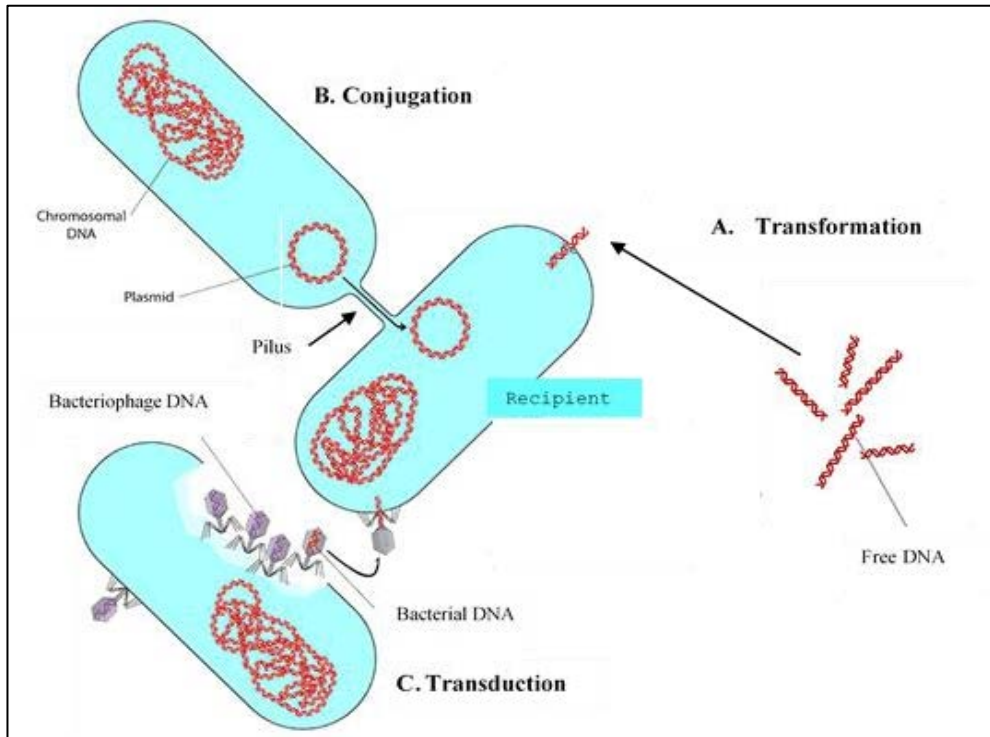
Figure 1.8: Lateral gene transfer (LGT) mechanisms. Briefly, the figure represents the three different methods of lateral gene transfer. (A) Transformation, a bacterium can uptake naked DNA. (B) Conjugation, the donor cell transfers genetic elements such as plasmids or integrative conjugative elements to a recipient through direct cell-to-cell contact. (C) Transduction, DNA transfer occurs from donor to recipient cells *via* an intermediate bacteriophage and can be either specialized or generalized in nature. In the case of generalized transduction, any part of the donor DNA can be transferred whereas, with specialized transduction, only DNA neighbouring the insertion site of the prophage DNA can be transferred (see text for more details). The figure is adapted from von Wintersdorff et al. 2016 (von Wintersdorff et al. 2016).

1.4.2 Conjugation

Conjugation is the process of DNA transfer through direct contact between donor and recipient cells (Thomas & Nielsen 2005). Typically, transfer of DNA from cells to cells is facilitated by either a self-transmissible or a mobilizable plasmid using a specialized apparatus termed conjugative pilus (Figure 1.8). Conjugation can also mediate the transfer of genetic elements called integrative conjugative elements (ICEs) that can excise from the donor genome and once transferred into the recipient, integrate into a specific genetic site (Burrus et al. 2002; Frost et al. 2005; Ochman, Lawrence & Groisman 2000). This genetic exchange mechanism is well known for bacteria that are antibiotic resistant with antibiotic resistance genes commonly found on plasmids and ICE's (Ochman, Lawrence & Groisman 2000). For example, the seventh pandemic *V. cholerae* strains encode multidrug resistance through the circulation of the SXT/R391 ICEs (Carraro et al. 2016; Carraro et al. 2014; Hochhut et al. 2001). Moreover,

other self-transmissible genetic elements such as conjugative plasmids (IncA/P) are commonly associated with multidrug resistance in *V. cholerae* (Carraro et al. 2014).

1.4.3 Transduction

Transduction is the transfer of DNA from a donor cell to a recipient cell through an intermediate bacteriophage (Figure 1.8) (Frost et al. 2005; Maiques et al. 2007). Prior to explaining the LGT process of transduction and generalized and specialized transduction (Figure 1.9), a brief introduction into bacteriophage lifecycles is required. Bacteriophages are viruses that infect and replicate within bacterial cells (Brüssow, Canchaya & Hardt 2004; Weinbauer 2004). Generally, a bacteriophage can be lytic or lysogenic. Following infection of the host bacterial cell, lytic bacteriophages proceed through a process called the lytic cycle allowing them to use the bacterial cell as a factory to rapidly produce phage progeny which then lyse the host cell and exit to being the process again (Figure 1.9). In contrast, lysogenic phages incorporate their genomic material into a specific site within the host genome (prophage) where it can remain latent and replicate as part of the bacterial genome for multiple generations. Temperate phages can enter into the lytic cycle by excising from the chromosome following a DNA-damaging trigger such as exposure to UV light or to mutagens (Zaneveld, Nemergut & Knight 2008).
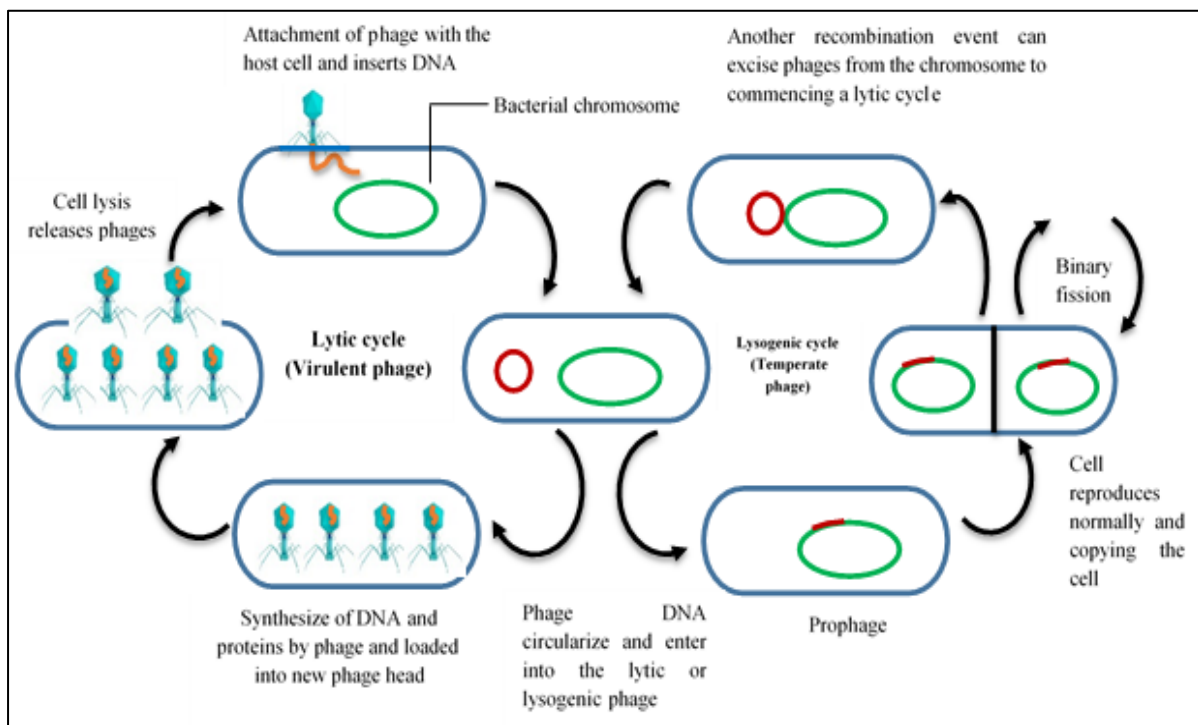
Figure 1.9: The lifecycles of bacteriophage (Lytic and lysogenic). Virulent phages undergo only the lytic lifecycle, whereas temperate phages undergo two phases in their lifecycle: lytic and lysogenic. In the case of the lysogenic cycle, the phage genome integrates into a specific site and replicates as part of chromosomal replication and cell division. In the lytic cycle, the cell is taken over and its machinery

used to make multiple copies of the phage genome and to express viral proteins that allow new phages to be made that lyse the cell and exit. Lysogenic phage can switch from the lysogenic cycle to the lytic cycle in response to certain SOS-inducing DNA damaging stressors such as UV-irradiation or certain mutagenic antibiotics (Fortier & Sekulovic 2013).

There are two forms of transduction called specialized and generalized transduction. Specialized transduction arises when prophage inserted at a particular DNA site imperfectly excises taking with it part of the host DNA adjacent to the insertion site. This is packaged into phage particles that due to imperfect excision are defective but can infect and deliver the DNA to a recipient cell. On the other hand, generalized transduction is the accidental random packaging of bacterial DNA fragments by phage during the lytic cycle. Whilst specialized transduction can only transfer host DNA adjacent to the phage insertion site, generalized transduction may transfer any component of the host DNA. Since bacteriophages are ubiquitous in the aquatic environment, it is likely that transduction is a common mechanism of LGT in *V. cholerae* (Jiang & Paul 1998).

Finally, lysogenic conversion is when a lysogenic phage containing genes that enhance a bacterium's adaptive or pathogenic capabilities inserts into the host genome. For example, lysogenic cholera toxin phages (CTX phages) that contain the *ctx* genes are responsible for producing toxigenic forms of *V. cholerae* (Kim et al. 2017) that cause cholera in humans.

1.4.4 Integration of laterally acquired DNA

The exchange of genetic elements between donor and recipient cell through the different processes of LGT (transformation, transduction and conjugation) does not ensure that the acquired genetic material is stably maintained in the recipient cell (Juhas et al. 2009; Ochman, Lawrence & Groisman 2000). For maintenance in the host and to confer a phenotype, the newly acquired DNA must be integrated into the recipient genome (or a resident replicon such as a plasmid) and the encoded genes must be transcribed and translated (Juhas et al. 2009; Zaneveld, Nemergut & Knight 2008). If the incoming DNA is a plasmid, it can replicate and maintain itself independently (Juhas et al. 2009), whereas other LGT-transferred DNA must be integrated (Juhas et al. 2009; Zaneveld, Nemergut & Knight 2008).

Integration mechanisms consist of a number of processes (Table 1.1) including homologous recombination, site-specific recombination, and transposition (Ochman, Lawrence & Groisman 2000; Thomas & Nielsen 2005; Zaneveld, Nemergut & Knight 2008).

Table 1.1: Mechanism of transferred DNA integration and maintenance in the bacterial cell

Mechanisms for integration and maintenance of transferred DNA	Example of transferred elements
1. Independent replication	Plasmids
2. Homologous recombination	
3. Site-specific recombination	Integron/gene cassette system, genomic islands, lysogenic bacteriophage
4. Transposition	Transposons

This table has been adapted from Stokes and Gillings, 2011 (Stokes & Gillings 2011).

1.4.4.1 Homologous recombination

Homologous recombination is an important DNA repair process in bacteria by mediating the exchange of highly similar or homologous DNA strands (Frost et al. 2005). However, homologous recombination has an important role in integration of DNA acquired by LGT. This complex process depends on the length of incoming DNA (around 25-200 bp) as well as high nucleotide similarity to the recipient cell genome of approximately 80 % or more (Thomas & Nielsen 2005). Briefly, the process involves the breaking and re-joining of paired DNA sequences carried out by single-stranded binding proteins (SSB) and the recombinase RecA (del Carmen Orozco-Mosqueda et al. 2009). Although this process requires highly similar or complete identical sequences, novel fragments of DNA can be recombined, if they are flanked by homologous DNA sequence(s).

1.4.4.2 Site-specific recombination

Site-specific recombination is the exchange of DNA strands between two DNA fragments by an integrase, a recombinase enzyme that recognizes specific sequences allowing recombination to occur. Site-specific recombinase enzyme cleave sequence specific DNA sequences, exchange the two DNA helices and re-join them (Fogg et al. 2010; Lee et al. 2007; Ochman, Lawrence & Groisman 2000). The integron/gene cassette system, genomic islands and lysogenic bacteriophage are ideal examples of genetic elements that utilize site-specific recombination.

1.4.4.3 Transposition

Transposition is a process where DNA fragments move from one genetic location to another through the action of an enzyme called transposase. Elements that undergo transposition are

called transposable elements or transposons and more colloquially known as 'jumping genes'. Transposons are diverse and can move in a cut-and-paste fashion or a copy-and paste fashion (called replicative transposition) (Bennetzen 2000). Transposases often make staggered cuts at a target site producing sticky ends, cuts out the DNA transposon and ligates it into a new target site. Target sites are dependent on the transposase and tend to be more varied than those targeted by site-specific recombination. Host DNA polymerase fills in the resulting gaps from the sticky ends and DNA ligase closes the sugar-phosphate backbone often resulting in target site duplication (Berg 1985; Muñoz-López & García-Pérez 2010). The circulation of transposable elements (TEs) with diverse array of catabolic genes, drug and metal resistance genes has shown to them to be major contributors of genomic diversity and fitness of bacterial hosts (McDonald 2012).

1.4.5 Mobile genetic elements (MGEs): a source of transferrable DNA

MGEs are genetic elements that can mediate movement and integration of DNA intracellularly (within genomes) or intercellularly (between bacterial cells) (Frost et al. 2005). Consequently, genes carried by MGEs significantly contribute to the diversification of bacteria impacting on the genome plasticity and play a vital role in the dissemination of antibiotic resistance and virulence associated genes as well as enhancing microbial fitness (Frost et al. 2005; Juhas et al. 2009). This section will briefly describe common MGEs present in *V. cholerae* that have contributed to shaping its genome.

1.4.5.1 Genomic islands (GIs)

Genomic islands are large, discrete chromosomal DNA fragments, usually ranging from 10 to 200 kb and possess a number of genes that are favourable for the bacterium in certain environments (Juhas et al. 2009). They are generally flanked by ~16-20 bp direct repeats (DR) and carry a number of functional and segmented insertion sequence (IS) elements and other mobility genes such as integrases, recombinase and transposases that help mediate their integration and excision from the host bacterial chromosome (Dobrindt et al. 2004; Gal-Mor & Finlay 2006; Juhas et al. 2009). As with most mobile DNA, the GC content of the GI is generally different from the rest of the chromosome and they are often inserted adjacent to a tRNA gene. Figure 1.10 represents the general features of a GI.
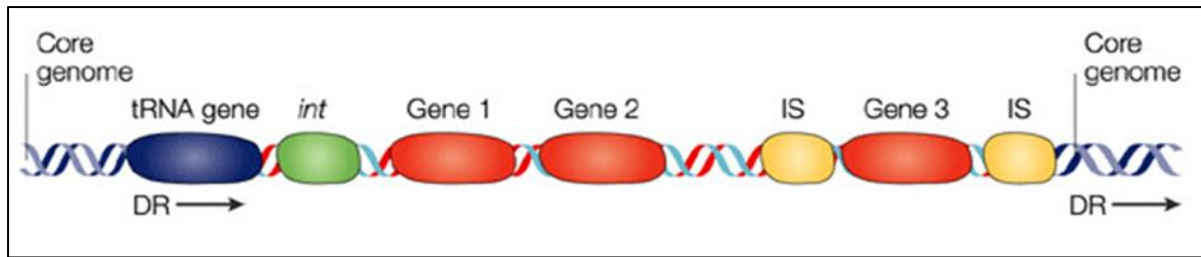
Figure 1.10: General features of genomic islands (GIs). Briefly, genomic islands are often inserted near a tRNA and flanked by direct repeats (DR). GIs harbor a number of genes that have adaptive potential (gene 1-3) for the bacterial host. Typically, GIs carry multiple functional and segmented insertion sequence (IS) elements and, other mobility genes such as integrase that aid in excision and integration of the GI. DNA between insertion sequences can translocate as a composite transposon. For example, the two insertion sequences in this figure could transfer and integrate gene 3 into the host genome or into other MGEs. This figure has been reproduced from Dobrindt et al. 2004 (Dobrindt et al. 2004) with permission from the publisher

GI's frequently possess one or multiple genes that are associated with increased fitness and adaptability of the host bacterium. Pathogenicity islands are a type of genomic island that generally contain genes that enhance virulence and consequently are commonly associated with pathogenic bacteria. However, many studies have reported that GIs can influences traits such as antibiotic or heavy metal resistance, degradation of pollutants and general adaptation of diverse bacterial populations (Dobrindt et al. 2004).

In *V. cholerae*, the most remarkable example of pathogenicity islands are *Vibrio* pathogenicity islands I and II (VPI-I and VPI-II) in pandemic causing *V. cholerae* strains. VPI-I is a 40 kb island that carries genes encoding TCP, one of the two main virulence factors. TCP provides the receptor for CTX phage that encodes genes necessary for the production of the cholera toxin (CT). Thus, VPI-I plays an important role in the lysogenic conversion of non-toxigenic *V. cholerae* strains by the CTX phage (Schmidt & Hensel 2004). *Vibrio* pathogenicity island II (VPI-II) is a 57.3 kb island that encodes several gene clusters including a neuraminidase (*nanH*) and genes required for amino sugar metabolism (*nan-nag* region). Sialic acid is extensive on the mucous membrane of the human gut and removal of sialic acids by neuraminidase unmasks GM1, the receptor for cholera toxin, resulting in increased binding and uptake of CT by susceptible host cells and causing massive diarrhoea (Almagro-Moreno & Boyd 2009; Galen et al. 1992). Both VPI-I and VPI-II have been shown to excise from their chromosomal insertion sites to form circular products with an integrase in VPI-II shown to be necessary for this process (Murphy & Boyd 2008; Rajanna et al. 2003).

Vibrio seventh pandemic I and II (VSP- and VSP-II) are genomic islands that are unique to seventh pandemic O1 El Tor and O139 strains (Dziejman et al. 2002). VSP-I and VSP-II are 16 kb and 7.5 kb regions of which their precise functions are yet to be determined. However, acquisition of these islands in pandemic-causing strains suggest that they may have a role in the environmental fitness and dissemination of these strains (Faruque & Mekalanos 2003). Similar to VPI-I and VPI-II, VSP-II can also excise to form a circular element and in the case of VSP-II, the contained integrase is required for this process (Murphy and Boyd, 2008).

1.4.5.2 **Plasmids**

Plasmids are circularized DNA fragments that are capable of replicating independently and many can transfer *via* a process called conjugation (Dobrindt et al. 2004). Plasmids are recognized by their smaller sizes in comparison to the chromosome and lack genes essential for core cellular functions and instead contain adaptive genes such as those encoding virulence and antibiotic resistance (Frost et al. 2005). In a strain of *V. cholerae* O139, a conjugative plasmid (pMRV150) isolated from China encodes multidrug resistance (at least six drugs) (Pan et al. 2008). The most common bacterial plasmids are circular dsDNA molecules however, linear double stranded plasmids have been described in a number of species such as *Borrelia* and *Streptomyces* (Hinnebusch & Tilly 1993; Stewart et al. 2005).

Plasmids can be self-transmissible and/or mobilizable. Self-transmissible plasmids are often called fertile or F-plasmids or conjugative plasmids and contain *tra* genes (transfer genes) and an *oriT* (origin of transfer). The *tra* genes encode proteins necessary for the formation of conjugative pili and proteins that direct the plasmid (*via* binding of the *oriT*) through the pili into a recipient cell. A mobilizable plasmid is not transferred by itself as it doesn't contain any or all of the *tra* genes but does contain an *oriT* allowing it to be transferred by Tra proteins produced by a co-resident F-plasmid (Frost et al. 2005). In addition to replication, plasmids must also control their copy number and guarantee their inheritance during cell division using a process known as partitioning and thus contain genes essential for this.

1.4.5.3 **Transposons**

Transposons are a distinctive class of MGE that can frequently change their location in DNA (Dobrindt et al. 2004; Frost et al. 2005). They are flanked by inverted repeat sequences (IR) and encode a transposase gene(s) whose product(s) acts as a catalyzing agent for transposition. The simplest transposon is an insertion sequence (IS) that relocates in a "cut and paste" fashion. The inverted repeats are recognized by the transposase and excise the IS and subsequently

allow it to relocate into a new DNA position (Salyers et al. 1995). Composite transposons are those where IS elements flanking DNA can mobilize as a single unit (Figure 1.11). For example, Tn*5* is a composite transposon consisting of two IS elements flanking a kanamycin resistance gene. However, transposons have been shown to carry genes that are involved in metabolism such as Tn*951*, which is involved in lactose fermentation (Michiels et al. 1987). Replicative transposons are those that carry an additional gene(s) encoding a "resolvase" that permits replicative transposons to move by first making a copy of themselves (Brown & Evans 1991) and then using the resolvase to allow one copy to remain in the original insertion site and the other to move to a new site (Willey, Sherwood & Woolverton 2011). Transposons can assist the movement of host DNA from one location to another such as into another MGE (e.g. plasmid) through transposition (i.e. composite transposon; Figure 1.11) or through homologous recombination between similar transposons in different locations. Movement of transposons between cells occurs when they are embedded in other MGEs such as plasmids, bacteriophages or integrative conjugative elements (ICEs) (Dobrindt et al. 2004; Frost et al. 2005) or *via* DNA transformation.
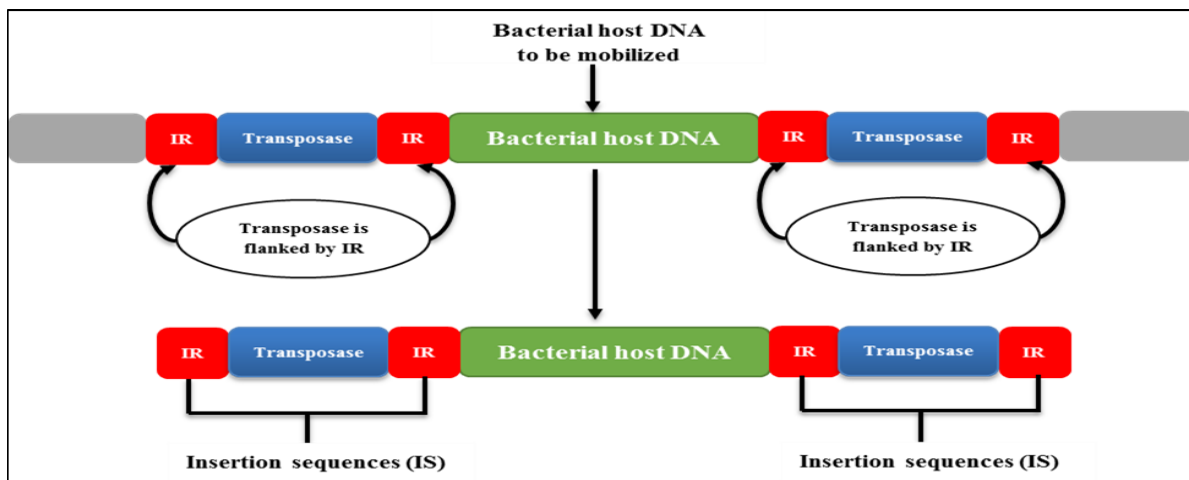
Figure 1.11: General feature of a simple insertion sequence and a composite transposon. This figure shows the distinctive features of a composite transposon where transposase gene (blue) is flanked by inverted repeats (IRs) (red), together this region is called insertion sequences (IS). The grey colour region is DNA surrounding the transposon and the DNA sequence that is mobilised illustrated in green.

1.4.5.4 **Integrative conjugative elements (ICEs)**

Integrative conjugative elements (ICEs) are clusters of gene sequences that exist in the bacterial chromosome (Wozniak & Waldor 2010). They can excise, circularize and transfer to neighbouring cells *via* conjugation (through presence of *tra* genes) and integrate into the recipient host DNA site-specifically (Frost et al. 2005; Thomas & Nielsen 2005; Wozniak & Waldor 2010). ICEs are ~100 kb in size and can contain genes for conferring antibiotic

resistance (Waldor & Mekalanos 1996) as well as a variety of other functions such as virulence, biofilm formation and metabolic processes (Hochhut et al. 1997; Wozniak & Waldor 2010). In *V. cholerae*, the SXT element was the first identified ICE and confers multiple antibiotic resistance genes (Hochhut & Waldor 1999).

1.4.5.5 Lysogenic bacteriophage

Whilst bacteriophages are viruses they can also act as MGEs. Integration of lysogenic phage DNA into the bacterial genome can contributes to the generation of genetic diversity and evolutionary fitness of recipient bacteria (Frost et al. 2005; Hassan et al. 2010). In *V. cholerae* for instance, the CTX phage genome provides the cholera toxin virulence genes (Faruque & Mekalanos 2003). In addition to CTX prophage, multiple additional phages have been characterized (Table 1.2) that can play critical roles in horizontal gene transfer among *V. cholerae.*

Table 1.2: A short list of representative vibrio phages with their functions associated with LGT.

Phage designation	Description and functions
CTXφ	A filamentous phage of *V. cholerae* that encodes CT. CTXφ can also integrates into the host chromosome and develops stable lysogen.
CP-T1	This phage is associated with generalized transduction and mediates the chromosomal fragments transfer among strains of *V. cholerae via* LGT.
RS1	A satellite phage genome that contains the gene encoding anti-repressor protein RstC, which stimulates the replication and transmission of CTXφ. In addition, RS1 uses the morphogenesis genes of CTXφ to form RS1 phage particles.
KSF-1φ	This phage (filamentous) can package RS1 as well as various heterologous DNA of *V. cholerae.*
TLCφ	TLCφ (Toxin linked cryptic related phage) is a satellite filamentous phage that promotes conversion of non-toxigenic *V. cholerae* to toxigenic strain by CTX phage integration through restoring the dif site (recombination site) of the dif negative non-toxigenic strains.
fs2	A filamentous phage that provides the genes to TLCφ for its morphogenesis (the formation of infectious phage particles).

1.4.5.6 The integron/gene cassette system

Integrons are genetic elements that integrate exogenous mobile elements called gene cassettes through site specific recombination. Integrons are defined by three components (Figure 1.12) including (i) an integrase gene (*intI*) encoding a recombinase protein called integrase (IntI); (ii) *attI*, a recombination site where gene cassettes are inserted; and (iii) a promoter (P_c), that drives transcription of inserted gene cassettes (Cambray, Guerout & Mazel 2010). In addition, the P_{int} promoter drive transcription of *intI* (Figure 1.12). Gene cassettes are small, discrete, circular, independent mobilisable DNA elements (approximately 300 –1500 bp) usually containing a single promoterless gene and, a recombination site called *attC* (Collis et al. 1993; Labbate, Case & Stokes 2009).
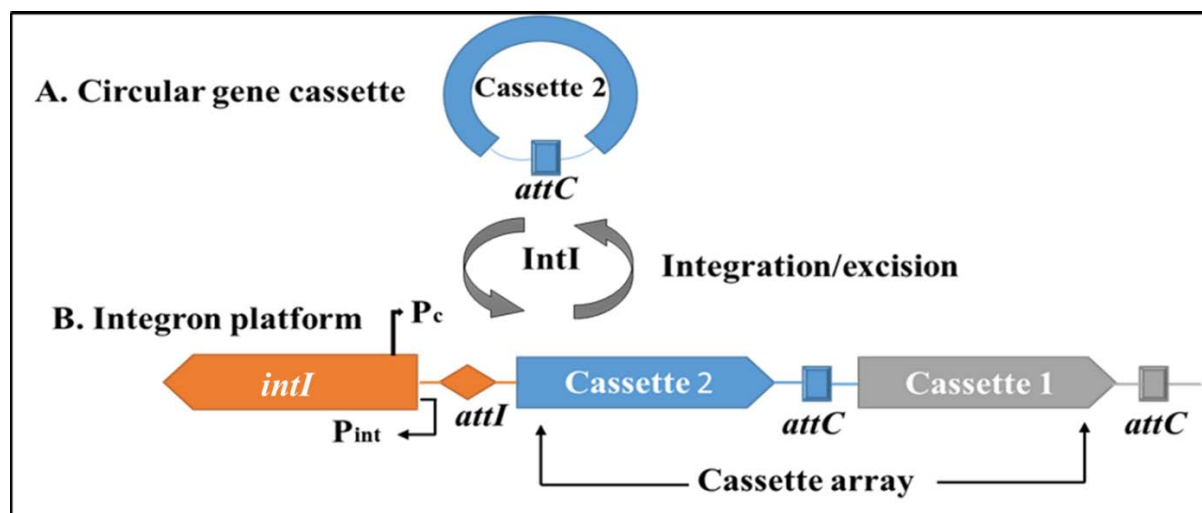
Figure 1.12: The integron/gene cassette system. A gene cassette (e.g. cassette 2) usually consists of a single gene and a recombination site called *attC* (A). The integron platform (B) contains three components including the integrase gene (*intI*) encoding an integrase (IntI) that catalyses integration, excision and shuffling of gene cassettes; *attI* a recombination site where gene cassettes insert, and a promoter (P_c) that drives downstream gene cassette transcription. P_{int} is the promoter that facilitates the expression of *intI*. A large contiguous cassette array can be produced by multiple insertion events.

Integration of the gene cassettes into the integron platform is facilitated by IntI and occurs *via* site specific recombination between *attI* and *attC* or less commonly between two *attC* sites (Collis et al. 1993). The length and sequence of the *attC* sites can vary but form a stem-loop like structure through conserved inverted repeat regions at their ends that is recognized by IntI for catalysing the site-specific recombination (Stokes et al. 1997). Due to the nature of the *attC* sites (Collis & Hall 1995), gene cassettes always insert in the same orientation with most of the gene cassette gene(s) in the array in the orientation that allows transcription from the P_c promoter. IntI also facilitate excision events and rearrangement of cassettes from one position to another (Hocquet et al. 2012). Since the identification of the class 1 integron in antibiotic

resistant Gram negative pathogens in 1989 (Stokes & Hall 1989a), over 100 divergent integrases have been characterized from environmental samples showing that the integron is a genetic platform that extends beyond clinical pathogens (Nield et al. 2001). Because of its importance in antibiotic resistance, the class 1 integron is the most extensively researched carrying small gene cassette arrays of up to approximately 8 cassettes that encode mostly antibiotic resistance determinants (Partridge et al. 2009). Class 1 integrons are commonly found on plasmids and transposons in Gram-negative bacteria and for this reason are sometimes referred to "mobile class 1 integrons" (Escudero et al. 2015; Stokes & Hall 1989b) however, class 1 integrons have been identified in the chromosomes of *Betaproteobacteria* and these are likely original source of the class 1 integron.

Other than class 1 integrons, integrons from other classes are present within bacterial chromosomes in approximately 10 % of sequenced bacterial genomes (Boucher et al. 2007). Cassette arrays in chromosomal integrons can vary ranging from 0 to greater than 200 cassettes (Partridge et al. 2009). Large tandem cassette arrays emerge from multiple gene cassette insertion events with *Vibrio* species commonly harbouring large arrays. Besides P_c at the front-end of the cassette array, diverse internal cassette associated promoters are present across large cassette arrays allowing transcription of gene cassettes distant from P_c (Michael & Labbate 2010). Additionally, some gene cassettes are known to be silent and can be mobilized in front of a promoter allowing transcription (Rowe-Magnus, Guerout & Mazel 2002).

Diversity of the gene cassette is catalysed by the activity of the integrase, which is induced by the bacterial SOS response (Guerin et al. 2009). The SOS response is a widespread regulatory cascade that is important in DNA repair and is triggered by several DNA damaging stresses including UV irradiation or specific antibiotics (e.g. fluoroquinolones, β lactams) (Erill, Campoy & Barbe 2007; Kelley 2006). When DNA damage stalls chromosomal replication, excess single-stranded DNA (ssDNA) collects in the cell and is the substrate for RecA polymerization. The formation of ssDNA-RecA nucleofilament triggers the autoproteolysis of the LexA repressor which causes de-repression of genes composing the SOS regulon (Aertsen & Michiels 2006;) of which *intI* is a member (Cambray et al. 2011; Guerin et al. 2009). This suggests that under specific conditions, bacteria may use the integron as a mechanism for creating genetic diversity that may allow for adaptation to a changing and/or stressful environment.

In addition to SOS-inducing DNA damage, DNA uptake processes such as conjugation and transformation results in the abnormal presence of ssDNA in the cell upregulating SOS and *intI* transcription thus linking the physical transfer of DNA with the integrative process of the integron (Baharoglu, Bikard & Mazel 2010; Baharoglu, Krin & Mazel 2012). In the case of *V. cholerae*, growth of this bacterium on chitin surfaces induce a natural state of competence that enables cells to uptake exogenous DNA (Blokesch 2012a; Hayes, Dalia & Dalia 2017) and therefore, induce integrase activity (Baharoglu, Krin & Mazel 2012). In the environment, *V. cholerae* exists in a biofilm state consisting of multiple species and strains, so the link of biofilm formation to competency in *V. cholerae* provides plenty of opportunity for DNA uptake and, DNA integration through induction of the integrase (Baharoglu, Krin & Mazel 2012) thus enhancing genetic diversity.

1.4.5.7 **What are the role of gene cassettes in *V. cholerae* adaptation?**

In *Vibrio* species, cassette arrays constitute a significant portion of the genomes (up to 3 %), and most of the genes in the cassette arrays are unique and novel with no identifiable functions. Genome sequence analyses of *Vibrio* species has revealed that 65 % of gene cassettes in vibrios do not have any homologues and 13 % are homologous to proteins with no known functions (Boucher et al. 2007). The remaining cassettes are involved in comprehensive non-specific functions such as metabolic process, cellular process and storage information (Elsaied et al. 2007; Koenig et al. 2008; Koenig et al. 2009; Stokes et al. 2001). This data shows that there is a significant knowledge gap in our understanding of the contribution of integron-associated gene cassette encoded products towards the general adaptation and evolution of vibrios. However, some gene cassettes from *Vibrio* species have been shown to provide adaptive functions other than antibiotic resistance and these are listed in Table 1.3.

Table 1.3: Functional ORF in gene cassettes other than antibiotic resistance

Cassette origin	Functions	Discovery of functions	References
Cassettes from *Vibrio* species			
V. cholerae	Sulfate-binding protein	Complementation of *E. coli* mutation	(Rowe-Magnus et al. 2001)
V. cholerae OP4G	Transcriptional regulation	Crystal structure determination and drug binding assay	(Deshpande et al. 2011)

V. cholerae GP156	Heat stable enterotoxin	Active in suckling mouse assay when expressed in *E. coli*	(Ogawa & Takeda 1993)
V. cholerae	Mannose–fucose resistant hemagglutinin	Mutagenesis *in vivo* and testing in infant mouse model	(Barker, Clark & Manning 1994; Franzon, Barker & Manning 1993)
V. cholerae	Lipoprotein	Act as transporters of hydrophobic molecules	(Barker & Manning 1997b)
V. cholerae	Toxin/antitoxin (TA) genes	Stabilization of 179 large cassette arrays	(Iqbal et al. 2015)
V. rotiferianus DAT722 (cassette21)	dNTP-pyrophosphohydrolase (iMazG)	Crystal structure determination. Expressed in *E. coli* and enzyme activity measured	(Robinson et al. 2007)
V. rotiferianus DAT722 (cassette11)	Porin regulation	Deletion of cassette *in vivo*	(Labbate et al. 2011)
V. rotiferianus DAT722 (multiple cassettes)	Surface polysaccharide modification	Deletion of cassettes *in vivo*	(Rapa et al. 2013)
Cassettes from metagenomic DNA			
Soil metagenomic DNA	Potential transport protein	Crystal structure determination	(Robinson et al. 2005)
Soil metagenomic DNA	ATPase activity	Expressed in *E. coli* and enzyme activity measured	(Nield et al. 2001)
Soil metagenomic DNA	Methyltransferase activity	Expressed in *E. coli* and enzyme activity measured	(Nield et al. 2001)
Deep submarine gas-hydrate-bearing core metagenomic DNA	Oxidoreductase and alky transferase activity	PCR amplification and homology searches	(Elsaied et al. 2014)
Human oral metagenomic DNA	Competence and motility related proteins	PCR amplification and homology searches	(Tansirichaiya et al. 2016)

The table has been adapted from Rapa et al. 2013. (Rapa et al. 2013)

In the case of *V. cholerae* O1 strain N16961 strain, there are 216 ORFs across 179 cassettes in the cassette array which are mostly (80-90 %) of unknown function (Heidelberg et al. 2000). All O1 strains are very closely related and share similar cassette arrays. A few studies have

characterized some gene cassettes in *V. cholerae* including three virulence factors namely, a heat-stable toxin (*sto*), a mannose-fucose-resistant haemagglutinin (*mrhA*), a lipoprotein (Barker & Manning 1997b; Franzon, Barker & Manning 1993; Ogawa & Takeda 1993), transcriptional regulation (Deshpande et al. 2011) and a sulphate binding protein (SBP) (Rowe-Magnus et al. 2001). Even with the limited understanding of gene cassettes functions of the *V. cholerae* integron, some studies on other *Vibrio* species and metagenomic DNA sample have extended our knowledge on how gene cassettes influence general adaptation. For example, gene cassettes encode many additional proteins that have homology to several enzymes involved in primary metabolism and cell surface properties (Boucher et al. 2006; Kim et al. 2003; Robinson et al. 2005; Rowe-Magnus 2009; Rowe-Magnus et al. 2003; Rowe-Magnus et al. 2001). Gene cassettes from diverse metagenomic DNA encoded the proteins that are highly homologous to potential pollution degrading enzymes (Koenig et al. 2009; Nemergut, Martin & Schmidt 2004), a protein related to Tfox which is responsible for competence and a protein encoding bacterial twitching motility (Tansirichaiya et al. 2016). In a study on gene cassettes isolated from coral mucus containing *Vibrio* spp., 12.5 % of gene cassettes were associated with biochemical functions possibly associated with conferring antibiotic resistance (Koenig et al. 2011).

In addition to the genetic diversity generated by acquisition of novel gene cassettes, deletion or rearrangement of gene cassettes in the cassette array may result in a variety of novel phenotypes. In *V. rotiferianus* strains DAT722 containing a large cassette array, deletion of large sections of cassettes resulted in modification in surface polysaccharide and biofilm formation (Rapa et al. 2013) . It was hypothesised that gene cassette products may modify surface polysaccharide (e.g. addition of functional groups) changing its properties and adhesion/biofilm capabilities. Alternatively, gene cassette products could manipulate the regulatory pathways of biofilm formation. For example, a gene cassette from *V. rotiferianus* DAT722 strains encodes a putative RNA binding protein and when expressed in *V. cholerae* S24, significantly enhances biofilm formation (Rapa 2014). Currently, there is no data on the exact mechanism(s) by which gene cassette products affect biofilm formation through manipulation of indigenous regulatory pathways in *V. cholerae*.

1.5 Project hypothesis and aims

V. cholerae is a causative agent of pandemic diarrhoeal disease called cholera. Biofilm formation by *V. cholerae* on abiotic or biotic surfaces including chitin surface act as a major reservoir in the environment that also induces the natural competency of this bacterium and allows the lateral gene transfer (LGT) through uptake of exogenous DNA. LGT processes of transformation and conjugation induces the integron integrase which is controlled by the bacterial SOS stress response that results in diversity in the cassette array of integron through integration of new gene cassettes or, deletion or movement of existing cassettes. However, the dynamics of gene cassette transfer into the *V. cholerae* integron platform and the functions of most gene cassette-encoded proteins are unknown.

Free-living heterotrophic protists called protozoa uptake bacteria including *V. cholerae* and digest them within their food vacuoles with the coordinated action of different chemicals and enzymes including ROS which are known to induce SOS and the integron-integrase. Studies have reported that *V. cholerae* survives and replicates within the food vacuoles of protozoa however, there is a significant knowledge gap in the understanding of *V. cholerae* resistance inside the protozoal phagosome and whether the phagosome environment induces processes such as LGT that drive genetic diversity.

The broad objective of the work presented in this thesis was to investigate the role of the *V. cholerae* integron/gene cassette system in biofilm formation and to investigate integron/gene cassette dynamics in protozoa. Thus, it is hypothesised that the *V. cholerae* integron/gene cassette system affects biofilm formation and is induced in the presence of bacterivorous protists.

Chapter 2 developed tools to investigate gene cassettes transfer frequency into the *V. cholerae* chromosomal integron. To accomplish this, two different artificial gene cassettes namely circular and synthetic linear gene cassettes were constructed and amplified and transformed into *V. cholerae*. Different *V. cholerae* mutant strains were constructed and complemented. Finally, engineered linear DNA was constructed to develop novel complementation assay in *V. cholerae* targeting *attI* site integration.

Chapter 3 investigated the role of gene cassettes in *V. cholerae* biofilm formation. For this, different strains with integrated circular gene cassettes were selected and subjected to biofilm

assay. Then, gene cassettes deletion mutants were constructed, complemented and subjected to different assays to confirm their role in biofilm formation.

Chapter 4 determined if integron-integrase gene expression and integrase-mediated LGT in *V. cholerae* is enhanced in the presence of bacteriovorist protozoa. In order to accomplish this, two protozoan models such as *T. pyriformis* and *A. castellanii* were used and co-incubate with *V. cholerae*. Different reporter strains and mutants were constructed, complemented and several assays were designed to understand the factors that trigger integrase expression inside the food vacuoles of protozoa and subsequent LGT.

Chapter 5 provides a general summary and discussions of the results reported in this work. Future directions and implications of this research are also presented.

Chapter 2 : Development of artificial gene cassettes for investigating cassette transfer dynamics into the *V. cholerae* integron

2.1 Introduction

The genome of *V. cholerae* contains an adaptive genetic element called the integron/gene cassette system constituting ~2% of its genome (Boucher et al. 2007). The integron platform is defined by three main components including: (i) an integrase gene (*intIA*) encoding a recombinase protein called integrase (IntIA); (ii) a recombination site called *attI* where mobile genetic units called gene cassettes are inserted; and (iii) a promoter (P_c), that drives transcription of inserted gene cassettes (Cambray, Guerout & Mazel 2010) (for more details see section 1.4.5.6). Gene cassettes are small circular mobilisable DNA elements that contain a recombination site called *attC* and usually contain a single promoterless open reading frame (ORF) (Collis et al. 1993; Labbate, Case & Stokes 2009).

Integration of gene cassettes into the integron platform occurs *via* integron integrase-mediated site-specific recombination between *attI* and *attC* or less frequently between two *attC* sites (Collis & Hall 1992; Collis et al. 2001). In *V. cholerae*, *attC* sites are 128-129 bp, are highly conserved (~90% nucleotide identity) and contain short regions of sequence similarity at their boundaries (R' and R" pairing forms the R box, while the L' and L" forms the L Box; Fig. 2.1) interrupted by a stretch of imperfect internal dyad symmetry. The *attC* sites form single stranded (ssDNA) imperfect hairpin structures (Figure. 2.1) through extrusion from supercoiled DNA or from ssDNA produced during replication (Loot et al. 2010) and are recognised by IntIA. Importantly, structural elements of the *attC* hairpin (e.g. extrahelical bases) make IntIA preferentially bind to the bottom strand to promote the excision of a gene cassette that inserts at *attI* in a specific orientation (Loot et al. 2010; Loot et al. 2017; Mukhortava et al. 2019; Nivina et al. 2016). Indeed, when ssDNA gene cassettes created with the *V. cholerae attC* on the top strand instead of the bottom strand, insertion into *attI* is reduced by several orders of magnitude (Nivina et al. 2016). Therefore, integron integrase-mediated recombination is a ssDNA process with the second strand produced by DNA replication.
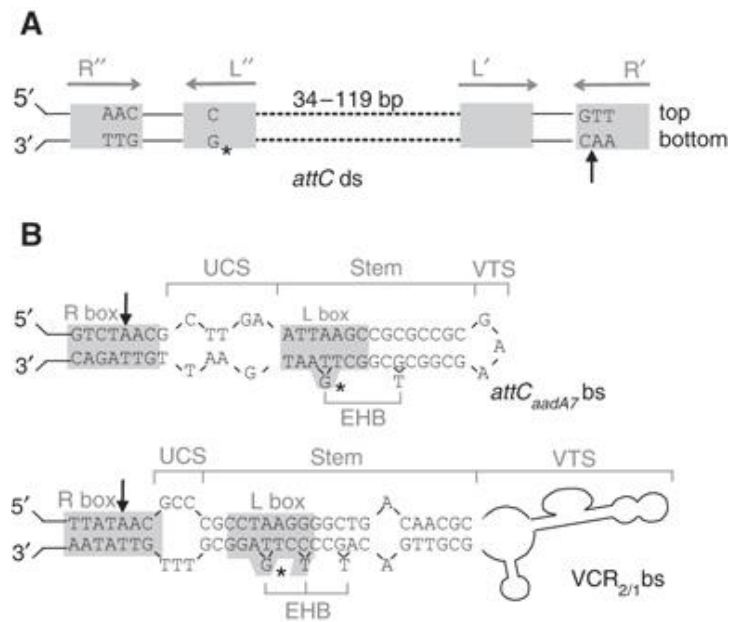
Figure 2.1: The *attC* site contains conserved ends with R' and R" pairing forming the R box and the L' and L" pairing forming the L Box with an intervening region of variable length (A). The arow in (A) indicates the site in the bottom strand that is cleaved during recombination. The *attC* sites form hairpin structures that facilitate IntI binding and recombination with the extra helical bases (EHBs), unpaired central spacer (UCS) and variable terminal structure (VTS) playing an important role in preferential binding of IntIA to the bottom strand (B). In *V. cholerae*, *attC* sites are 128-129 bp forming hairpin structures with complex branched structures (lower in B) compared to a shorter *attC* site (upper in B). Image reproduced from (Loot et al. 2010) and used with the permission from the publisher.

In comparison to other bacteria, *Vibrio* species harbour large cassette arrays (median of 100) (Loot et al. 2017) through multiple gene cassette insertion events and, due to the specific binding of IntIA to the bottom strand of *attC* sites, gene cassette ORFs are almost always in one orientation such that they can be transcribed from P_c. However, this promoter is unlikely to drive transcription beyond the first 5-6 gene cassette therefore, as has been identified in *V. rotiferianus* DAT722, transcription of most cassettes probably relies on diverse promoters across the array (Michael & Labbate 2010). In *Vibrio* species, large cassette arrays are favoured for a couple of reasons that minimise cassette excision and promote array stability. First, relative to chromosomal replication, the bottom strand of *attC* sites are present on the leading strand minimising the presence of ssDNA (lagging strand contains more ssDNA due to formation of Okazaki fragments) and the likelihood of hairpin structures required for IntIA binding and recombination (Loot et al. 2017). Second, compared to other bacteria, *Vibrio attC* sites contain a long intervening sequence between the conserved boundaries that likely form complex branched structures that prevent IntIA binding and/or recombination (Loot et al. 2017).

Diversity in the *V. cholerae* cassette array is driven by integron integrase-mediated integration of new gene cassettes or, deletion or movement of existing cassettes. However, the presence of paralogous cassettes across the array could also allow homologous recombination events. The *intIA* promoter is called P_{int} and is controlled by the SOS response (Guerin et al. 2009), a regulatory cascade that is triggered by DNA damage induced by stresses such as UV irradiation or certain antibiotics (e.g. fluoroquinolones, β-lactams). This co-ordinated response to DNA damage is controlled by a repressor called LexA and an activator which is the RecA recombinase (Erill, Campoy & Barbe 2007; Walker 1984). In non-stress conditions, LexA binds to a specific sequence in the promoter region of genes in the SOS regulon called the SOS box. In *Escherichia coli*, the SOS regulon comprises of at least 43 unlinked genes (Courcelle et al. 2001; Fernández de Henestrosa et al. 2000). DNA damage induces the SOS response through stalled chromosomal replication resulting in excess single-stranded DNA (ssDNA) causing the formation of ssDNA-RecA nucleofilaments. These ssDNA-RecA nucleofilaments induce autoproteolysis of LexA allowing transcription of these LexA-repressed genes (Little 1991). The *V. cholerae intIA* promoter is known to contain a functional SOS box and is therefore part of the SOS regulon (Guerin et al. 2009). In addition to DNA damage, external DNA uptake from lateral gene transfer (LGT) processes such as conjugation and transformation result in the abnormal presence of ssDNA in the cell and is capable of upregulating the SOS response and therefore, *intIA* transcription linking the process of DNA transfer with DNA integration (Baharoglu, Bikard & Mazel 2010; Baharoglu, Krin & Mazel 2012).

Transformation in *V. cholerae* (Blokesch 2012a) and other *Vibrio* species (Gulig et al. 2009; Pollack-Berti, Wollenberg & Ruby 2010) is enhanced when grown on chitin through induction of a natural state of physiological competence that enables the uptake of exogenous DNA. Specifically, competency induces the expression of type IV pili (Tfp) that bind DNA and then retract to help it cross across the outer membrane (Seitz & Blokesch 2013). The ComEA proteins assist the transport of DNA into the periplasmic space and through an inner membrane channel (ComEC) into the cytoplasm as a single DNA strand with the other degraded (Seitz & Blokesch 2013).

In this study, it was aimed to develop artificial gene cassettes that could be used to investigate cassette integration into the *V. cholerae* integron using natural chitin-induced transformation. Circular and linear gene cassettes were developed and shown to both integrate into the *V.*

cholerae integron. Additionally, we show that the integron is a novel site for adding DNA for complementation in *V. cholerae*.

2.2 Method and materials

2.2.1 Bacterial strains and growth conditions

All strains and plasmids used in this study are shown in Table 2.1. The naturally competent *V. cholerae* O1 strain A1552 (Marvig & Blokesch 2010) was used to investigate gene cassette transfer into the integron platform. The two A1552 chromosomes are sequenced (NZ_CP025936 and NZ_CP025937) and the integron was found to contain 176 gene cassettes, of which 96 are different types of paralogous cassettes (see Appendix 2.1 for a list of cassettes in the A1552 cassette array and Appendix 2.2 for paralogous sets). Compared to the first annotated *V. cholerae* N16961 genome which contains 178 gene cassettes (Labbate et al. 2007), A1552 lacks the first, second and 45th cassettes however, all other cassettes are in their expected relative positions. The gene cassettes are annotated according to their position in the N16961 array so VCH3 is the first gene cassette in A1552 (identified in this thesis). *Escherichia coli* DH5α λ-pir was used for cloning experiments and for the cultivation of artificial circular gene cassettes containing an R6K origin of replication and a kanamycin resistance gene marker (*nptII*). Luria-Bertani medium supplemented with 0.5 % (w/v) NaCl (LB5) was routinely used for cultivating *V. cholerae* and *E. coli* strains. Kanamycin (Kan), spectinomycin (Spc) and carbenicillin (Cab) were added at 50 μg ml^{-1}, 100 μg ml^{-1} and 100 μg ml^{-1} respectively for both *V. cholerae* and *E. coli*. Chloramphenicol (Cm) was added at 5 μg ml^{-1} for *V. cholerae* and 25 μg ml^{-1} for *E. coli*. In order to induce gene expression in an arabinose-inducible vector carrying strains, a final concentration of 0.2 % (w/v) L-arabinose was supplemented to the growth medium.

Table 2.1: Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and phenotype	Source
E. coli **strains**		
DH5α-λ pir	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r$_K^-$, m$_K^+$) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
E. coli S17-1 λ pir	TpR Sm^R recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir	ATCC 47055
V. cholerae **strains**		
A1552	Wild type *V. cholerae* O1 EI Tor A1552, smooth variant	(Yildiz & Schoolnik 1999)
Chitin transformant pKC01⁺ 1	A1552, insertion of pKC01⁺ between VCH 35 andVCH 36; Kan^R	This study
Chitin transformant pKC01⁺ 2	A1552, insertion of pKC01⁺ at *attI*; Kan^R	This study
Chitin transformant pKC01⁺ 3-4	A1552, insertion of pKC01⁺ between VCH 35 andVCH 36; Kan^R	This study
Chitin transformant pKC01⁺ 5	A1552, insertion of pKC01⁺ at *attI;* Kan^R	This study
Chitin transformant pKC01⁺ 6	A1552, insertion of pKC01⁺ between VCH 35 andVCH 36; Kan^R	This study
Chitin transformant pKC01⁺ 7	A1552, insertion of pKC01⁺ at *attI;* Kan^R	This study
Chitin transformant pKC01⁺ 8-9	A1552, insertion of pKC01⁺ between VCH 35 andVCH 36; Kan^R	This study
Chitin transformant pKC01⁺ 10	A1552, insertion of pKC01⁺ at *attI;* Kan^R	This study
Chitin transformant pKC01⁻ 11	A1552, insertion of pKC01⁻ between VCH 60 andVCH 61; Kan^R	This study
Chitin transformant pKC01⁻ 12-15	A1552, insertion of pKC01⁻ at *attI;* Kan^R	This study
Chitin transformant pKC01⁻ 16-19	A1552, insertion of pKC01⁻ between VCH 60 andVCH 61; Kan^R	This study
Chitin transformant pKC01⁻ 20	A1552, insertion of pKC01⁻ at *attI;* Kan^R	This study
Chitin transformant gc_kan 1-10	A1552, insertion of gc_kan at *attI;* Kan^R	This study
A1552 Δ*intIA::cat*	A1552, partial deletion of *intIA* and *intIA* interrupted with FRT-*cat*-FRT from pKD3, Cm^R	This study
A1552 Δ*intIA*	A1552, partial deletion of *intIA*	This study
A1552 Δ*recA*	A1552, defective for SOS induction and homologous recombination; Spc^R	This study
A1552 Δ*intIA*Δ*recA*	A1552, defective for *intIA* and SOS induction, and homologous recombination; Spc^R	This study
A1552 *ΔhmgA*	A1552, in-frame deletion of VC1345 (*hmgA*)	This study
A1552 *ΔhmgA*_gc_hmgA_kan	A1552, Δ*hmgA,* gc_kan-*hmgA* at *attI*, Kan^R	This study

C6706_TnVCH35	*V. cholerae* O1 EI Tor biotype, smooth variant carrying TnFGL3 insertion with + orientation at 102 nucleotides in VCH35	(Cameron, Urbach & Mekalanos 2008)
C6706_TnVCH60	*V. cholerae* O1 EI Tor biotype, smooth variant carrying TnFGL3 insertion with – orientation at 125 nuclotides in VCH60	(Cameron, Urbach & Mekalanos 2008)
Plasmids		
pKC01$^+$	Derived from TnFGL3 transposon (Cameron, Urbach & Mekalanos 2008)	This study
pKC01$^-$	Derived from TnFGL3 transposon (Cameron, Urbach & Mekalanos 2008)	This study
pKD3	Contains chloramphenicol resistance gene (*cat*) abutted by FRT sites, Cm^R	(Datsenko & Wanner 2000)
pOriVn$_{700}$	Source of spectinomycin resistance gene, Spc^R	(Le Roux, Davis & Waldor 2011)
pBR-flp	FLP+, λ cI857+, λ *p*R from pCP20 integrated into *Eco*RV site of pBR322, Cab^R	(Silva & Blokesch 2010)
pSU-pBAD	Arabinose inducible expression vector, Cm^R	Laboratory collection

Kan^R: Kanamycin resistant, Cm^R: Chloramphenicol resistant, Spc^R: Spectinomycin resistant, Rif^R: Rifampicin resistant, Cab^R: Carbenicillin resistant, Sm^R: Streptomycin resisant

2.2.2 Primers, PCR, DNA extractions and, genome sequencing and bioinformatics

All primers used in this study are shown in Table 2.2. In order to identify the location of integrated artificial gene cassettes across the cassette array, 21 pairs of primers targeting only the unique cassettes were designed using the online program Primer 3 (Koressaar & Remm 2007; Untergasser et al. 2012). Each primer pair was designed to give an amplicon size of no more than 10-kb. Genomic DNA (gDNA) was extracted using the Wizard® Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instructions. Plasmid and PCR fragment extractions were done using the Wizard® Plasmid Isolation kit (Promega, USA) and Wizard® SV Gel and PCR Clean-Up System (Promega, USA) respectively according to the manufacturer's instructions. Long range PCR was carried out using Velocity polymerase (Bioline, USA), a proofreading DNA polymerase. PCR reactions were set up using 5.0 μl of 5x Hi-Fi reaction buffer, 2.5 μl of dNTP mix, 1.25 μl each of forward and reverse primer (10 μM stock), 1.0 μl of template DNA and molecular biology water (MBW) to a final volume of 25 μl. The PCR cycling (35 cycles) conditions were as follows: an initial denaturation of 98 °C for 30 seconds followed by an appropriate annealing temperature for 30 seconds, an extension of 72 °C for 30 seconds/kb, and a final extension time of 10 minutes at 72 °C (see Table 2.2 for specific annealing temperatures and extension times for primer sets). PCR products were subjected to electrophoresis on 1 % (w/v) agarose gel at 90 V for 1 h and stained in 25 μg/ml of ethidium bromide solution for 15-30 min prior to visualisation and imaging on a transilluminator (InGenius3 gel imaging system, USA).

To enhance assembly of *V. cholerae* cassette arrays containing repeat *attC* sites and gene cassettes, genome sequencing of *V. cholerae* strains was done using both long-read Oxford Nanopore (ONP) MiniIon and short-read Illumina technologies at the ithree institute, University of Technology Sydney (UTS). All long and short reads were compiled to a single file using the cat* command and then subjected to a hybrid assembly by Unicycler (Wick et al. 2017) using normal mode. During hybrid assembly, SPAdes v3.13.0 was utilised to assemble the short reads; Miniasm and Racon were employed to assemble the long reads and to circularise the genomes. Polishing was done by Pilon and Samtools v1.0. The assembly graphs were later investigated by Bandage. Assembled genomes were annotated with PROKKA (Seemann 2014) and cassette arrays aligned with MAUVE (Darling, Mau & Perna 2010).

Table 2.2: Primers used in this study

Primer	Sequence (5`-3`)	Target	Annealing temperature (°C)	Extension time (min)	Source
ML147 F	CAGCAATCGTGACTTGGAAA	5' end of VCH35	67.5	3.5	This study
ML148 R	TGAAACATAAAGCGCTGCAA	3' end of VCH36			
ML149 F	GCTTGTTCTCGCAAGCTTTT	5' end of VCH60	60	3.5	This study
ML150 R	CCAACAAATCAGGGTGATCC	3' end of VCH61			
ML15 F	ATTTTGCCAAAGGGTTCGTG	5' end of artificial kanamycin resistant gc_kan	50	1.0	This study
ML16 R	CATCAGCCCATCCTGTTGAA	3' end of artificial kanamycin resistant gc_kan			
IntIA_F	CGCGAACACTTAACAAAAACTGG	5' end of the integron-integrase of *V. cholerae*	62	0.5	This study
VCH_3_R	AACGGCCAATACCACCCATT	5' end of VCH1 in *V. cholerae* A1552 integron			
VCH_3_F	CCAGCTTGGAGTAGGTAGCA	3' end of VCH1 in *V. cholerae* A1552 integron	62	2.5	This study
VCH_10_R	AATCCTGGGCGAACACAGTT	3' end of VCH8 in *V. cholerae* A1552 integron			
VCH_10_F	GTTATTACTGGCGGTAGCCGA	5' end of VCH8 in *V. cholerae* A1552 integron	68	4.0	This study
VCH_20_R	AATATCGCCGCCATTCTCGT	3' end of VCH18 in *V. cholerae* A1552 integron			
VCH 20_F	ATTGATGCCGGTGAGTCAGG	5' end of VCH18 in *V. cholerae* A1552 integron	73	2.5	This study
VCH_27_R	TCCCTTTTGAGCTGCGAGTT	3' end of VCH25 in *V. cholerae* A1552 integron			
VCH_26_F	GCAGCTATGGGATGTCGCAA	5' end of VCH24 in *V. cholerae* A1552 integron	72	4.0	This study
VCH_38_R	GCATGCCTGCTCATATAGCCT	3' end of VCH36 in *V. cholerae* A1552 integron			
VCH_38_F	AAATTCTGACTGGGGAGCGT	5' end of VCH36 in *V. cholerae* A1552 integron	66	4.0	This study
VCH_49_R	CTTGCGCCTAAAACAGCACC	3' end of VCH47 in *V. cholerae* A1552 integron			
VCH_48_F	TGTCGATAACTACCGAGCCG	5' end of VCH46 in *V. cholerae* A1552 integron	73	3.5	This study
VCH_57_R	ATAGGTCCACCAACCCACTG	3' end of VCH55 in *V. cholerae* A1552 integron			
VCH_57_F	ATGTGCGATGCAGAGGGAAA	5' end of VCH55 in *V. cholerae* A1552 integron	68	2.5	This study
VCH_64_R	CCACTGTTTTCACATACAGCGA	3' end of VCH62 in *V. cholerae* A1552 integron			
VCH_64_F	TGAGTTATCCCAAGCACCACC	5' end of VCH62 in *V. cholerae* A1552 integron	64	4.0	This study
VCH_69_R	AGGTAGAACCAAGGCACACC	3' end of VCH67 in *V. cholerae* A1552 integron			
VCH_69_F	AATGGTGTGGTGAGTGGACG	5' end of VCH67 in *V. cholerae* A1552 integron	72	4.5	This study
VCH_82_R	ATGTGAGCAGCTAGCATCGT	3' end of VCH80 in *V. cholerae* A1552 integron			
VCH_81_F	AACCAATGCACGCTTTGAGG	5' end of VCH79 in *V. cholerae* A1552 integron	68	4.0	This study
VCH_91_R	TGAACTCGGGATGAACAGGG	3' end of VCH89 in *V. cholerae* A1552 integron			
VCH_91_F	GCAAGCCGAAACGCTGTATG	5' end of VCH89 in *V. cholerae* A1552 integron	76	3.5	This study

VCH_102_R	AGCGTAAGGACAACGCTCAG	3' end of VCH100 in *V. cholerae* A1552 integron			
VCH_101_F	GGCTCGAAAATGAGGGCTTC	5' end of VCH99 in *V. cholerae* A1552 integron	72	4.3	This study
VCH_114_R	GCCGAGCAATTCCGTTTTGT	3' end of VCH112 in *V. cholerae* A1552 integron			
VCH_113_F	AAGCGATTGATGGGGCTCAA	5' end of VCH111 in *V. cholerae* A1552 integron	72	3.0	This study
VCH_122_R	TCAGACATCGCCTTTTGCCA	3' end of VCH120 in *V. cholerae* A1552 integron			
VCH_121_F	GGTTATCCGGCGGGTGTTAT	5' end of VCH119 in *V. cholerae* A1552 integron	72	3.0	This study
VCH_128_R	CGCACGTGTGAAAGATGCTC	3' end of VCH126 in *V. cholerae* A1552 integron			
VCH_127_F	GGCTATTTGGCTCTTTGTCGC	5' end of VCH125 in *V. cholerae* A1552 integron	74	3.5	This study
VCH_135_R	ATCGATGCCTGGAGCAACAT	3' end of VCH133 in *V. cholerae* A1552 integron			
VCH_135_F	AAAATGCTGCATCGGTGCTT	5' end of VCH133 in *V. cholerae* A1552 integron	72	4.5	This study
VCH_146_R	GTTAGCAGTACGCTCAGGCT	3' end of VCH144 in *V. cholerae* A1552 integron			
VCH_146_F	AGCCCCTTTACCCAGATGGA	5' end of VCH144 in *V. cholerae* A1552 integron	66	4.5	This study
VCH_159_R	GATAACCCGCCGTACGAAGT	3' end of VCH157 in *V. cholerae* A1552 integron			
VCH_159_F	TTACCTTGTGCCAGCGGAAA	5' end of VCH157 in *V. cholerae* A1552 integron	74	3.5	This study
VCH_168_R	AAGCGTCAACACCTTGGGAA	3' end of VCH166 in *V. cholerae* A1552 integron			
VCH_167_F	GCCACTAAGATCCTTGCCGA	5' end of VCH165 in *V. cholerae* A1552 integron	72	4.0	This study
VCH_175_R	ACTCAAGCCTGCAACGACAT	3' end of VCH173 in *V. cholerae* A1552 integron			
VCH_175_F	CGTGGGTCCAGGTAAAGAGT	5' end of VCH173 in *V. cholerae* A1552 integron	72	1.5	This study
VCH_Ch_R	ACCATCAGGGTTACGAGCTG	Immediate sequence from chromosome 2 after VCH177			
Kan_F	ATTCAACGGGAAACGTCTTG	Kanamycin resistant gene (*nptII*) in artificial circular cassettes pKC01+ and pKC01- and, gc_kan	62	0.5	This study
Kan_R	CGAGCATCAAATGAAACTGC	Kanamycin resistant gene (*nptII*) in artificial circular cassettes pKC01+ and pKC01- and, gc_kan			
GFP_R	GCGATACCGTAAAGCACGAG	Used with intIA-F and, ML147_F or ML149_F to determine the orientation of integrated circular gene cassttes	62	0.5	This study
ompW_*F*	CACCAAGAAGGTGACTTTATTGTG	5' end of *ompW* in *V. cholerae*	60	0.5	(Nandi et al. 2000a)
ompW_*R*	GGTTTGTCGAATTAGCTTCACC	3' end of *ompW* in *V. cholerae*			
intIA_up_F	ATAATGCCTCGCGTAAAACG	Binds within *intIA*, used with IntIA-up-R to amplify *intIA* upstream region	62	1.0	This study
intIA_up-R	<u>TCCAGCCTACACTG</u>GGGGCTGATATACGTACC	Binds within *intIA*, used with IntIA-up-F to amplify *intIA* upstream region			
intIA-down_F	<u>GGAGGATATTCATATG</u>TTGCATAAATTCGCGAACAC	Binds within *intIA*, used with IntIA-down-R to determine whether any cassettes have inserted into *attI*	62	1.0	This study

intIA_down_R	CCCATTGCGTTAAATACCAT	Binds within VCH3, used with IntIA-down-F to amplify *intIA* downstream region			
intIA_Del_R	GGTTCGGGGTTAAGGTTGAT	Binds internal to *intIA* in the deleted region, used with IntIA-up-F to confirm gene deletion	60	1.0	This study
Cat_F	GTGTAGGCTGGAGCTGCTTC	Used with Cat_R to amplify the FRT-cat-FRT cassette from pKD3	60	0.5	(Datsenko & Wanner 2000)
Cat_R	CATATGAATATCCTCCTTAG	Used with Cat_F to amplify the FRT-cat-FRT cassette from pKD3			
Cat_Del_R	GCGTGTTACGGTGAAAACCT	Binds internal to *cat*, used with Cat_F to confirm presence of *cat*	60	0.5	This study
hmgA_up_F	TTCTCTGCTTGCACCTTACTCA	Used with hmgA_up_R to amplify the upstream region of *hmgA* in *V. cholerae*	60	0.5	This study
hmgA_up_R	<u>CTCCAGCCTACAC</u>GTTCGTAAATCGC CTGCTCT				
hmgA_down_F	<u>GATATTCATATG</u>CCATGCGCTACAGT TTAGTGAC	Used with hmgA_Down_R to amplify the downstream region of *hmgA* in *V. cholerae*	58	1.0	This study
hmgA_down_F	CTCATCAATGTCGCAAAAGAAA				
Cat_F1	<u>CGATTTACGAAC</u>GTGTAGGCTGGAG CTGCTTC	Used with Cat_R1 to amplify the FRT-cat-FRT cassette from pKD3	60	0.5	This study
Cat_R1	<u>CTGTAGCGCATGG</u>CATATGAATATCC TCCTTAG				
recA-up-F	TTCAGGCGGTCGAGCATAAT	Used with recA-up-R to amplify the upstream region of *recA* in *V. cholerae*	65	1.00	This study
recA-up-R	<u>AGATCGTTTT</u>GCATGATGGAGCCTTT ACCA				
recA-down-F	<u>GGGCGAATTGC</u>TGATCGCTGAAACA TCTTCTG	Used with recA-down-R to amplify the downstream region of *recA* in *V. cholerae*	64	1.00	This study
recA-down-R	GAAGCCGTAGGTGTCGTAGAGT				
aadA7-F	<u>TCCATCATGC</u>AAAACGATCTCAAGA AGATC	Used with *aadA7*-R to amplify the spectinomycin resistance gene from pOriVn$_{700}$	56	1.00	This study
aadA7-R	<u>CAGCGATCAG</u>CAATTCGCCCTATAGT GAGT				
RecA_F	ATTGAAGGCGAAATGGGCGATAG	5' end of *recA*	60	0.5	(Marashi et al. 2013)
RecA_R	TACACATACAGTTGGATTGCTTGAGG	3' end of *recA*			

attC_Up_F	TTAACCCTCACTAAAGGAGCTA	Used with attC_R to amplify the upstream region of synthetic linear DNA	60	0.5	This study
attC_Up_R	CAAATCCTTGATTTCCTGTGTGAAAT TGTTATC	Used with attC_F to amplify the upstream region of synthetic linear DNA			
hmgA_F	ACACAGGAAATCAAGGATTTGGTGA AGGTAAC	Used with hmgA_R to amplify the hmgA gene (VC 1345) of *V. cholerae* A1552	65	1.0	This study
hmgA_R	ATTAATTGCGTCGCTAATTTCATATC GCCCTAC	Used with hmgA_F to amplify the hmgA gene (VC 1345) of *V. cholerae* A1552			
Kan_attC_F	TGAAATTAGCGACGCAATTAATGTG AGTTAGC	Used with Kan-attC_R to amplify the downstream region of the synthetic linear gene cassette gc_kan	60	1.0	This study
Kan_attC_R	ACGACTCACTATAGGGGCATA	Used with Kan-attC_F to amplify the downstream region of the synthetic linear gene cassette gc_kan			
pSU-pBAD-F	AGTCTAGACAGCGCTTTTCC	Used with pSU-pBAD-R to amplify the backbone of vector pSU-pBAD	58	2.30	This study
pSU-pBAD-R	GTTTCACTCCATCCAAAAAAAC	Used with pSU-pBAD-F to amplify the backbone of vector pSU-pBAD			
Cloning intIA_F	TTTTGGATGGAGTGAAACGCTAAAG ACGGGATAATGGGCTTA	Used with cloning intIA_R to amplify the *V. cholerae intIA*	60	1.0	This study
Cloning intIA_R	AAAAGCGCTGTCTAGACTATGAAAT CCCAGTTTTTGTTAAGTG	Used with cloning intIA_F to amplify the *V. cholerae intIA*			
Cloning recA_F	TTTTGGATGGAGTGAAACGCGAGAA TAAACAGAAGGCACTTG	Used with cloning recA_R to amplify the *V. cholerae recA*	60	1.0	This study
Cloning recA_R	AAAAGCGCTGTCTAGACTAGCCTGC CGATTAAAACTCTTC	Used with cloning recA_F to amplify the *V. cholerae recA*			

2.2.3 Construction of artificial gene cassettes

Two plasmids (pKC01$^+$ and pKC01$^-$) doubling as circular gene cassettes were constructed. These plasmids/gene cassettes contain an R6K origin of replication (oriR6K) and are capable of replicating in *E. coli* λ-pir strains allowing for easy amplification and purification however, they cannot replicate in *V. cholerae* A1552. pKC01$^+$ and pKC01$^-$ were constructed using PCR amplification of two transposon mutants with TnFGL3 inserted within gene cassettes VCH35 and VCH60 respectively (Cameron, Urbach & Mekalanos 2008). The transposon in these two mutants are in opposite orientations (Cameron, Urbach & Mekalanos 2008) (Figure 2.2A). Specifically, 5' phosphorylated primers ML147F/ML148R and ML149F/ML150R (Table 2.2) were used to amplify fragments of approximately 6.7 kb (Supplementary Figure S1.1) containing VCH35 and VCH60 interrupted with TnFGL3 respectively. The amplicons were gel purified and self-circularised by ligation. Briefly, ligation reaction mixtures containing 2 μl of 10x T4 DNA ligation buffer, 50 ng of purified DNA, 1 μl of T4 DNA ligase and dH$_2$O to a final volume of 20 μl was incubated overnight at room temperature and the reaction inactivated at 70 °C for 15 min. The ligation mixture was transformed into chemically competent *E. coli* DH5α λ-pir cells using a heat shock method (Sambrook & Russell 2006). Transformants were plated on LB5 plates containing kanamycin and single colonies isolated and confirmed to contain plasmids pKC01$^+$ and pKC01$^-$ by PCR using primers KanF/KanR (Table 2.2). Both plasmids/gene cassettes contain a kanamycin resistance gene (*nptII*), promoterless *gfpmut3* and *lacZ* and, a single *V. cholerae*-specific *attC* site with, pKC01$^+$ and pKC01$^-$ containing *gfpmut3-lacZ*-oriR6K-*nptII* downstream of the GTTRRRY *attC* recombination site however, pKC01$^-$ contains these same genes in the opposite orientation (i.e. *nptII*-oriR6K-lacZ-*gfpmut3*) relative to pKC01$^+$ (Figure 2.2B). As a result of the approach taken to amplify artificial gene cassettes from transposon mutants with TnFGL3 inserted within VCH35 and VCH60, partial sequences of gene cassettes VCH35 (395 bp, 54 bp), VCH 36 (107 bp) and VCH 60 (313 bp, 64 bp), VCH 61 (67 bp) flank the *attC* sites of both pKC01$^+$ and pKC01$^-$ respectively (Figure 2.2B and 2.2C)

Additionally, a synthetic 1.5 kb linear kanamycin-resistant gene cassette (gc_kan; Figure 2.2D) was designed and synthesised at Integrated DNA Technologies containing a *lac* promoter from pCR-XL-TOPO-plasmid (http://www.transomic.com/Vectors-and-Maps/pCR-XL-OPO.aspx) in front of a kanamycin resistance ORF (*nptII*) from pUC4K (https://www.addgene.org/browse/sequence_vdb/4506/) and an *rrnB* transcriptional terminator

from the iGEM part BBa_B0015 repository (http://parts.igem.org/Terminators) after *nptII*. The linear fragment is abutted by two *V. cholerae*-specific *attC* sites (taken from VCH59 and VCH60) and primer (ML15/ML16) binding sites for high yield PCR amplification (Figure 2.1D). Successful amplification of gc_kan for transformation experiments is shown in Supplementary Figure 2.1B.
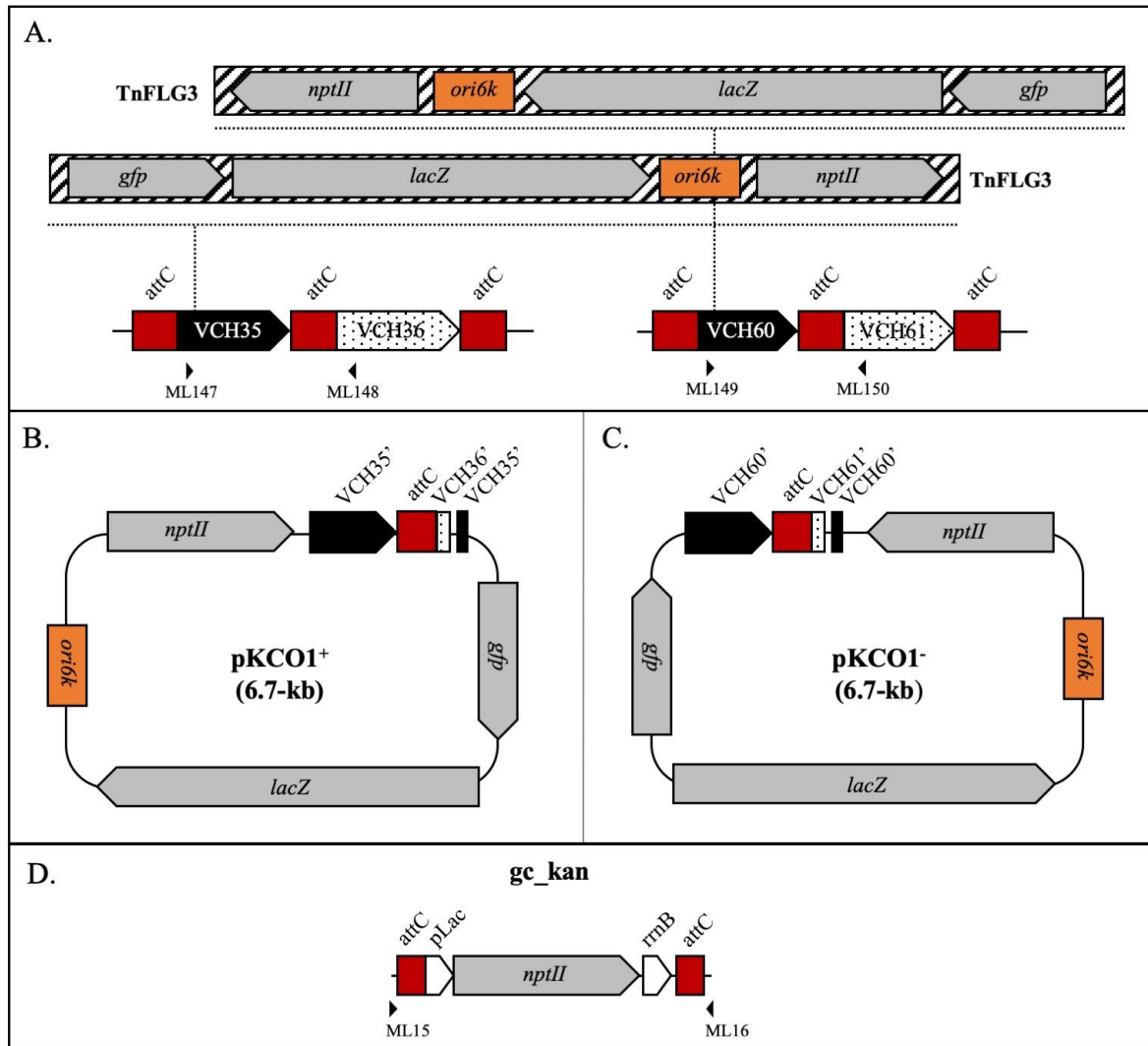
Figure 2.2: Genetic architecture of elements used in this study. *V. cholerae* C6706 TnFLG3 mutants used in this study showing insertion of TnFLG3 in VCH35 and VCH60 in opposite orientations (A). Primer binding sites are shown as black triangles and were used to amplify the entire TnFLG3 along with a *V. cholerae attC* site (shown in red) and self-ligated to create the kanamycin-resistant artificial gene cassettes pKC01⁺ (B) and pKC01⁻ (C). Partial sequences of gene cassettes on either side of the *attC* are shown in rectangle boxes. A linear gene cassette (gc_kan) was designed and synthesised containing *nptII* under the control of a pLac promoter and with an *rrnB* transcriptional terminator from the iGEM repository (part BBa_B0015) (D). The linear construct is abutted by *V. cholerae attC* sequences and ML15 and ML16 primer binding sites (shown as black triangles) for easy amplification. ORFs are shown as arrows and indicate their direction. Genetic elements are not drawn to scale.

2.2.4 Chitin transformation of *V. cholerae* A1552 with artificial donor gene cassettes

Purified artificial donor gene cassettes (pKC01$^+$ and pKC01$^-$ and, gc_kan) were transformed into *V. cholerae* O1 A1552 using chitin transformation according to a previously published method (Marvig & Blokesch 2010). Briefly, 10 mL of *V. cholerae* O1 A1552 was grown at 37 °C in fresh LB5 broth following inoculation from an overnight culture (1:1000 dilution) until it reached an OD$_{600}$ of 0.4-0.5. Cells were pelleted by centrifugation (4000 x *g* for 10 min) and washed once with 1 mL of sterile 0.7% Sea Salts Solution (SSS; Sigma-Aldrich, buffered to pH 7) before final resuspension in 0.5 mL SSS. Sterile chitin flakes (50-70 mg) were mixed with 900 µl of sterile SSS by vortexing before adding 100 µl of the resuspended *V. cholerae* cells and incubating overnight at 30 °C statically to promote biofilm formation on the chitin flakes. Following incubation, the chitin flakes settle to the bottom of the tube allowing for careful aspiration and discarding of 500 µl of supernatant. Donor DNA (0.2-1.0 µg) was then added to facilitate DNA transformation. The tubes were incubated statically for 2 h and then rigorously vortexed for 2 min to detach the cells from the chitin flakes before plating on LB5 agar supplemented with kanamycin. Transformation frequencies were measured by calculating the number of kanamycin resistance CFUs/total number of CFUs (Marvig & Blokesch 2010). To confirm that the chitin-transformed strains were *V. cholerae*, PCR using *V. cholerae* species-specific primers were used (*ompW*_F/*ompW*_R) (Nandi et al. 2000b) (Table 2.2). Successful transformants were confirmed using *nptII*-specific primers (KanF/KanR) (Table 2.2).

2.2.5 PCR cartography of the cassette array structure

To determine the insertion point of the artificial gene cassettes in the cassette array, purified gDNA from chitin transformants were subjected to PCR cartography using the 21 primer pairs listed in Table 2.2 (Figure 2.3). Insertion of a gene cassette in a specific region of the array targeted by a primer pair was indicated by no amplicon (i.e. amplicon to large to amplify) or a different sized amplicon compared to the wild-type.
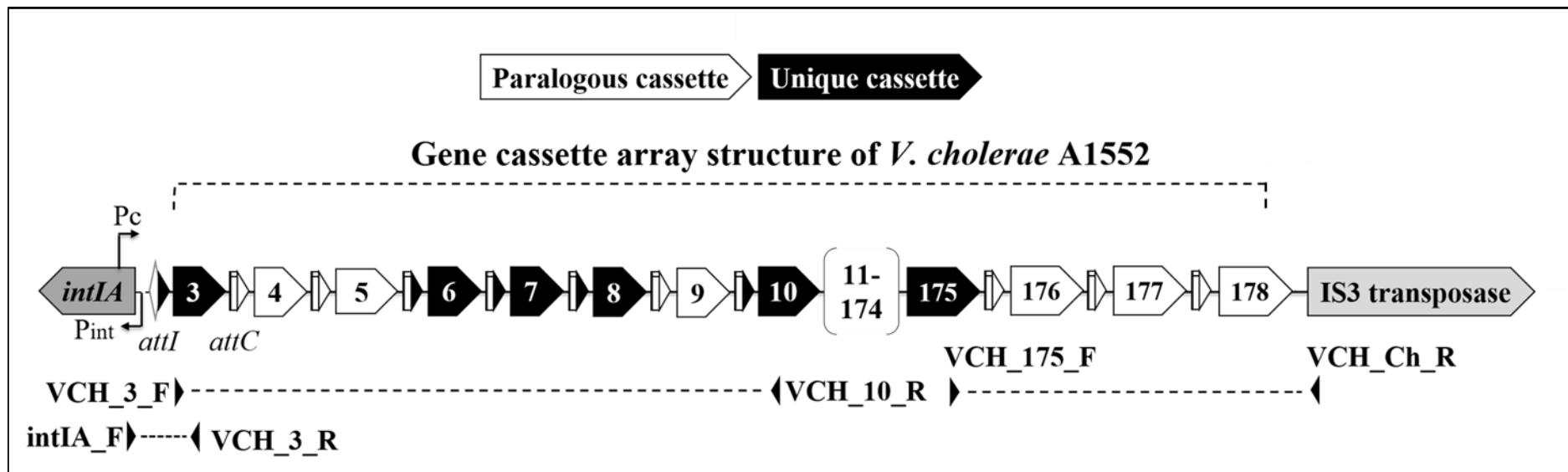
Figure 2.3: PCR cartography of the *V. cholerae* O1 A1552 cassette array structure. Cassettes are labelled with numbers relating to their position in the array relative to *attI*. Compared to the first genome annotated *V. cholerae* N16961 strain, A1552 lacks the first (VCH 1), second (VCH2) and 45th (VCH45) cassettes with all other cassettes in the expected relative positions. For simplicity, the numbering scheme for *V. cholerae* N16961 was used for A1552. In A1552, VCH3 is the first gene cassette among a total 175 gene cassettes (see Appendix 2.1). Twenty-one primers targeting the front (intIA_F) and back ends (VCH_Ch_R) of the array and, the unique cassettes (black) were designed to investigate the integration of artificial gene cassettes into the cassette array. Paralogous cassettes (white) were not targeted due to the likelihood of multiple PCR products being amplified. For example, insertion of cassettes at *attI* were determined using primers intIA_F and VCH_3_R. Absence (i.e. amplicon to large to amplify) or a different sized amplicon relative to the wild-type indicates potential integration of the artificial gene cassette in the region targeted by the primer set.

2.2.6 Construction of *V. cholerae* mutants

The *V. cholerae* Δ*intIA::cat* and *V. cholerae* Δ*hmgA::cat* mutants were constructed by performing an in-frame deletion of the integron-integrase gene (*intIA*) and interrupting it with a chloramphenicol resistance cassette (*cat*) abutted by FRT sites. The mutant allele was constructed via splicing-by-overlap-extension-PCR (SOE-PCR) (Silva & Blokesch 2010). pKD3 which contains the FRT-*cat*-FRT cassette (Datsenko & Wanner 2000) was purified and used as a template in PCR amplification. In brief, approximately 600 bp regions of DNA upstream and downstream of and, including part of the target gene (*intIA* and *hmgA*) were amplified using primers intIA_Up-F/R and intIA_Down-F/R, and hmgA_Up-F/R and hmgA_Down-F/R respectively (Table 2.2) and gel purified separately. Oligonucleotides with overlapping ends were constructed with the online free software Genome Compiler (https://designer.genomecompiler.com/app) to assist the correct orientation and successful binding of the three fragments (upstream fragment, FRT-*cat*-FRT and downstream fragment). Then two step SOE-PCR (splicing-by overlap-extension-PCR) was conducted, where the first round works to anneal the three fragments together without the addition of primers at 50 °C for 2.5 h and in the second round, upstream forward and downstream reverse primers were added to allow the amplification of the fused fragments. The second round PCR cycling conditions were as follows: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, an appropriate annealing temperature for 30 s, an appropriate extension time of 20 s/kb at 72 °C, and a final extension time of 2 min at 72 °C (See Table 2.2 for specific annealing temperature and extension times for each primer pairs). The fused PCR construct was then gel purified and 500 ng was chitin-transformed into *V. cholerae* A1552. Following chitin transformation, transformants were selected on selective LB5 agar containing chloramphenicol and incubated overnight at 37 °C. To confirm transformants carried the mutant allele, PCR was carried out using primers intIA_Up-F/VCH_3-R (Table 2.2) that bind in the deleted region of *intIA* and the first gene cassette (VCH 3) and primers hmgA_up-F/ hmgA_down_R (Table 2.2) that is expected to give reduction in size of the amplicon compared to the wild type strain.

The FRT-*cat*-FRT cassettes was excised from the *V. cholerae* Δ*intIA::cat* and *V. cholerae* Δ*hmgA::cat* mutants via FLP-mediated recombination according to a previously described method with some modifications (Blokesch 2012b). Briefly, the pBR-flp plasmid was chitin transformed into A1552 *ΔintIA::cat* and transformants selected on LB5 agar plates containing carbenicillin at 30 °C. From a single transformant, the thermosensitive promoter of the *flp* gene

was activated by growth at 37 °C for 8 h resulting in flippase expression that excises of the FRT-flanked *cat* cassette. Chloramphenicol sensitive clones were cured of the pBR-flp plasmid by overnight growth in LB5 broth (no antibiotics) at 37 °C and subsequently plated on LB5 agar. The antibiotic sensitivity of clones was measured by re-streaking single colonies on LB5 agar containing chloramphenicol and LB5 agar containing carbenicillin. The FRT-*cat*-FRT excision was confirmed using PCR to amplify the *intIA* and *hmgA* using the primer pair intIA-up-F/intIA-down-R and hmgA_up_F/hmgA_down_R, repectively (Table 2.2) with a reduction in the size of the amplicon compared to wild type confirming a mutated *intIA* and *hmgA* allele with *cat* excised.

V. cholerae Δ*recA* and *V. cholerae* Δ*intIA* Δ*recA* (double mutant) were constructed by an in-frame deletion of *recA* from wild type *V. cholerae* and *V. cholerae* Δ*intIA*. Using splicing-by-overlap-extension-PCR (SOE-PCR), a DNA fragment with *recA* interrupted with a spectinomycin resistance gene (*aadA7*; sourced from pOriVn$_{700}$) (Le Roux, Davis & Waldor 2011) was constructed. Oligonucleotides with overlapping ends were constructed with Genome Compiler and then two step SOE-PCR was carried out as described above. The upstream and downstream regions of *recA* and, *aadA7* were amplified using oligonucleotides described in Table 2.2 with annealing temperatures and extension times for each oligonucleotide sets also listed in Table 2.2. The gel purified PCR construct was chitin transformed into wild type A1552 and the Δ*intIA* mutant to create *V. cholerae* Δ*recA* and *V. cholerae* Δ*intIA* Δ*recA*. PCR confirmations was carried out on each transformant colony using oligonucleotides (Table 2.2) that bind to upstream and downstream of the deleted gene, and compared with the wild type. Homologous recombination at the target site was confirmed when no amplification of target gene was observed.

2.2.7 Complementation of *V. cholerae* mutations

To clone *intIA* and *recA* for complementation studies, the arabinose inducible expression vector, pSU-pBAD, was used. The vector backbone was PCR amplified using the primers, pSU-pBAD_F and pSU-pBAD_R (Table 2.2). *intIA* and *recA* were amplified using primers (Table 2.2) designed to have 20 bp homologous overlaps with the vector using Genome Compiler (https://designer.genomecompiler.com/app). The PCR cycling conditions were as follows: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, a variable annealing temperature for 30 s, a variable extension time of 20 s/kb at 72°C, and a final extension time of 5 min at 72 °C (See Table 2.2 for specific annealing temperature and extension times for

each primer pairs). The PCR products were gel-purified separately using ISOLATE II PCR and Gel Kit (Bioline, England) and then the fragments were assembled using the Gibson master mix (New England Biolabs, Ipswich, MA) following the manufacturer's instructions and transformed into *E. coli* S17-1λ pir on LB5 to obtain the plasmid pSU-pBAD::*intIA* and pSU-pBAD::*recA*. These plasmids were propagated in overnight in LB5 broth supplemented with chloramphenicol and 1% glucose (to repress the induction of arabinose promoter). The extracted plasmids were confirmed by PCR to contain *intIA* and *recA* using primers pSU-pBAD_F /cloning intIA_R and pSU-pBAD_F /cloning recA_R, respectively (Table 2.2). Complementation of plasmids (pSU-pBAD::*intIA* and pSU-pBAD::*recA*) into *V. cholerae* Δ*intIA* and *V. cholerae* Δ*recA* were performed using the chitin transformation method and then plating on selective LB5 agar.

2.2.8 Construction of a linear gene cassette containing *hmgA* and complementation of a *V. cholerae hmgA* mutant

To construct a linear gene cassette containing *hmgA* (gc_hmgA-kan), the upstream (*attC-lac* promoter) and downstream (*nptII-rrnB-attC*) regions of gc_kan were amplifed using primers (attC_Up_F/R) and primers (Kan_attC_F/R), respectively (Table 2.2) and, *hmgA* (VC1345) was amplified from *V. cholerae* A1552 using primers hmgA_F/R (Table 2.2). All amplicons were gel purified. The primers were constructed to have overlapping ends (Table 2.2) using the online free software Genome Compiler (www.genomecompiler.com) to assist the correct orientation and successful fusion of the three fragments using two steps SOE-PCR as described above. After the first round facilitating fragment fusion at 50 °C for 2.5 h, the upstream forward and downstream reverse primers were added to allow amplification of the fused fragments with the PCR conditions of 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, 72 °C for 4 mins and a final extension at 72 °C for 10 min. The gc_hmgA-kan PCR construct was then purified and quantified. One microgram of purified gc_hmgA-kan construct was chitin-transformed into *V. cholerae* A1552 Δ*hmgA* with transformants selected on LB agar plates containing kanamycin and incubation at 37 °C. After 24 h incubation, the colonies of the *V. cholerae* A1552 Δ*hmgA* complemented strain were compared with the wild-type A1552 colonies. To confirm that gc_hmgA-kan inserted into *attI*, six kanamycin resistant colonies were subjected to PCR using primers flanking *attI* (intIA-F/VCH 3R; Table 2.2) and fragment size estimated via gel electrophoresis.

2.2.9 Statistical analysis and DNA sequences access

Statistical analyses were performed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com). Two-tailed student's t-tests were used to compare means between experimental samples and controls. For experiments with multiple samples, one-way ANOVAs were used with Tukey's or Dunnett's Multiple Comparison Test post-hoc comparisons of means. *P* values of <0.05 was considered to indicate statistical significance.

Annotated DNA sequences of the *V. cholerae* A1552 integron and cassette array, pKCO1+, pKCO1-, gc_kan and gc_hmgA-kan are provided as GenBank files and can be accessed at the following link:

https://www.dropbox.com/sh/mzevtyqstes3qv9/AAAilVraFs0EumaH2pN_yJwOa?dl=0

Assembled genomes of six pKCO1[+] chitin transformants are available at the following link:
https://www.dropbox.com/sh/rfi0pn7ox3upkls/AADplFhssTs7r7YGyQqo3P3ta?dl=0

2.3 Results

2.3.1 Transformation frequency of artificial gene cassettes inserted into the *V. cholerae* integron

In order to measure cassette integration dynamics during chitin transformation, artificial gene cassettes containing a kanamycin resistance marker were created including i) artificial circular gene cassettes (pKC01$^+$ and pKC01$^-$) containing an R6K origin of replication permitting replication as a plasmid in *E. coli* λ-*pir* strains for easy amplification and harvesting and ii) a linear gene cassette containing a single kanamycin resistance gene abutted by two *attC* sites and primer binding sites for easy amplification by PCR. The artificial gene cassettes cannot replicate in *V. cholerae* and must integrate into the *V. cholerae* chromosome to replicate and be passed onto progeny.

In chitin transformation experiments using 1 μg of DNA, kanamycin resistant colonies were obtained for all gene cassettes with the transformation frequency almost 10-fold higher when using the circular gene cassettes (pKC01$^+$ and pKC01$^-$) compared to the linear gene cassette (gc_kan) (Figure 2.4). No significant difference was observed between the transformation frequency of pKC01$^+$ and pKC01$^-$ (Figure 2.4). As expected, no kanamycin resistance colonies were detected in the no DNA control (Figure 2.4). Thirty (30) kanamycin resistant colonies were randomly selected (10 for each artificial gene cassette) and confirmed to contain the *V. cholerae* species-specific *ompW* and the kanamycin resistance gene in the artificial gene cassettes (data not shown).
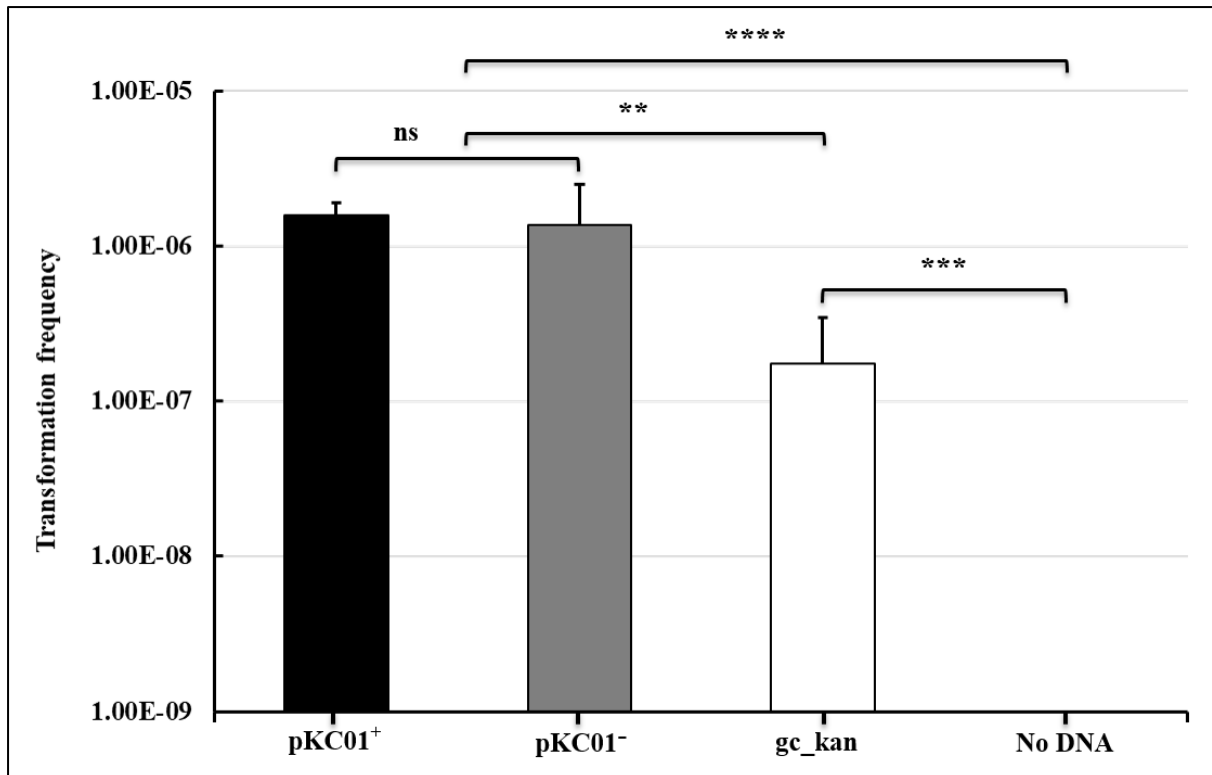
Figure 2.4: Transformation frequency of artificial circular and linear gene cassettes in *V. cholerae* A1552. Error bars represent the standard deviation. Significance was calculated using one-way ANOVA, Tukey's multiple comparisons test (ns= not significant, **p<0.01, ***p<0.001, ****p<0.0001).

2.3.2 Determination of integration site and orientation of the transformed circular and linear gene cassettes

To determine where the artificial gene cassettes had inserted into the integron, we selected 10 kanamycin resistant colonies from each of the pKC01$^+$, pKC01$^-$ and gc_kan chitin transformations and subjected them to PCR cartography. Any changes in amplicon size or absence of the target cassettes compared to the wild type indicates probable insertion of the donor gene cassette within the array. The PCR results showed that colonies from gc_kan chitin-transformants were identical, only showing an increased amplicon size using primers IntIA_F and VCH_3_R and indicating insertion at *attI* (Figure 2.5D). However, the pKC01 chitin-transformants showed an absence of amplicons with IntI_F and VCH_3_R (Figure 2.5D) and with VCH_26_F and VCH 38_R for pKC01$^+$ and VCH_57_F and VCH 64_R for pKC01$^-$ (Appendix 2.3). Additionally, a larger amplicon of ~1500 bp was obtained using intIA_F/VCH 3_R for transformant 4 with insertion of pKC01$^+$ and transformant 8 with insertion of pKC01$^-$ indicating a small insertion at *attI*. No other changes in the gene cassette array were found.
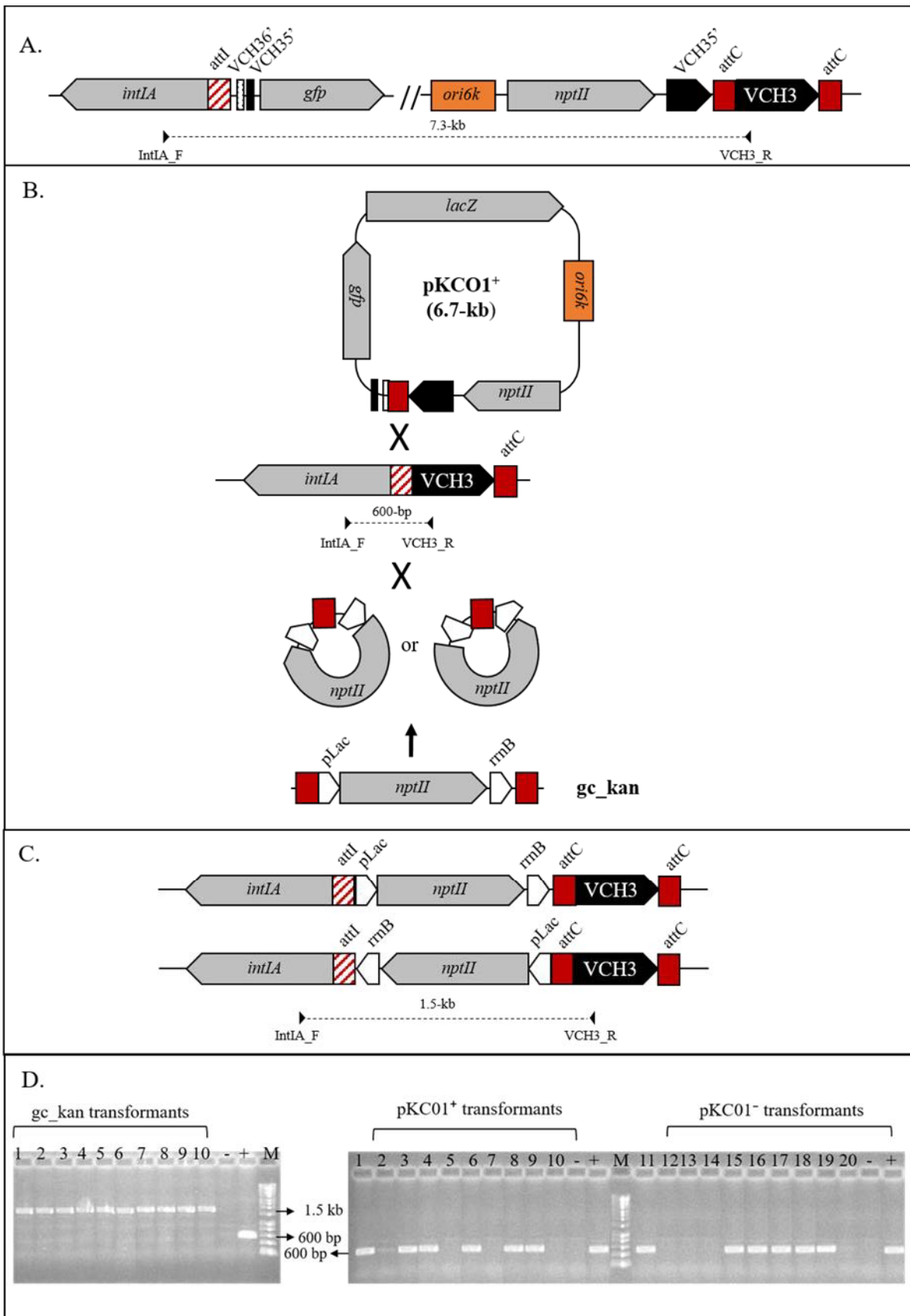
Figure 2.5: Expected genetic architecture of pKC01⁺ and gc_kan insertion into *attI* of the *V. cholerae* integron *via* site-specific integration with *attC*. Site-specific recombination of the artificial gene cassettes into *attI* are demonstrated in (B) with the X symbol showing site-specific recombination with pKC01⁺ (upper) and gc_kan (lower). Genetic architecture of pKC01⁺ insertion into *attI* in shown in (A).

Depending on the DNA strand that is transformed into the *V. cholerae* cell, gc_kan is hypothesised to circularise in one of two orientations inside the cell before integration into *attI* resulting in insertion of gc_kan into *attI* in one of two orientations (C). With no insertion into *attI*, PCR with primers IntIA_F and VCH3_R (primer binding sites shown as black triangles) gives a product of ~600-bp whereas insertion of the gc_kan and pKC01 gene cassettes would result in product sizes of ~1.5-kb and absence of product respectively (D). ORFs are shown as arrows and indicate their direction. Genetic elements are not drawn to scale.

Unexpectedly, PCR using primers intIA_F/Kan-F showed that the linear gene cassette (gc_kan) always inserted in one orientation with the kanamycin gene reading toward *attI* and never away from it (see Figure 2.5C – lower image). In contrast, the circular gene cassettes (pKC01$^+$ and pKC01$^-$) inserted in the expected orientation as confirmed by PCR using primers (intIA_F or ML147_F/Kan-R and intIA_F or ML149_F/Kan-F) (data not shown).

2.3.3 Genome sequences analysis

In order to validate the PCR results and to check any other potential changes in the cassette array due to insertion of pKC01, six chitin transformants (chitin transformants 1-4, 6 and 10 in Table 2.1) with insertion of pKC01$^+$ at different sites in the gene cassette array were selected and subjected to short- (Illumina) and long-read (Nanopore) genome sequencing. Following assembly of reads with Unicycler, each genome gave two large contigs of approximately 3.3 Mb and 1.1 Mb consistent with the large and small chromosomes of *V. cholerae* respectively (Appendix 2.4). The gc_kan chitin transformants were not considered for genome sequencing due to the lack of diversity with all showing insertion at *attI*. Following sequence assembly and annotation, cassette arrays were aligned and visualised using MAUVE (Figure 2.6). Consistent with the PCR results, the genome sequence analysis showed that pKC01$^+$ was integrated between VCH35 and VCH36 for chitin transformants 1, 3, 4 and 6, while transformant 2 and 10 contained pKC01$^+$ inserted at *attI*. In addition to integration of pKC01 in different position in the cassette array, genome sequence analysis showed that transformant 4 contained a gene cassette duplication of VCH 66 (877 bp) integrated at *attI* (Figure 2.6). This result is also consistent with the PCR result described above where a larger amplicon was obtained using intIA_F/VCH 3_R for transformant 4 with insertion of pKC01$^+$.
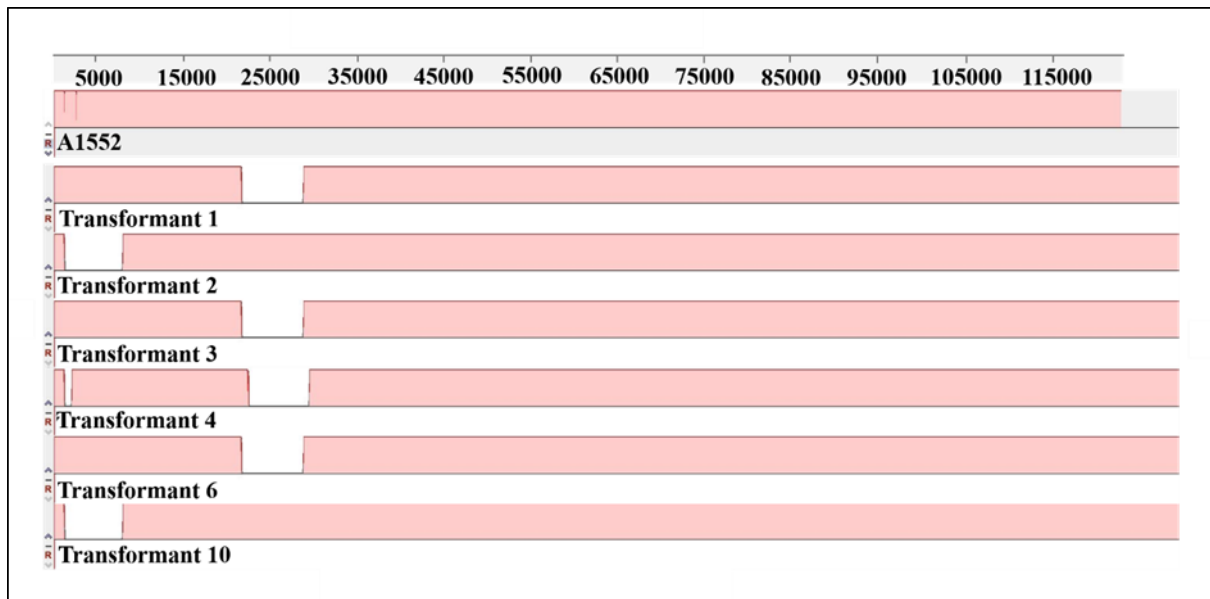
Figure 2.6: Mauve alignment of the *V. cholerae* A1552 gene cassette array with those from pKC01⁺ chitin transformants. Each cassette array is laid out in a pink horizontal track with the larger white gaps indicating an insertion relative to the wild-type and confirmed as the integration of pKC01⁺ in the cassette array of the different chitin transformants. Transformants 1, 3, 4 and 6 contain pKC01⁺ inserted between VCH 35 and VCH 36, while transformants 2 and 12 contained pKC01⁺ inserted at *attI*. An insertion at *attI* in transformant 4 shown as a smaller white gap was found to be due to duplication of VCH66 and insertion at *attI*.

2.3.4 Integrase (IntIA) and the SOS response induction is required for site-specific recombination of synthetic gene cassettes into the *V. cholerae* integron

To determine whether integration of gene cassettes requires the integron-integrase gene, a strain lacking *intIA* was created and subjected to chitin transformation using the artificial gene cassettes (Figure 2.7). No transformants were obtained when the linear gc_kan was used indicating that *intIA* is essential for its integration however, transformants were obtained for the circular gene cassettes although at an efficiency of 2706- and 2481-fold lower than the wild-type for pKC01⁺and pKCO1⁻ respectively. As *recA* is required for induction of *intIA* through the SOS response, the Δ*recA* mutant was also tested in a chitin transformation giving no kanamycin resistant colonies for all artificial gene cassettes (Figure 2.7). It was hypothesized that integration of the circular gene cassettes in the Δ*intIA* mutant was due to RecA-mediated homologous recombination through the short VCH35 and VCH60 sequences in pKC01⁺ and pKCO1⁻ respectively (see Figure 2.2B and 2.2C showing the genetic architecture of pKC01⁺ and pKC01⁻ respectively) and, explaining the specific insertion of the circular gene cassettes between VCH35 and VCH36 (pKC01⁺), and VCH60 and VCH61 (pKC01⁻). Indeed, when the Δ*intIA, ΔrecA* double mutant was tested removing both IntIA-

mediated site-specific recombination and RecA-mediated homologous recombination possibilities, no kanamycin resistant colonies were obtained (Figure 2.7).
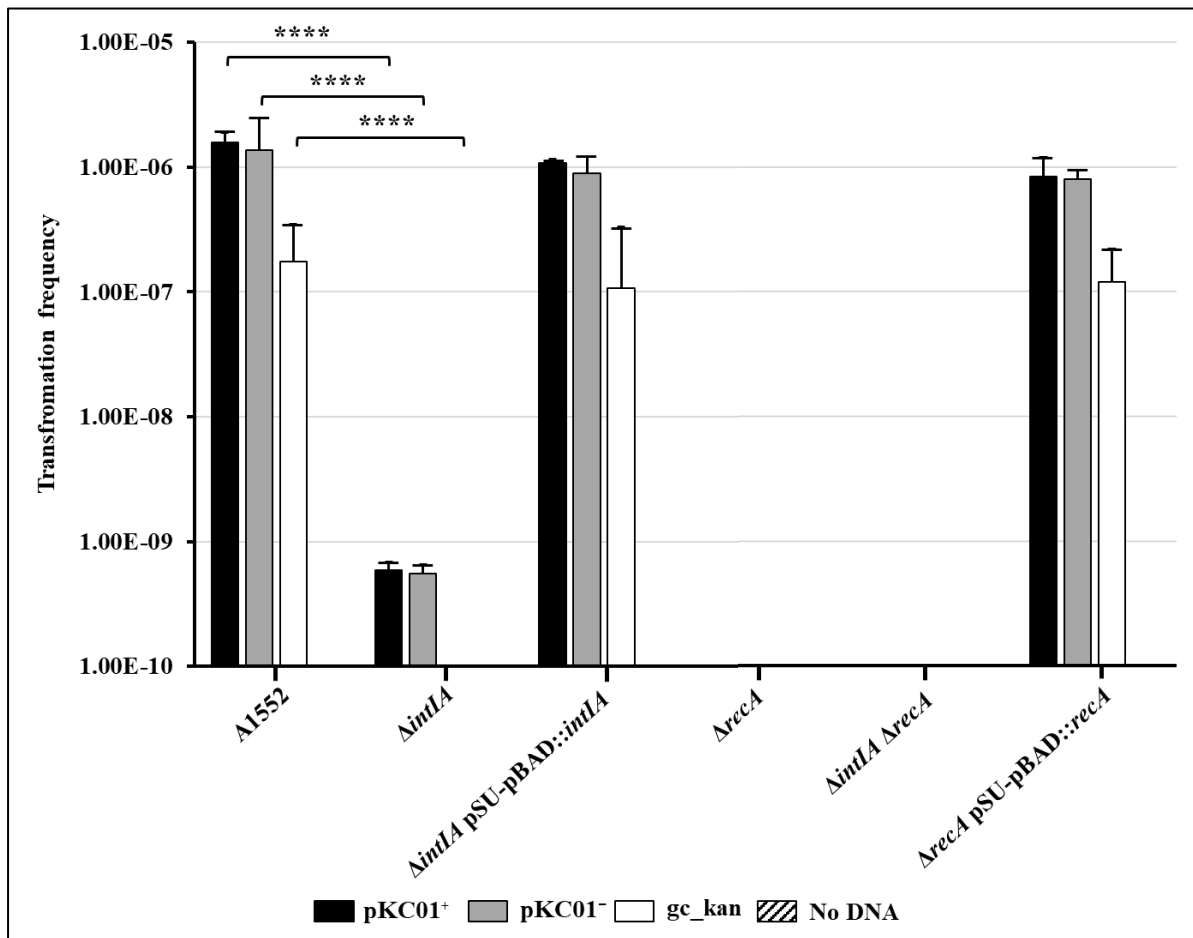
Figure 2.7: Transformation frequency of artificial circular and linear gene cassettes in the *V. cholerae* Δ*intIA*, Δ*recA*, Δ*intIA* Δ*recA* and A1552 wild type strains. Transformation frequency of artificial circular and linear gene cassettes in the *V. cholerae* Δ*intIA* and Δ*recA* strains followed by complementation of *intIA* and *recA,* respectively. Error bars represent the standard deviation. Significance was calculated using one-way ANOVA, Tukey's multiple comparisons test (ns= not significant, ****$p<0.0001$).

To determine if the transformation frequency of artificial circular and linear gene cassettes could be restored by complementation, *intIA* and *recA* were cloned into an arabinose inducible pSU-pBAD expression vector and introduced into the Δ*intIA* and Δ*recA* backgrounds restoring the transformation frequency back to wild type levels (Figure 2.7).

2.3.5 Synthetic linear gene cassettes: Tools that can be used to complement *V. cholerae* mutations

Chitin transformation in *V. cholerae* A1552 with the synthetic gc_kan showed that insertion always occurs at *attI* site providing an opportunity to use linear gene cassettes as a tool for

inserting DNA into *attI* for experiments such as complementation of mutants. To address this possibility, a 2,728 bp engineered linear gene cassette carrying *hmgA* and a kanamycin resistance marker under the control of a *lac* promoter (gc_*hmgA*-kan) was chitin transformed into a *V. cholerae* A1552 Δ*hmgA* mutant. The Δ*hmgA* mutant was selected due to its production of an easily observable pigment that is reversed when complemented. Following transformation, six kanamycin resistant colonies were randomly selected and subjected to PCR using primers IntI_F and VCH_3 to determine if gc_*hmgA*_kan had inserted at *attI*. Of the 6 colonies, 2 (colony 3 and 5) showed an expected increased amplicon size of 3,328 bp (size is 2,728 bp for gc_*hmgA*_kan + 600 bp for the *intIA* - VCH3 region) indicating insertion at *attI* (Figure 2.8A) and when streaked on fresh LB5 plates, no pigment production was observed (Figure 2.8B). Using primers hmgA_up_F/ hmgA_down_R, the PCR results of the other 4 colonies showed an increased amplicon length compared to the Δ*hmgA* mutant strain indicating homologous recombination of gc_*hmg*_kan at *hmgA* (Supplementary Figure S2.2).
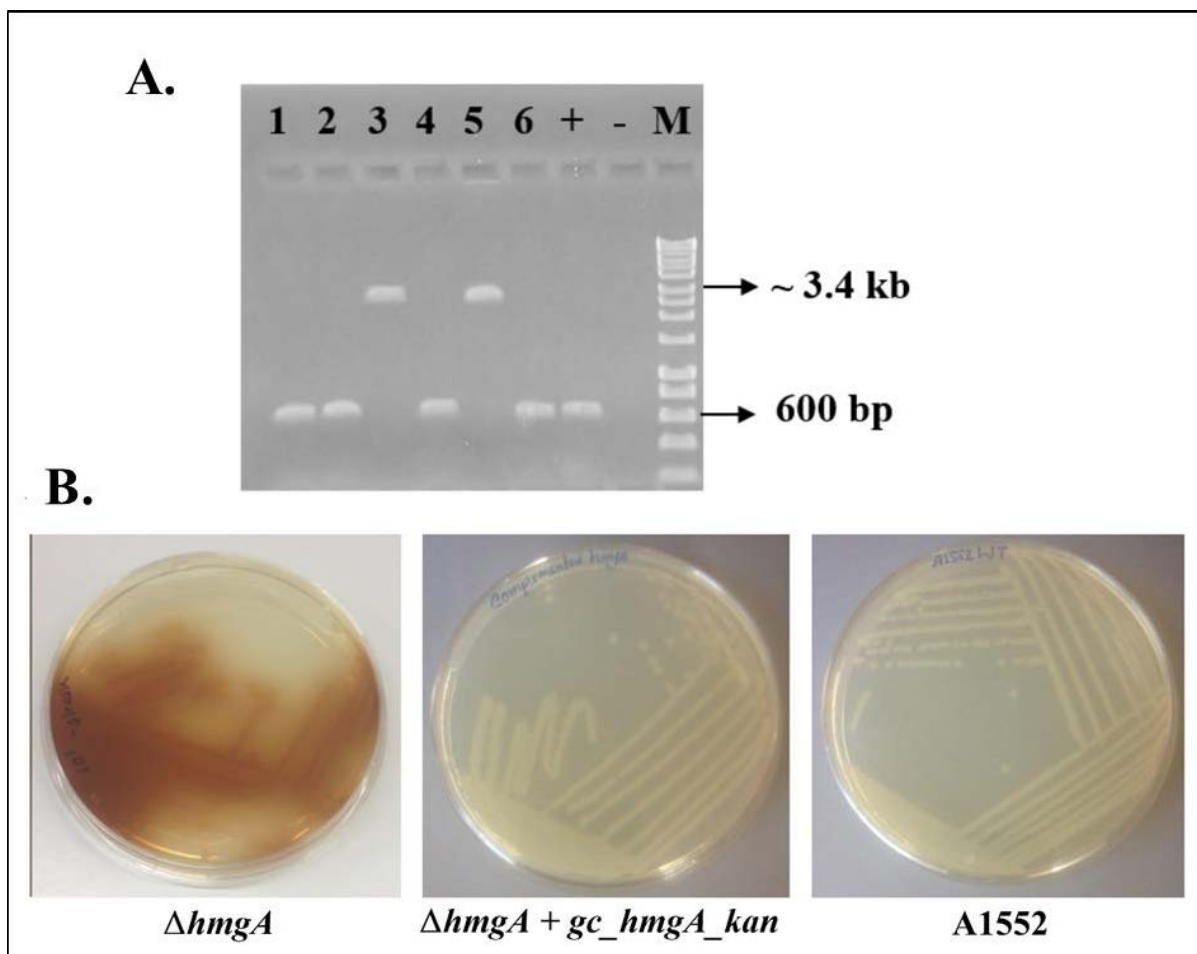
Figure 2.8: PCR confirmation of insertion site of gc_*hmgA*-kan and phenotypic changes after complementation with gc_*hmgA*-kan. PCR amplification to confirm the insertion of gc_*hmgA*-kan at *attI* using intIA-F/VCH 3_R (A). Lanes 1 - 6 are PCR of 6 randomly selected gc_*hmgA*_kan kanamycin resistant transformants with lanes labelled +, - and M showing the *V. cholerae* A1552 Δ*hmgA* gDNA

positive control, dH$_2$O negative control and DNA ladder, respectively. Pigment production by the Δ*hmgA* mutant, Δ*hmgA* when complemented using gc_*hmgA*-kan through insertion at *attI*, and wild type (A1552), respectively (B). Colonies were grown on LB5 for 24 h at 28 °C.

2.4 Discussion

The human pathogen *V. cholerae* is an aquatic bacterium that exists with a variety of other bacterial species in biofilms formed on chitinous surfaces where it can become naturally competent and acquire novel genetic material (Hall-Stoodley, Costerton & Stoodley 2004). Based on the link between chitin induced transformation and *intIA* transcription, circular and linear gene cassettes were constructed to investigate gene cassette transfer into the *V. cholerae* chromosomal integron of chitin-competent cells. Compared to the circular gene cassettes, transformation of the linear cassette was ~10-fold lower despite equal amounts of DNA being added to the transformation. This has also been observed in *V. fischeri* where transformation of a plasmid was 40-fold more efficient that PCR-generated linear DNA (Pollack-Berti, Wollenberg & Ruby 2010). With the linear gene cassette being almost 1/5th smaller, a higher amount of gene cassette copies would be present and so the reduced transformation efficiency is unusual but could be explained by reasons such as its susceptibility to exonucleases such as Dns that digest linear DNA (Blokesch & Schoolnik 2008) or the absence of methylation in the linear cassette compared to the circular cassette (cultivated in *E. coli*). Despite the difference in efficiency, this result shows that linear gene cassettes can serve as transforming material that specifically recombines into *attI*.

Whilst the circular gene cassettes inserted at *attI,* they also inserted at sites that correlate with homologous regions present in the artificial gene cassettes (e.g. VCH35 and VCH60). This indicates that the circular gene cassette are inserting *via* integrase-mediated site-specific recombination and homologous recombination. Homologous recombination is supported by the presence of transformants in the Δ*intIA* mutant that is defective in integrase-mediated recombination with almost 3000-fold less transformation than the wild-type. This substantial drop in transformants in the Δ*intIA* mutant suggests that the vast majority of circular gene cassette integration is integron integrase-mediated. In an Δ*intIA* Δ*recA* double mutant defective in integrase-mediated and homologous recombination, no transformants were observed consistent with the idea that these methods of recombination are responsible for integration of the circular gene cassettes. Of the 20 random circular gene cassette transformants, almost half had insertions between VCH35/VCH36 and VCH60/VCH61 which based on the insertion site specificity would indicate insertion by homologous recombination. As integration through

homologous or integrase-mediated recombination gives the same genetic architecture, it's not possible to ascertain from the genome sequences which method of integration is responsible. Exclusive insertion at these sites from homologous recombination is inconsistent with the ~3000-fold reduction in transformation efficiency in the Δ*intIA* mutant. This suggests that the homologous DNA sequence may be guiding the gene cassettes to these sites but that integration is being mostly facilitated by integrase-mediated recombination. For future experiments aimed at measuring integrase-mediated insertion, creating a circular gene cassette with no homologous regions and a single *attC* site is ideal. However, the circular gene cassette constructed here are still highly useful for investigating transformation and integrase-mediated integration of DNA. Additionally, transformation of an artificial circular gene cassette resulted in a gene cassette duplication event and its integration in *attI*. This may have occurred due to the movement of a gene cassette between two chromosomes in one cell during replication (Achaz et al. 2003; Levinson & Gutman 1987). Also, such events may explain the numerous paralogous cassettes present in the arrays of *Vibrio* species (Rowe-Magnus et al. 2003).

In terms of the orientation of integrated gene cassettes, the structure of the *attC* sites are such that (Loot et al. 2010; Mukhortava et al. 2019; Nivina et al. 2016) the integrase preferentially uses the bottom strand of the gene cassette for mediating its insertion in one orientation (Loot et al. 2010; Mukhortava et al. 2019; Nivina et al. 2016). Consistent with this, the circular gene cassettes always integrated in the expected orientation. For the linear gene cassette, integration occurred in only one orientation and in an orientation that suggests the top strand was used for integration. As the top strand is less efficiently (Loot et al. 2010; Nivina et al. 2016) recombined, this may also explain why the transformation rate of the linear gene cassette was less than the circular gene cassette. It's unclear as to why only the top strand was used for recombination as transformation should allow cells to accept each strand of the cassette in a 50:50 ratio. It's possible that the *rrnB* terminator which forms an elaborate secondary structure and positioned just before the *attC* site could be responsible by affecting integrase binding of the bottom strand. Another possibility is that the *rrnB* terminator makes one of the DNA strands difficult to import. In this scenario, only the top DNA strand would be taken up and would produce a circular gene cassette that would result in integration in the observed orientation. Further studies are needed to explore the mechanism behind this result such as creating artificial gene cassettes with removal of the *rrnB* terminator or with the *lac-nptII-rrnB* elements constructed in the inverse orientation.

Finally, the utility of the *attI* site for complementation of mutations in *V. cholerae* using an engineered linear gene cassette was shown. Complementation of mutations is often done using multicopy plasmids that can be detrimental to cell growth through over-expression or be unstable (Dong, Nilsson & Kurland 1995; Elvin et al. 1990; Tabor & Richardson 1985). By using the *attI* site, DNA can be stably inserted into a chromosomal location in single copy. Although we used the native *hmgA* promoter to drive transcription, it would be possible to engineer a linear gene cassette that would permit insertion of genes in an orientation that would allow transcription from the native P_c promoter.

2.5 Supplementary information
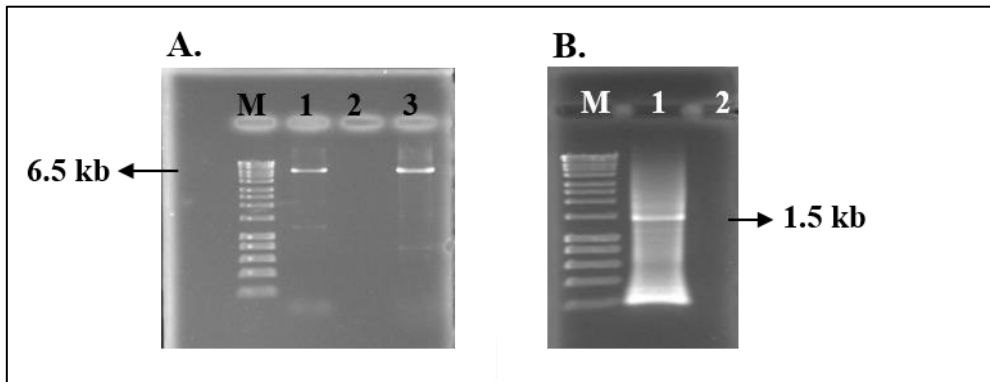
Supplementary Figure S2.1. PCR amplification of pKC01 gc_kan. Panel A. Amplicon length of pKC01, where M indicates 10 kb ladder; Lane 1: pKC01$^+$; Lane 2: blank; Lane 3: pKC01$^-$. Panel B. Amplicon gc_kan, where M indicates 10 kb ladder; Lane 1: gc_kan; Lane 2: blank.
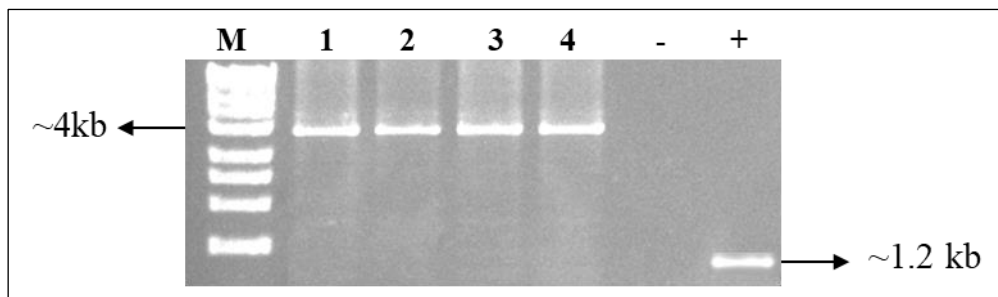
Supplementary Figure S2.2. PCR amplification of *hmgA* region in kanamycin resistant colonies 1-4 that had no insertion of gc_*hmgA*-kan at *attI*. M indicates 10 kb ladder; lane 1-4 are amplicons derived from colonies 1, 2, 4 and 6. label (-) and (+) indicates the negative control (dH$_2$O) and Δ*hmgA* mutant strain positive control.

Chapter 3 : Investigation into the role of gene cassettes in biofilm formation by *V. cholerae*

3.1 Introduction

The chromosomal integron of *V. cholerae* contains a large cassette array with strains generally containing gene cassettes of greater than 150. Although 80-90 % of gene cassettes encode proteins of unknown or uncharacterised functions (Boucher et al. 2007; Mazel et al. 1998), some studies have characterised functionality in a minority of gene cassettes in this species. Specifically, *V. cholerae* gene cassettes are known to encode a number of factors including a heat-stable toxin (*sto*), a mannose-fucose-resistant haemagglutinin (*mfrhA*) and a lipoprotein that are linked with pathogenicity (Barker & Manning 1997a; Franzon, Barker & Manning 1993; Ogawa & Takeda 1993) and, other adaptive factors including a transcriptional regulator (Deshpande et al. 2011) and a sulphate binding protein (Rowe-Magnus et al. 2001). In other *Vibrio* species such as *V. rotiferianus* strain DAT722, deletion of gene cassettes affected biofilm formation and binding of a polysaccharide stain to the surface of the cell indicating that gene cassettes modify surface polysaccharide structure (Rapa et al. 2013). In support of the role of gene cassettes in surface polysaccharide modification, a transposon mutagenesis study on encapsulated *V. vulnificus* 1003(O) showed that an insertion in a gene cassette affected capsular polysaccharide (CPS) synthesis of this strain (Smith & Siebeling 2003). In *V. cholerae*, the exopolysaccharide, VPS (Vibrio polysaccharide), is an extracellular matrix and is required for the formation of three dimensional biofilm structures. It is a major component of the biofilm contributing 50 % of the biofilm matrix (Teschler et al. 2015) and is hyper-produced when *V. cholerae* undergoes a transition from smooth to rugose colony morphology – a transition controlled by the intracellular signalling molecule, cyclic dimeric guanosine monophosphate (c-di-GMP) (Bomchil, Watnick & Kolter 2003; Tischler & Camilli 2004).

In *V. rotiferianus* DAT722, diverse gene promoters are present across the array within cassettes and in *attC* sites (Michael & Labbate 2010) leading to the hypothesis that rearrangement and deletion of cassettes in *Vibrio* species could create phenotypic diversity due to modification of gene cassette transcription (Rapa & Labbate 2013). As insertion of the artificial gene cassettes may affect transcription of downstream gene cassettes due to polar effects such as those exhibited by transposon mutagenesis (Calos & Miller 1980; Cameron, Urbach & Mekalanos 2008; Hutchison et al. 2019; van Opijnen, Bodi & Camilli 2009) or through upregulation from

insertion of an upstream promoter, it was investigated whether integration of the pKC01$^+$ or pKC01$^-$ cassettes in *V. cholerae* affected biofilm formation and/or surface polysaccharide.

3.2 Methods and materials

3.2.1 Bacterial strains and growth conditions

All strains and plasmids used in this study are shown in Table 3.1. *V. cholerae* and *E. coli* strains were routinely cultivated on Luria-Bertani medium supplemented with 0.5 % NaCl (LB5) at 37 ºC. The antibiotics kanamycin (Kan) and ampicillin (Ap) were used at 50 μg ml^{-1} and 100 μg ml^{-1} respectively. Chloramphenicol (Cm) was used at 5 μg ml^{-1} for *V. cholerae* and 25 μg ml^{-1} for *E. coli*. In order to induce gene expression in strains carrying the arabinose-inducible pSU-pBAD vector, a final concentration of 0.2 % (w/v) L-arabinose was added to the growth medium.

3.2.2 Construction of *V. cholerae* deletion mutants

According to the insertion point of the artificial circular gene cassette (pKC01$^+$ or pKC01$^-$) into the *V. cholerae* integron, at least three gene cassettes downstream of these artificial gene cassettes were targeted for deletion. This is because gene cassette orientation is such that pKC01 insertion would affect transcription of downstream cassettes. These included, VCH3-5 for pKC01$^+$ integration into *attI*, VCH36-38 for pKC01$^+$ integration into the VCH35 *attC* site, and VCH61-63 for pKC01$^-$ integration into the VCH60 *attC* site. Three *V. cholerae* mutants (ΔVCH3-5::*cat*, ΔVCH36-38::*cat*, ΔVCH61-63::*cat*) were made by constructing an artificial allele consisting of an in-frame deletion of the gene cassettes in question (i.e. VCH3-5, VCH36-38, VCH61-63) interrupted with a chloramphenicol resistance cassette (*cat*) abutted by FRT sites amplified from pKD3 (Datsenko & Wanner 2000). pKD3 was purified (Wizard® plasmid isolation kit, Promega, USA) and used as a template in PCR amplification. The mutant alleles were constructed via splicing-by-overlap-extension-PCR (SOE-PCR) (Silva & Blokesch 2010). Primers with overlapping ends were constructed with Genome Compiler (https://designer.genomecompiler.com/app) to assist the correct orientation and successful binding of the three fragments (upstream fragment, FRT-*cat*-FRT and downstream fragment). In brief, approximately 1-kb regions of DNA upstream and downstream of and, including part of the target gene cassettes (VCH3-5, VCH36-38 and VCH61-63) were amplified using primers listed in Table 3.2. PCR was performed using Q5® Hot-Start Hi-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) which provides high fidelity amplification

of the template with a low error rate. PCR reactions were set up using 12.5 µl 2x Q5 reaction buffer, 1.25 µl each of forward and reverse primers (10 µM), 1.25 µl of template DNA and molecular biology grade water (MBW) to a final volume of 25 µl. The PCR cycling conditions were as follows: an initial denaturation of 98 °C for 30 s followed by 30 cycles of 98 °C for 30 s, an appropriate annealing temperature for 30 s, an appropriate extension time of 20 s/kb at 72°C, and a final extension time of 2 min at 72 °C (See Table 3.2 for specific annealing temperature and extension times for each primer pairs). The PCR constructs were then gel purified.

One µl of each upstream and downstream fragment was mixed with 2 µl of *cat* fragment, 25 µl Q5® Hot Start High-Fidelity Master Mix (New England Biolabs, Ipswich, MA), and MBW to a final volume of 45 µl for SOE-PCR. Then two step SOE-PCR was conducted, where the first round anneals the three fragments together without the addition of primers at 50 °C for 2.5 h and in the second round, upstream forward and downstream reverse primers were added to facilitate the amplification of the fused fragments. The second round PCR cycling conditions were as follows: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, an appropriate annealing temperature for 30 s and an appropriate extension time of 20 s/kb at 72 °C, and a final extension time of 2 min at 72 °C (See Table 3.2 for specific annealing temperature and extension times for each primer pairs). The fused PCR constructs were then gel purified and 500 ng was chitin-transformed into *V. cholerae* A1552 (as described in chapter 2, section 2.2.4). Following chitin transformation, transformants were selected on selective LB media plates containing chloramphenicol and incubated overnight at 37 °C. To confirm that the transformants carried the mutant allele, PCR was carried out using primers (VCH3-5_Up-F/ VCH3-5_Down-R, VCH36-38_Up-F/ VCH36-38_Down-R and VCH61-63_Up-F/ VCH61-63_Down-R) (Table 3.2) that is expected to give a smaller sized fragment compared to the wild type due to the deletion of target region. The FRT-*cat*-FRT cassettes was excised from the ΔVCH3-5::*cat*, ΔVCH36-38::*cat*, ΔVCH61-63::*cat* via FLP-mediated recombination according to the method described in chapter 2, section 2.2.6.

Table 3.1: Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and phenotype	Source
***E. coli* strains**		
DH5α-λ pir	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r$_K^-$, m$_K^+$) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
S-17-1 λ pir	TpR Sm^R *recA*, *thi*, pro, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir; Kan^R, Tet^R	(Val et al. 2008)
***V. cholerae* strains**		
O1 A1552S	Wild type *V. cholerae* O1 EI Tor A1552, smooth variant	(Yildiz & Schoolnik 1999)
pKC⁺_*attI*	*V. cholerae* O1 EI Tor A1552::insertion of pKC01⁺ at *attI* site of integron platform, Kan^R	This study
pKC⁺_VCH35	*V. cholerae* O1 EI Tor A1552::insertion of pKC01⁺ between VCH 35 and VCH 36, Kan^R	This study
pKC⁻_VCH60	*V. cholerae* O1 EI Tor A1552::insertion of pKC01⁻ between VCH 60 and VCH 61, Kan^R	This study
O1 A1552SΔVCH3-5	*V. cholerae* O1 EI Tor A1552 carrying deletion of gene cassettes (VCH3-5), Cm^R	This study
O1 A1552SΔVCH36-38	*V. cholerae* O1 EI Tor A1552 carrying deletion of gene cassettes (VCH36-38), Cm^R	This study
O1 A1552SΔVCH61-63	*V. cholerae* O1 EI Tor A1552 carrying deletion of gene cassettes (VCH61-63), Cm^R	This study
Plasmids		
p*vpvABC*(R)	pACYC177::VC2456 operon from rugose, includes VC2454, VC2455 and VC2456, Ap^R	(Beyhan et al. 2007)
pACYC177	Multicopy number cloning vector, Ap^R	New England Biolabs
pSU-pBAD	Arabinose inducible expression vector, Cm^R	Laboratory collection
pSU-pBAD::VCH3-5	Arabinose inducible expression vector pSU-pBAD carrying VCH3-5, Cm^R	This study
pSU-pBAD::VCH36-38	Arabinose inducible expression vector pSU-pBAD carrying VCH36-38, Cm^R	This study
pSU-pBAD::VCH61-63	Arabinose inducible expression vector pSU-pBAD carrying VCH61-63, Cm^R	This study

Kan^R, Kanamycin resistant; Cm^R, Chloramphenicol resistant; Ap^R, Ampicillin resistant; Sm^R, Streptomycin resistant, Tet^R, Tetracylcine resistant

Table 3.2: Primers used in this study

Primer	Sequence (5`-3`)	Target cassette	Annealing temperature (°C)	Extension time (min)	Reference
VCH3-5-Up-F	AACACCGCTTGCACCTCTAT	Used with VCH3-5-Up-R to amplify the VCH3-5 upstream region	62	1.00	This study
VCH3-5-Up-R	CTCCAGCCTACACGGAAAAATGAAAGCGGAACC	Used with VCH3-5-Up-F to amplify the VCH3-5 upstream region			
Cat-VCH3-5-F	GCTTTCATTTTTCCGTGTAGGCTGGAGCTGCTTC	Used with Cat-VCH3-5-R to amplify the chloramphenicol resistance gene	62	1.00	This study
Cat-VCH3-5-R	AGGGGTGAACAACGCATATGAATATCCTCCTTAG	Used with Cat-VCH3-5-F to amplify the chloramphenicol resistance gene			
VCH3-5-Down-F	GAGGATATTCATATGCGTTGTTCACCCCTTAATGC	Used with VCH3-5-Down-R to amplify the VCH3-5 downstream region	63	1.00	This study
VCH3-5-Down-R	TCGGACGATTTATGTTGTGT	Used with VCH3-5- Down -F to amplify the VCH3-5 downstream region			
VCH36-38-Up-F	GTTCACGAATCAGCCTTTGC	Used with VCH36-38-Up-R to amplify the VCH36-38 upstream region	64	1.00	This study
VCH36-38-Up-R	GCTCCAGCCTACACATGAAACATAAAGCGCTGCA	Used with VCH36-38-Up-F to amplify the VCH36-38 upstream region			
Cat-VCH36-38-F	GCTTTATGTTTCATGTGTAGGCTGGAGCTGCTTC	Used with Cat-VCH36-38-R to amplify the chloramphenicol resistance gene	62	1.00	This study
Cat-VCH36-38-R	GGCAGACAACGCTACCATATGAATATCCTCCTTAG	Used with Cat-VCH36-38-F to amplify the chloramphenicol resistance gene			

VCH36-38-Down-F	<u>GAGGATATTCATATGG</u>TAGCGTTGTCTGCCCCTTA	Used with VCH36-38-Down-R to amplify the VCH36-38 downstream region	63	1.00	This study
VCH36-38-Down-R	GCCCATAAGTAAAGGCACCA	Used with VCH36-38- Down -F to amplify VCH36-38 downstream region			
pSU-pBAD_F	AGTCTAGACAGCGCTTTTCC	Used with pSU-pBAD_R to amplify the backbone of vector pSU-pBAD	58	2.30	This study
pSU-pBAD_R	GTTTCACTCCATCCAAAAAAAC	Used with pSU-pBAD_F to amplify the backbone of vector pSU-pBAD			
Cloning_VCH3-5_F	<u>TTTTGGATGGAGTGAAAC</u>GTTAGGCTAATCAGGGAGATA	Used with Cloning_VCH3-5_R to amplify the VCH3-5 region	54	1.00	This study
Cloning_VCH3-5_R	<u>AAAAGCGCTGTCTAGACT</u>CCAGACCTAACACTTTCATT	Used with Cloning_VCH3-5_F to amplify the VCH3-5 region			
Cloning_VCH36-38_F	<u>TTTTGGATGGAGTGAAAC</u>AAAGAGTGGATGCCACTAATTGTA	Used with Cloning_VCH36-38_R to amplify the VCH36-38 region	56	1.00	This study
Cloning_VCH36-38_R	<u>AAAAGCGCTGTCTAGACT</u>GCACCAATTTGTTCTTGATACC	Used with Cloning_VCH36-38_F to amplify the VCH36-38 region			
Cloning_VCH61-63_F	<u>TTTTGGATGGAGTGAAAC</u>GGGGTTCACATCCATGCTCTTAT	Used with Cloning_VCH61-63_R to amplify the VCH61-63 region	55	1.00	This study
Cloning_VCH61-63_R	<u>AAAAGCGCTGTCTAGACT</u>TTGAAAATCACAATTCGAGTACA	Used with Cloning_VCH61-63_F to amplify the VCH61-63 region			

3.2.3 Complementation of *V. cholerae* gene cassette deletion mutants and gene cassette overexpression in the wild-type

To clone the deleted gene cassettes (VCH3-5, VCH36-38 and VCH61-63) for complementation studies, the arabinose inducible expression vector, pSU-pBAD, was used. Primers (Table 3.2) were designed such that vector and inserts would contain overlaps for cloning following the method described in chapter 2, section 2.2.7. The PCR cycling conditions were as follows: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, an appropriate annealing temperature for 30 s and an appropriate extension time of 20 s/kb at 72 °C, and a final extension time of 5 min at 72 °C (See Table 3.2 for specific annealing temperature and extension times for each primer pairs). The PCR products were gel purified separately using the ISOLATE II PCR and Gel Kit (Bioline, England) and the fragments assembled using the Gibson master mix (New England Biolabs, Ipswich, MA) following the manufacturer's instructions and transformed into *E. coli* S17-1λ pir on LB5 containing chloramphenicol and 1% glucose (to repress the induction of arabinose promoter) to obtain the plasmids pSU-pBAD::VCH3-5, pSU-pBAD::VCH36-38 and pSU-pBAD::VCH61-63. These plasmids were propagated overnight in LB5 broth supplemented with chloramphenicol and 1 % glucose. The extracted plasmids were confirmed by PCR to contain the gene cassettes VCH3-5, VCH36-38 and VCH61-63 using primers pSU-pBAD_F /cloning VCH3-5_R and pSU-pBAD_F /cloning VCH36-38_R and pSU-pBAD_F/cloning VCH61-63_R, respectively (Table 3.2). Complementation of plasmids (pSU-pBAD::VCH3-5, pSU-pBAD::VCH36-38 and pSU-pBAD::VCH61-63) into *V. cholerae* ΔVCH3-5, *V. cholerae* ΔVCH36-38 and *V. cholerae* ΔVCH61-63 were performed using the chitin transformation method and then plating on selective LB5 agar.

3.2.4 Gene cassettes bioinformatics

The deleted gene cassettes (VCH3-5, VCH36-38, VCH61-63) sequences were subjected to BlastX (https://www.ncbi.nlm.nih.gov/) to identify the putative conserved domains in the proteins encoded by these gene cassettes (Kojima & Jurka 2011). The most precise bacterial protein localization prediction tool, PSORTb (version 3.0) (Yu et al. 2010) was used to predict the subcellular localization of gene cassettes products, where cut-off score was 7.5 and below 7.5 score was considered as "unknown" localization. Functional annotation of the gene cassettes products was determined by using the Universal Protein Resource (Consortium 2014).

3.2.5 Colony morphology and growth curve assays

V. cholerae strains stored at -80 °C were streaked onto LB5 agar to obtain isolated colonies. A single colony was then inoculated into liquid LB5 and incubated for 24 h overnight at 37 °C with shaking at 200 rpm before being serially diluted and an appropriate dilution of each culture plated onto LB5 agar to obtain well isolated single colonies. These colonies were photographed using a Nikon ECLIPSE Ti stereomicroscope at a magnification of 10X using a DS-Qi2 camera and images were collected using Image Analysis software (NIS elements viewer, Nikon). Growth curves of *V. cholerae* A1552 wild type and mutant strains were carried out in 24 well microtitre plates (Nunclon Delta surface, Thermo Fisher Scientific, Denmark) containing 1 mL of LB5 medium per well. The inoculum was prepared from overnight cultures grown in LB5 broth and then diluted to OD_{600} of 1.0 using 0.5 % NaCl. Growth curve cultures were inoculated at 1:100 and growth measured using a microplate reader (Spark microplate reader, TECAN, Switzerland) at OD_{600}.

3.2.6 Crystal-violet biofilm assay

V. cholerae strains were grown overnight in LB5 medium and then diluted (1:100) in fresh LB5 medium before 500 µl aliquots were added to triplicate wells of a plastic 24-well microtitre plate (Nunclon Delta surface, Thermo Fisher Scientific, Denmark). Plates were incubated for 24 h at 37 °C with shaking at 200 rpm to allow biofilm formation. Following incubation, unattached planktonic cells were carefully aspirated and the wells washed twice with 2 % (w/v) NaCl solution. The washed wells were stained for 15 min through the addition of 500 µl 0.2 % (w/v) crystal violet and then washed three times with PBS (phosphate buffered saline, pH 7.4). To measure the biomass of adhered cells, the crystal violet was solubilised with 30 % (v/v) acetic acid and transferred to a clean microtitre plate for OD_{600} measurement using a microplate reader (Spark microplate reader, TECAN, Switzerland). Each assay was repeated at least three times.

3.2.7 Congo red binding assays

Congo red is a dye which interacts with a range of polysaccharides through hydrogen bonding between hydroxyl groups of polysaccharides and amino groups of the dye (Puchtler & Sweat 1965) and was used in a solid and liquid medium to assess the ability of bacterial cells to bind to the stain. Any changes in the sugar component of polysaccharide can modify Congo red binding which can be detected by visualizing the colony morphology on solid medium and changes on optical density (OD) of the supernatant in liquid medium.

Changes in colony morphology were assessed by streaking *V. cholerae* strains on LB5 plates or marine minimal medium (2M) (Rapa et al. 2013) with 0.2 % (w/v) glucose plates and, 0.001 % (w/v) Congo red (Sigma) and incubated for 7 d at room temperature. Colonies were observed using a Nikon ECLIPSE Ti stereomicroscope at a magnification of 10X using a DS-Qi2 camera and images were collected using Image Analysis software (NIS elements viewer, Nikon). The Congo red liquid binding assay was conducted following a published method (Colvin et al. 2011). Briefly, 30 µl of overnight cultures of the strains were added into triplicate tubes containing 3 ml of LB5 and 0.04 % (w/v) sterile Congo red (Sigma) in 15 ml falcon tubes in triplicate and incubated for 24 h at 37 °C with shaking at 200 rpm. After 24 h of incubation, cells were pelleted and 100 µl of supernatants were taken to clean microtitre plates for measuring OD_{495} using a Spark microplate reader (Spark microplate reader, TECAN, Switzerland). A lower optical density in the supernatant indicates more binding of Congo red to the cells. Each assay was repeated at least three different times.

3.2.8 Conversion of *V. cholerae* to rugose morphology

Conversion of *V. cholerae* strains to rugose morphology was done according to a method described previously (Beyhan et al. 2007). The strains harbouring pKC01[+] and pKC01[-] and the mutant strains were subjected to a switch to rugose morphology through chitin transformation with the purified expression vector p*vpvABC*(R) and control vector pACYC177 (Beyhan et al. 2007). The plasmid p*vpvABC*(R) contains the *vpvC* allele cloned from a rugose genetic background which contains a single nucleotide change in *vpvC* resulting in increased c-di-GMP and VPS synthesis (Beyhan et al. 2007). Isolation and selection of the colonies were performed on LB5 agar plates containing kanamycin (50 µg ml^{-1}) and ampicillin (100 µg ml^{-1}). After 24 h of incubation, the colonies were photographed using Nikon ECLIPSE Ti stereomicroscope DS-Qi2 camera (NIS elements viewer, Nikon).

3.2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com). Two-tailed student's t-tests were used to compare means between experimental samples and controls. For experiments with multiple samples, one-way ANOVAs were used with Tukey's or Dunnett's Multiple Comparison Test post-hoc comparisons of means.

3.3 Results

3.3.1 Insertion of the artificial pKC01 gene cassette and gene cassette deletions alter biofilm formation in *V. cholerae*

Strains (chitin transformants) with insertion of pKC01$^+$ at *attI* (pKC$^+$_*attI*), between VCH35 and VCH36 (pKC$^+$_VCH35), and insertion of pKC01$^-$ between VCH60 and VCH61 (pKC$^-$_VCH60) were subjected to a biofilm assay. The results showed that the selected chitin transformants carrying pKC01$^+$ and pKC01$^-$ produced significantly higher (p<0.001, p<0.0001) biomass (>2 fold) (Figure 3.1A). To determine if this was due to a polar effect on genes downstream of the insertion or the artificial gene cassette (pKC01) containing a promoter enhancing transcription of downstream gene cassettes, the 3 gene cassettes immediately downstream of the insertion were first deleted in the wild-type (ΔVCH3-5, ΔVCH36-38 and ΔVCH61-63) and tested in the biofilm assay. Except for ΔVCH61-63, the deletion strains yielded lower biomass relative to the wild-type (Figure 3.1A) indicating one or more of the VCH3-5 and VCH36-38 gene cassettes have a role in biofilm formation. To determine where the biofilm results were not due to a growth defect, all cassette integrants and deletion mutants were subject to growth assays with no defects observed (Supplementary Figure S3.1). Complementation of ΔVCH3-5 and ΔVCH36-38 restored the biofilm phenotype to wild-type levels with no significant (p<0.05) difference in biofilm formation observed with the wild type and mutant strains carrying empty vector (Figure 3.1A). Overexpression of the deleted gene cassettes (VCH3-5, VCH36-38 and VCH61-63) in the wild type background all resulted in significantly (p<0.001) increased biofilm formation compared to the wild-type and the wild-type containing the empty vector (Figure 3.1A). These data indicate that pKC01 contains an internal promoter(s) and that insertion is upregulating transcription of downstream gene cassettes that increase biofilm formation. In the case of VCH61-63, deletion did not decrease biofilm formation indicating that transcription is low in the wild-type and so the biofilm phenotype is only observed when transcription is artificially increased.

Biofilm formation in *V. cholerae* is enhanced when it undergoes a shift to rugosity leading to increased VPS production. To determine if the results observed in the smooth variant were consistent with a rugose variant background, all strains were subjected to rugose phenotype conversion by transforming p*vpvABC*(R). All strains harbouring the plasmid p*vpvABC*(R) switched to a rugose phenotype compared to the colonies harbouring the control pACYC177 vector (Figure 3.1C). In the crystal violet biofilm assay, all rugose strains containing the

inserted pKC01 cassette (pKC$^+$_*attI*_R, pKC$^+$_VCH35_R, and pKC$^-$_VCH60_R) produced >3 fold higher biofilm compared to the smooth isogenic strains (pKC$^+$_*attI,* pKC$^+$_VCH35, and pKC$^-$_VCH60). Compared to the rugose wild type strain (A1552_R), the strains pKC$^+$_*attI*_R, pKC$^+$_VCH35_R, and pKC$^-$_VCH60_R produced 4-5 fold more biofilm (Figure 3.1B). The rugose deletion strains (ΔVCH3-5_R, ΔVCH36-38_R, ΔVCH61-63_R,) produced lower biofilm compared to the isogenic rugose versions (Figure 3.1B) although similar to the smooth strains, the ΔVCH61-63 result was not as pronounced. Complementation for all deletion strains resulted in biofilm formation at wild type rugose levels (Figure 3.1B). Overexpression of the deleted gene cassettes (VCH3-5, VCH36-38 and VCH61-63) in the wild type rugose strain resulted in substantially more biofilm formation compared to the wild type rugose strain and wild-type containing the empty vector (Figure 3.1B).

C.
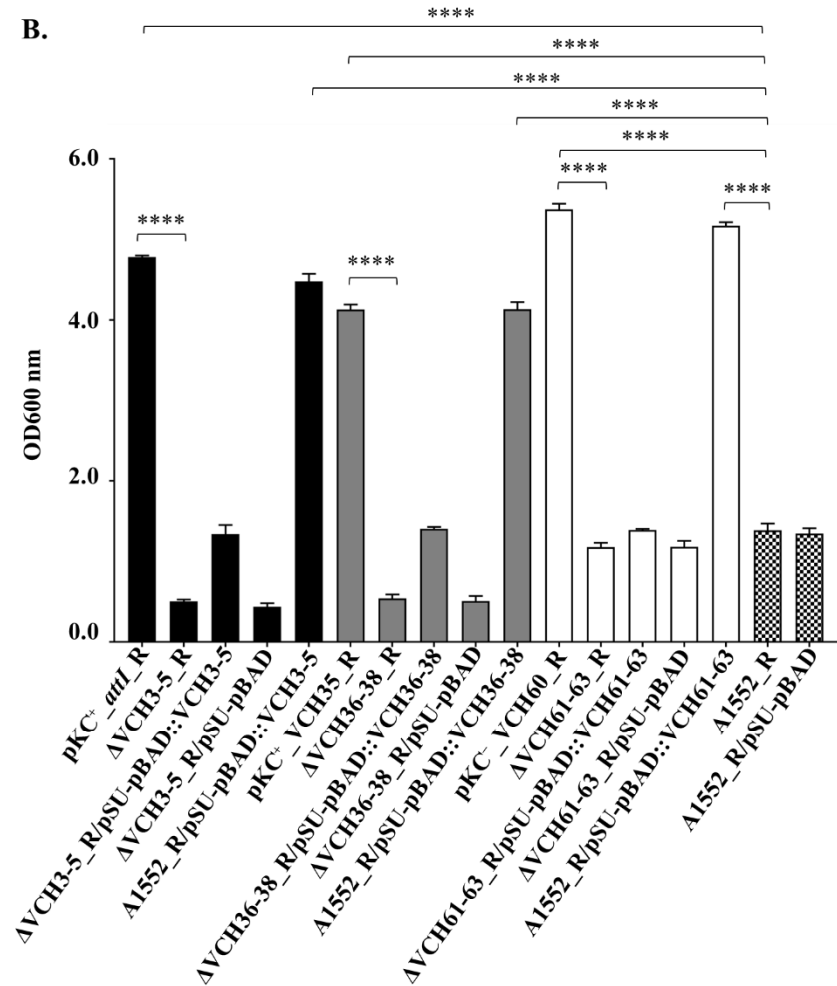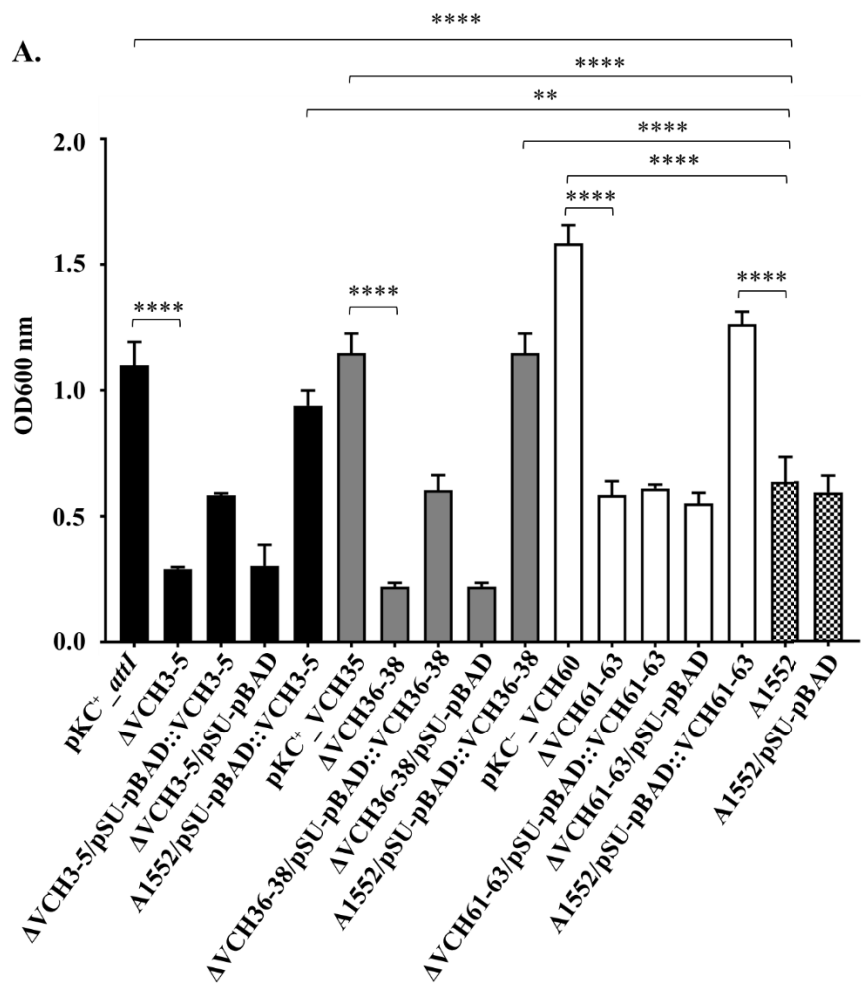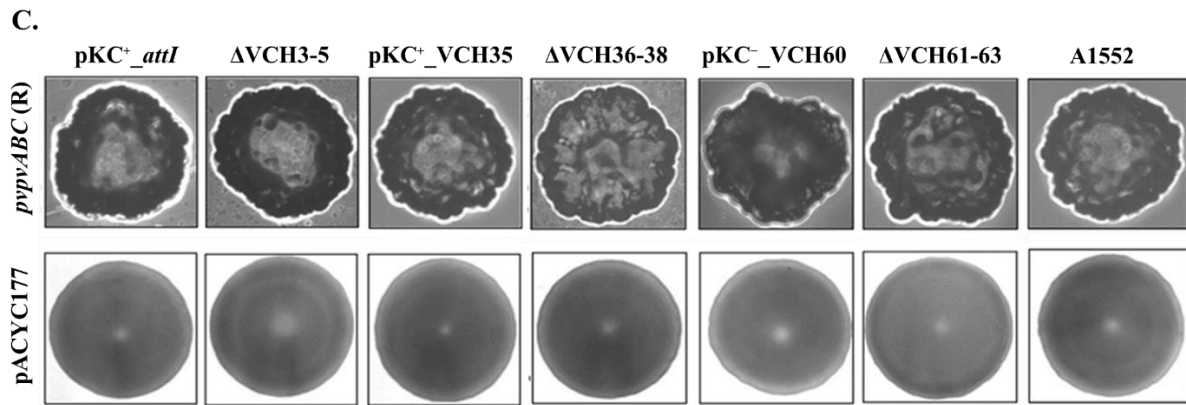
Figure 3.1: Biofilm formation of *V. cholerae* strains. (A). Strains with insertion at *attI* and downstream from VCH35 and VCH60, show increased biofilm formation. Except for ΔVCH61-63, deletion of cassettes downstream of the insertion points showed decreased biofilm formation. Complementation of ΔVCH3-5 and ΔVCH36-38 resulted in restoration of biofilm formation to wild type levels and enhanced biofilm formation when overexpressed in the wild type background. (B). Rugose strains with insertion at *attI* and downstream from VCH35 and VCH60, show increased biofilm formation. Gene cassette deletion strains in the rugose background showed decreased biofilm formation. Complementation of ΔVCH3-5_R, ΔVCH36-38_R and ΔVCH61-63_R resulted in restoration of biofilm formation to wild type rugose levels and enhanced biofilm formation when overexpressed in the wild type strain. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA, Tukey's multiple comparisons test (**$p<0.01$, ****$p<0.0001$). (C). Colony morphologies of strains carrying pKC01+, pKC01-, gene cassette deletion strains and wild type strain harbouring p*vpvABC*(R) and control vector pACYC177. All colonies were grown for 24 h on LB5 agar plates at 30 °C.

3.3.2 Insertion of the artificial pKC01 gene cassette and gene cassette deletion in *V. cholerae* alters binding of the polysaccharide stain Congo red

To determine whether insertion of pKC01+/pKC01- or deletion of cassettes modifies surface polysaccharide, colony morphology and Congo red binding assays were conducted. In the Congo red liquid binding assay, all strains showed increased binding affinity to Congo red (indicated by a lower OD495 nm of Congo red in the supernatant) compared to the A1552 wild-type indicating a change to surface polysaccharide properties (Figure 3.2A). Following complementation of ΔVCH3-5, ΔVCH36-38 and ΔVCH61-63, all the mutant strains restored their Congo red binding phenotype in liquid medium to the wild type level (Figure 3.2A). Interestingly, when VCH3-5, VCH36-38 and VCH61-63 were overexpressed in the wild type, binding to Congo red increased compared to the wild type and wild type carrying the empty vector (Figure 3.2A).

In the rugose background, all strains carrying pKC01+ and pKC01- had decreased binding to Congo red compared to the isogenic smooth strains (Figure 3.2A and 3.2B). Compared to the rugose wild type strain (A1552_R), all strains carrying pKC01+ and pKC01- in the rugose

background showed decreased binding to Congo red except the strain pKC_VCH35_R, which showed similar Congo red binding as the wild type rugose strain (Figure 3.2B). The deletion mutants in the rugose background (ΔVCH3-5_R, ΔVCH36-38_R and ΔVCH61-63_R) showed decreased Congo red binding compared to the isogenic smooth versions (Figure 3.2A and 3.2B). Compared to the rugose wild-type, ΔVCH3-5_R showed decreased Congo red binding whereas the other rugose deletion mutants showed increased binding. Following complementation of ΔVCH3-5_R, ΔVCH36-38_R, ΔVCH61-63_R, it was observed that all the mutant strains with rugose version restored their Congo red binding phenotype to wild type rugose level (Figure 3.2B). Overexpression of gene cassettes VCH3-5 and VCH61-63 in wild type rugose strain resulted in decreased Congo red binding, whereas VCH36-38 overexpression showed no difference compared to rugose wild type and rugose wild type carrying the empty vector (Figure 3.2B).
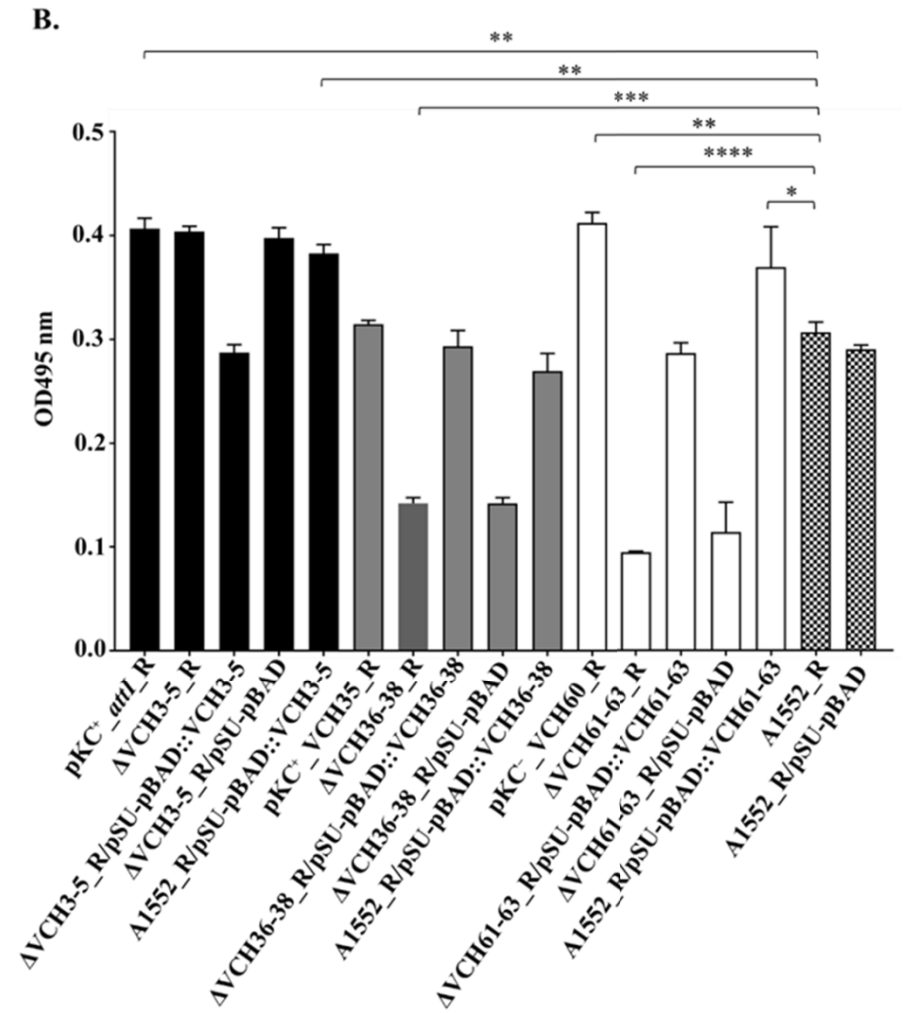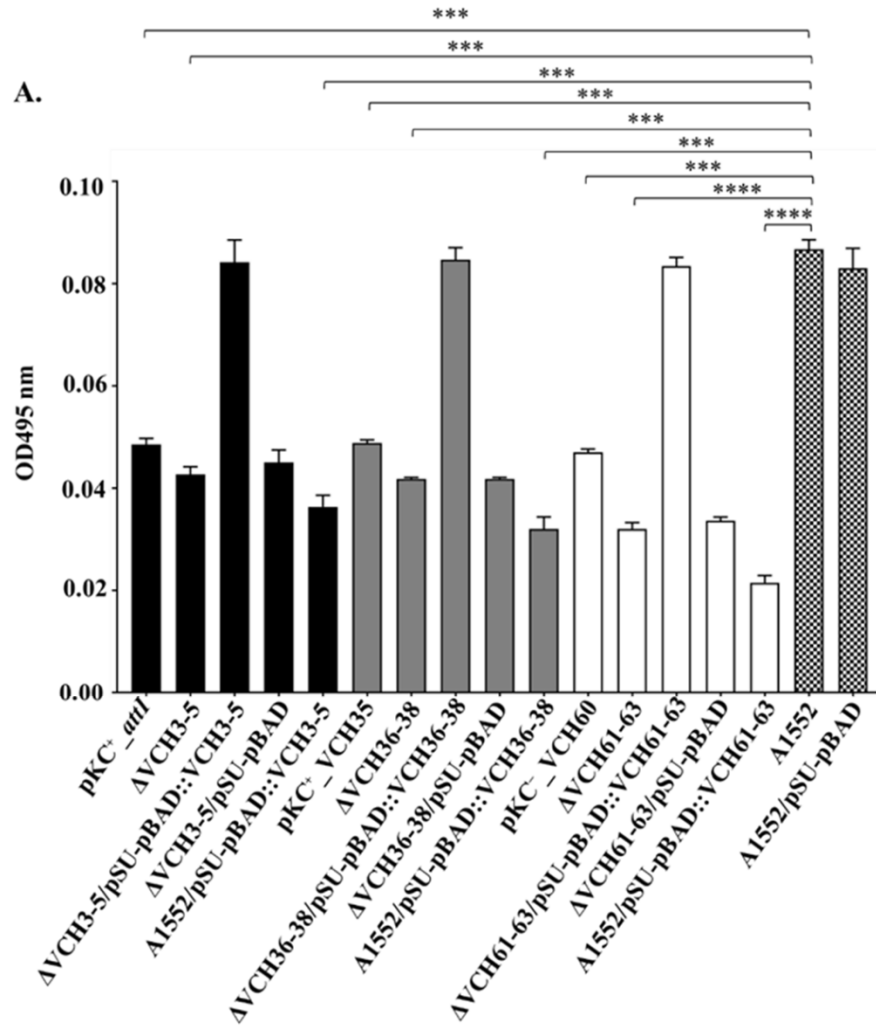
Figure 3.2: Congo red liquid binding of *V. cholerae* strains. Increased binding was observed for all smooth strains with pKCO1 inserted in *attI*, VCH35 and VCH60 or when cassettes VCH3-5, VCH36-38 and VCH61-63 were deleted compared to the wild type. When complemented, all deletion mutants had levels of Congo red binding restored to wild type. Overexpression of cassettes VCH3-5 and VCH61-63 in the wild type background enhanced Congo red binding whereas, no different was observed with overexpression of VCH36-38 (A). Congo red liquid binding showed decreased binding for all rugose strains with pKCO1 inserted in *attI*, VCH35 and VCH60 or when cassettes VCH36-38 and VCH61-63 were deleted compared to the rugose wild-type. ΔVCH3-5 showed decreased binding compared to the rugose wild-type. When complemented, all deletion mutants had their Congo red binding restored to rugose wild-type levels. Overexpression of VCH3-5 and VCH61-63 in the rugose wild-type background reduces Congo red binding whereas, no different was observed when VCH36-38 was over-expressed (B). The lower the optical density indicates higher the binding of Congo red to the cell surface and less in the supernatant. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA, Tukey's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3.3.3 Insertion of artificial pKC01 gene cassette and gene cassette deletion in *V. cholerae* does not alter colony morphology

Due to the changes observed in Congo red binding in the pKC01[+] and pKC01[-] insertion and, deletion strains and the impact that changes to polysaccharide can have on colony morphology, the colony morphology of all strains was inspected on LB5. No obvious changes were observed (Figure 3.3A). To help identify finer changes in colony morphology, Congo red was added to LB5 and 2M + 0.2% glucose agar with no obvious modified colony morphologies on both media (Figure 3.3B).
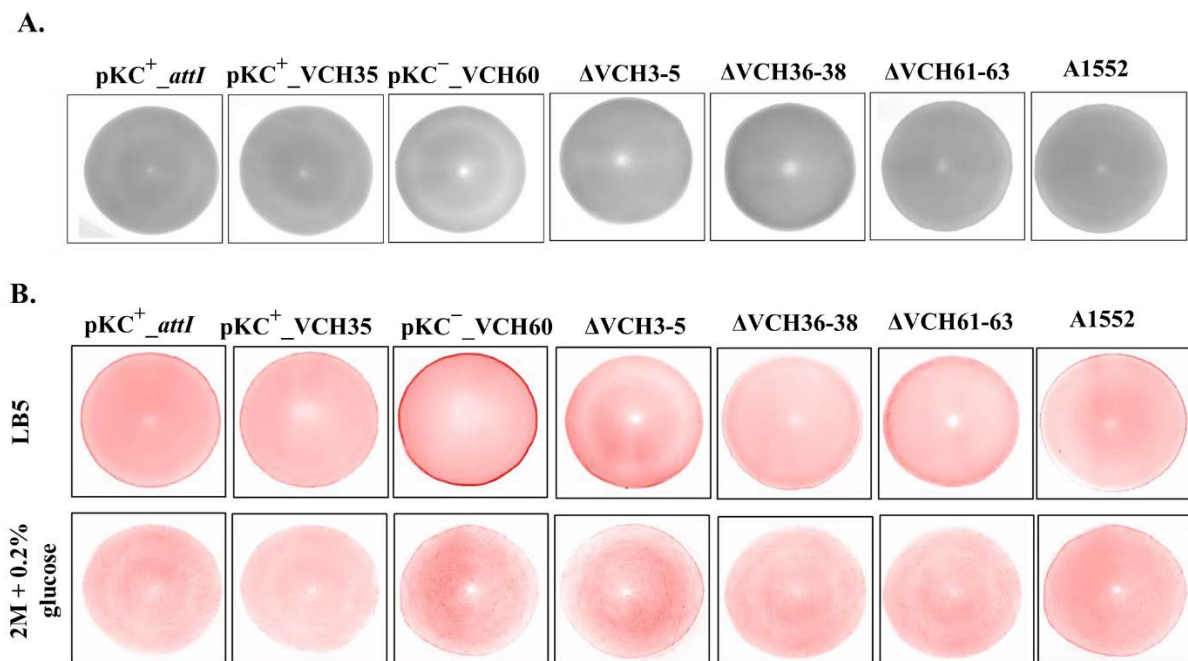
Figure 3.3: Colony morphology of strains carrying pKC01[+] and pKC01[-] , deletion strains and *V. cholerae* A1552 WT grown on LB5 agar plates for 24 h at 37 °C (A) and, on LB5 and 2M + 0.2% glucose supplemented with 0.001% Congo red for 72 h at 37 °C (B).

3.3.4 Bioinformatic analysis of deleted gene cassettes

The blastX analysis did not identify known putative conserved domains in the VCH3-5, VCH36, VCH38, VCH62 and VCH63 gene cassette encoded products however, the VCH37 encoded products showed homology to a CopG family transcriptional regulator and type II toxin-antitoxin system conserved domain and, the VCH61encoded product showed homology to a ribbon-helix-helix (RHH) transcriptional regulator conserved domain (Table 3.3; Appendix 3.1).

The bacterial protein localization tool PSORTb predicted localization of cassette encoded VCH3, VCH36, VCH62 and VCH63 as being located in the cytoplasmic membrane, cytoplasm, cytoplasm and cytoplasmic membrane, respectively (Table 3.3; Appendix 3.2). Functional analysis of the encoded proteins using UniProt showed that VCH3, VCH37 and VCH61 encodes proteins as being transmembrane, a transcriptional regulator and type II toxin-antitoxin system and, RHH transcriptional regulator respectively (Table 3.3).

Table 3.3: Putative conserved domains, sub-cellular localization and functional annotation of deleted gene cassettes products

Gene cassettes	Putative conserved domains	Sub-cellular localization	Functional annotation
VCH3	No putative conserved domain	Cytoplasmic membrane	Transmembrane, an integral part of cell membrane
VCH4	No putative conserved domain	Unknown	Unknown
VCH5	No putative conserved domain	Unknown	Unknown
VCH36	No putative conserved domain	Cytoplasmic	Unknown
VCH37	CopG family transcriptional regulator & Ribonuclease toxin, BrnT, of type II toxin-antitoxin system	Unknown	CopG transcriptional regulator; Type II toxin-antitoxin system
VCH38	No putative conserved domain	Unknown	Unknown
VCH61	Transcriptional regulator, contains Arc/MetJ-type RHH (ribbon-helix-helix) DNA-binding domain	Unknown	Transcriptional regulator
VCH62	No putative conserved domain	Cytoplasmic	Unknown
VCH63	No putative conserved domain	Cytoplasmic membrane	Unknown

3.4 Discussion

Prior studies investigating the role of gene cassettes in *Vibrio* physiology have suggested that gene cassettes products affects surface polysaccharide and biofilm formation (Rapa & Labbate 2013; Rapa et al. 2013). In this study, investigation was carried out to determine whether insertion of artificial gene cassettes into the cassette array affected biofilm formation. Integration of pKC01$^+$ and pKC01$^-$ into different positions of the cassette array (*attI*, VCH35 *attC* and VCH60 *attC*) increased biofilm formation compared to their parental *V. cholerae* O1 strain A1552 in both the smooth and rugose backgrounds in the absence of a growth defect. To explore the reason for this phenotype, it was hypothesised that insertion of the artificial gene cassettes impacted transcription of downstream gene cassettes by either introducing a polar effect similar to that introduced by transposons (Calos & Miller 1980; Hutchison et al. 2019; van Opijnen, Bodi & Camilli 2009) or, increasing transcription due to the read-through of a promoter in the artificial gene cassettes (Hutchison et al. 2019). To address the first possibility, the three downstream gene cassettes from the insertion point of the artificial gene cassette were deleted producing deletion mutants ΔVCH3-5, ΔVCH36-38, ΔVCH61-63 and subjected them to the crystal violet biofilm assay. For ΔVCH3-5, ΔVCH36-38, a decreased biofilm in the absence of a growth defect was observed indicating that the increased biofilm formation in the pKC$^+$_*attI* and pKC$^+$_VCH35 integrants is likely due to increased transcription of gene cassettes downstream from the pKC01 insertion point. For ΔVCH61-63, no change in biofilm formation was observed in the smooth background although a modest decrease was observed in the rugose background. Interestingly, overexpression of VCH3-5, VCH36-38 and VCH61-63 in the smooth and rugose wild-type backgrounds substantially increased biofilm formation. These data combined indicate that VCH3-5 and VCH36-38 are well expressed in the wild-type backgrounds whereas, VCH61-63 is not well expressed explaining why their deletion did not affect biofilm formation. That overexpression of all gene cassette groups increased biofilm formation in both the smooth and rugose backgrounds indicates that pKC01 contains a promoter(s) that is driving transcription of downstream gene cassettes. There is likely more than one promoter given that in pKC$^-$_VCH60, the artificial gene cassette is inserted in the opposing orientation to pKC$^+$_*attI* and pKC$^+$_VCH35. Due to time constraints, it was not possible to investigate increased transcription of cassettes downstream of pKC01 insertion using reverse transcription qPCR (RT-qPCR) but recommend this as a future experiment.

To try and explain how pKC01 insertion and deletion of downstream, gene cassettes affected *V. cholerae* biofilm formation, it was addressed whether there was a change in bacterial surface

polysaccharide. Whilst no major difference in colony morphology was observed between the wild-type and the other strains in the smooth background, the pKC01$^+$ and pKC01$^-$ integrants and gene cassette deletion mutants all showed increased binding to Congo red. In the rugose background, the results were different than in the smooth background. For all integrants and deletion strains, Congo red binding increased in the smooth background relative to the smooth wild-type however, for the pKC$^+$_attI, ΔVCH3-5 and pKC-VCH60 Congo red binding decreased in the rugose background relative to the rugose wild-type. For pKC$^+$_VCH35, Congo red binding was unchanged and increased in the ΔVCH36-38 and ΔVCH61-63 strains in the rugose background relative to the wild-type. Complementation of all mutants restored phenotypes to isogenic wild-type levels. Additionally, there was no obvious correlation between the Congo red assay and biofilm formation. For example, Congo red binding was the same in the pKC$^+$_attI and ΔVCH3-5 in the smooth and rough backgrounds whereas, biofilm formation was lower in the ΔVCH3-5 for both backgrounds. This data is hard to explain but it should be pointed out that the Congo red binding is a crude assay and that Congo red is capable of binding to protein as well as polysaccharide which could explain the variation between the smooth and rugose backgrounds (Reichhardt, Jacobson, et al. 2015). Conversion from smooth to rugose does more than just increase VPS production (Beyhan & Yildiz 2007) such as downregulation of flagellar production (Yildiz et al. 2004). Additionally, structural changes to the bacterial cell surface may compromise Congo red binding. Nevertheless, it's fair to say that the integrants and deletion mutants have modified surface properties in both the smooth and rugose backgrounds that could be affecting biofilm formation. Another enquiry of interest would be investigating the amount of VPS being produced in the cassette integrants and deletion mutants.

How gene cassette products are affecting biofilm formation in this study remains unclear. Increased VPS production and biofilm formation in *V. cholerae* is controlled by a complex integrated regulatory network including transcriptional activators, repressors, alternative sigma factors, a set of small regulatory RNAs and small nucleotide signalling molecules (Teschler et al. 2015). Therefore, there are two possibilities that could explain how gene cassette products are modifying surface properties and biofilm formation. First, it's possible gene cassette products are affecting the regulation of VPS. Secondly, gene cassette proteins could be enzymatically modifying VPS or other surface properties changing biofilm formation. With support of the former possibility, the bioinformatic analysis of deleted gene cassettes showed that VCH37 encodes a possible CopG transcriptional regulator and a ribonuclease toxin of type

II toxin-antitoxin system and VCH61 encodes a product with a RHH (ribbon-helix-helix) DNA binding domain. Prior studies have shown that toxin-antitoxin system (TA) to be involved in biofilm formation and persister cell formation in *E. coli* through controlling the c-di-GMP production (Wang & Wood 2011; Zhang et al. 2020).

In conclusion, multiple gene cassettes were found to affect *V. cholerae* biofilm formation however, to identify the specific gene cassette(s) responsible, single gene cassette deletion or complementation is required. Nevertheless, the results presented in this study have revealed that gene cassettes products contribute to *V. cholerae* biofilm formation and can modify surface properties.

3.5 Supplementary information
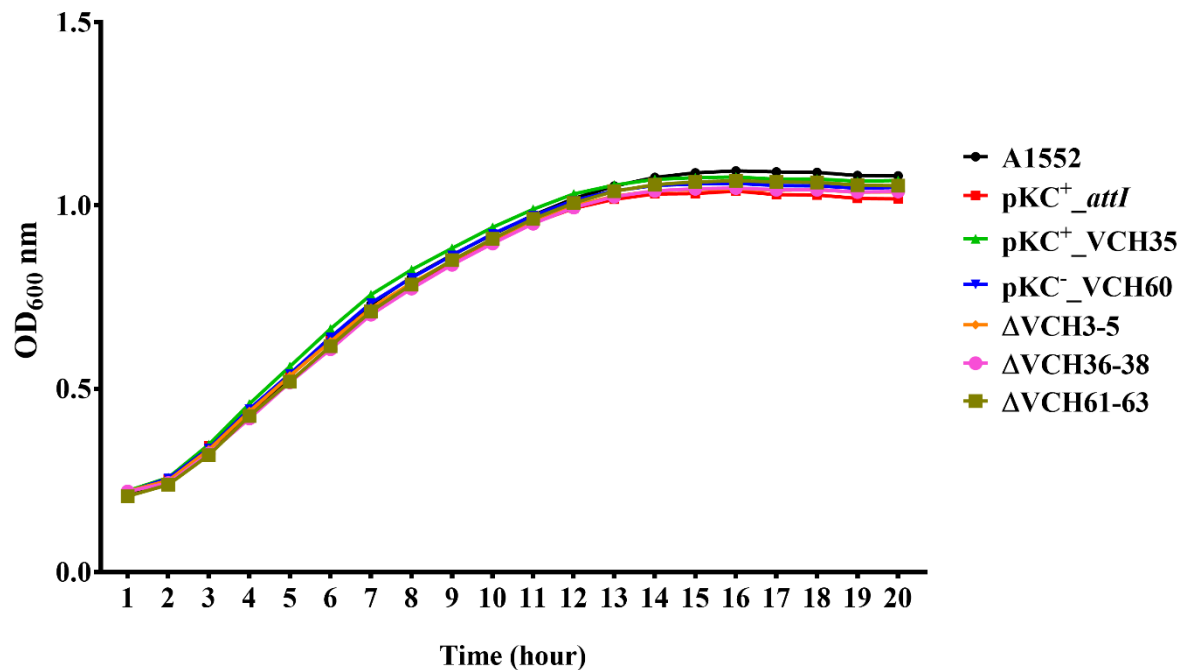
Supplementary Figure S3.1. Growth curve of *V. cholerae* strains. Growth curve of strains carrying pKC01$^+$ and pKC01$^-$, deletion strains and, *V. cholerae* A1552 WT grown in LB broth supplemented with 0.5 % NaCl for 20 h at 37 °C. Y-axis represents the optical density at 600nm (OD$_{600}$ nm) and X-axis represents the time intervals (hour). Error bars represent the standard deviations of three independent experiments.

Chapter 4 Integron-integrase-mediated lateral gene transfer in *V. cholerae* is enhanced in the presence of bacteriovorist protozoa

4.1 Introduction

V. cholerae is a natural inhabitant of coastal, estuarine and brackish water environments (Domman et al. 2017), where it persists through attachment and biofilm formation on abiotic or biotic surfaces including aquatic plants, phytoplankton and zooplankton that act as environmental reservoirs (Matz et al. 2005). As a member of the natural bacterioplankton community, *V. cholerae* is an integral part of the pelagic microbial food web and is constrained in its growth and survival by the predatory action of free-living heterotrophic protists called protozoa (Jürgens & Matz 2002; Sherr & Sherr 2002). The distribution of bacterivorous protozoa are ubiquitous in all ecosystems (Pernthaler 2005). Following uptake by protozoa, bacteria are packaged into the phagosome (food vacuoles) of protozoa that become filled with several toxic components including acidic proteolytic enzymes, antimicrobial peptides (AMPs), acidic pH, reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in digestion of bacterial prey (Elliott & Clemmons 1966; Guerrier et al. 2017; Nusse 2011).

Resistance to predation allowing for survival and replication of bacteria, including *V. cholerae,* within protozoa has been reported. For example, different strains of *V. cholerae* including O1 and O139 strains can survive and multiply within the trophozoites/cysts of *A. castellanii* (Abd et al. 2007). Other reports have also shown that in addition to the multiplication within protozoa, *V. cholerae* can escape into the extracellular environment either as free or encased cells through access into the secretory system of *A. castellanii* (Van der Henst et al. 2016). Similarly, *V. cholerae* can reproduce and survive inside the food vacuoles of *T. pyriformis* with food vacuoles being expelled and acting as a protective environment and a potential vector for disease transmission (Espinoza-Vergara et al. 2019). Several studies have attempted to understand the mechanisms that *V. cholerae* uses to survive and grow inside protozoa and have identified the PrtV protease (Vaitkevicius et al. 2006), chitin-induced production of ammonia (Sun et al. 2015) and the pigment pyomelanin (Noorian et al. 2017) as factors. Despite these studies, there is still a major knowledge gap in the understanding of *V. cholerae* resistance inside the protozoal phagosome.

Within the phagosome, ROS production is considered a key feature of killing causing DNA damage leading to mutagenesis (Baharoglu & Mazel 2011; Colin et al. 2014; Srinivas et al. 2019). Mutagenesis causes stalling of DNA replication generating an excess of single stranded DNA (ssDNA) that initiates the SOS response through the DNA repair protein RecA inactivating the LexA repressor of the SOS regulon (Baharoglu & Mazel 2011; Taddei, Matic & Radman 1995). The SOS response is considered a fundamental mechanism of bacterial adaptation and evolution that can be achieved through genetic changes such as gene mutation, re-arrangement of genes or lateral gene transfer (LGT) (Walker 1996). LGT is one of the most important mechanisms driving bacterial evolution potentially providing a quick and drastic evolutionary "jump" in ecological and pathogenic fitness through acquisition of novel genetic traits (Ochman, Lawrence & Groisman 2000).

Chromosome 2 of *V. cholerae* carries an adaptive genetic machinery called the integron/gene cassette system which, contains more than 150 mobile elements called gene cassettes in an array. Diversity in the cassette array is driven by a recombinase called the integron-integrase driving integration of new gene cassettes or, deletion or movement of existing cassettes and whose expression is controlled by the SOS response (Cambray et al. 2011; Guerin et al. 2009). Therefore, it was hypothesised that following internalization and packaging of *V. cholerae* into the food vacuoles of protozoa, that ROS might induce the SOS response leading to integrase expression and gene cassette recombination. In addition, another hypothesis was that due to the indiscriminate feeding behaviour of protozoa, different bacterial species could co-localise in the same food vacuoles where they could exchange genetic material.

4.2 Method and materials

4.2.1 Bacterial strains and growth conditions, plasmids and oligonucleotides

Bacterial strains and plasmids used in this study are listed in Table 4.1. All oligonucleotides are listed in Table 4.2. *E. coli, Pseudomonas aeruginosa* and *V. cholerae* strains were grown at 37 °C in Luria Bertani (LB) medium supplemented with 0.5 % NaCl. Antibiotics were used at the following concentrations for *V. cholerae*: Kanamycin (Kan), 50 µg ml^{-1}; chloramphenicol (Cm), 5 µg ml^{-1}; spectinomycin (Spc), 100 µg ml^{-1}. For *E. coli*, 25 µg ml^{-1} of chloramphenicol (Cm) was used. Diaminopimelic acid (DAP) was supplemented at a final concentration of 0.3 mM for *E. coli* WM3064. Plasmid DNA was extracted from *E. coli* strains using the PureYield™ Plasmid Miniprep Systems kit (Promega, Wisconsin, USA). PCR DNA fragments were gel purified using the ISOLATE II PCR and Gel Kit (Bioline, London, England). In order to induce gene expression in strains carrying the arabinose-inducible pSU-pBAD vector, a final concentration of 0.2 % (w/v) L-arabinose was added to the growth medium.

Table 4.1: Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and relevant phenotype[†]	Reference or source
Bacterial strains		
V. cholerae		
A1552	Wild type, O1 EI Tor, smooth variant	(Yildiz & Schoolnik 1999)
A1552 *intIA*::*gfp*	Contains *gfp* in transcriptional fusion with *intIA;* Cm^R	This study
A1552 *intIA*::*gfp*, Δ*recA*	A1552 *intIA*::*gfp* with an in-frame deletion of *recA;* Cm^R, Spc^R	This study
A1552 Δ*intIA*	Integron-integrase gene interrupted with a chloramphenicol resistance gene	Chapter 2
A1552 Δ*recA*	In-frame deletion of *recA;* Spc^R Defective for SOS induction	Chapter 2
A1552 Δ*hcp1*,Δ*hcp2*	Deletion of alleles VC1415 and VCA0017 encoding Hcp that polymerize to form the nanotube of the type VI secretion system.	(Ishikawa et al. 2009)
E. coli		
WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*], Sm^R	(Saltikov & Newman 2003)
β2163	Used to cultivate p4640; Cm^R, Kan^R	(Baharoglu, Bikard & Mazel 2010)
Plasmids		

p4640	*gfp* in transcriptional fusion with *intIA*, Cm^R, Kan^R	(Baharoglu, Bikard & Mazel 2010)
pKC01$^+$	*oriV*$_{R6K}$ and a *V. cholerae attC* site; Km^R	Chapter 2
pKD3	Source of chloramphenicol resistance gene flanked by FRT sites; Cm^R	(Datsenko & Wanner 2000)
pOriVn$_{700}$	Source of spectinomycin resistance gene, Spc^R	(Le Roux, Davis & Waldor 2011)
Protozoan strains		
T. pyriformis	Wild type	ATCC 205063
A. castellanii	Wild type	ATCC 30234

Cm^R, chloramphenicol resistant, Spc^R, spectinomycin resistant; Kan^R, kanamycin resistant, Sm^R, Streptomycin resistant

Table 4.2: Primers used in this study

Primer	Sequence (5`-3`)	Target	Annealing temperature (℃)	Extension time (min)	Reference
ompW_F	CACCAAGAAGGTGACTTTATTGTG	Used with ompW_R to amplify *ompW* in *V. cholerae*	62	0.30	(Nandi et al. 2000a)
ompW_R	GGTTTGTCGAATTAGCTTCACC	Used with ompW_F to amplify *ompW* in *V. cholerae*			
Kan_F	ATT CAA CGG GAA ACG TCT TG	Used with Kan_R to amplify the Kanamycin resistance gene (*nptII*)	62	0.30	This study
Kan_R	CGA GCA TCA AAT GAA ACT GC	Used with Kan_F to amplify the Kanamycin resistance gene (*nptII*)			
intIA-F	TCAAGCGCATACACTCCATC	Used with GFP_R to amplify the fused region of *intIA* with *gfp* in *V. cholerae* and to determine its orientation	60	1.00	This study
intIA_R	TTCAACGCTCGCAACTAGAA	Used with GFP_R to amplify the fused region of *intIA* with *gfp* in *V. cholerae* and to determine its orientation	60	1.00	
GFP_R	TGTGTCCGAGAATGTTTCCA	Used with intIA_F/R to amplify the fused region *intIA* with *gfp* in *V. cholerae* and to determine its orientation	60	1.00	(Baharoglu, Krin & Mazel 2012)
GyrA_F	CACGAACTCTTGGCAGACCT	Used with GyrA_R to amplify *gyrA* in *V. cholerae*	60	0.30	(Noorian et al. 2017)
GyrA_R	CAATACCAGATGCGCCGTTG	Used with GyrA_F to amplify *gyrA* in *V. cholerae*			
intIA-1F	TCAAGCGCATACACTCCATC	Used with intIA-1R to amplify *intIA* in *V. cholerae*	60	0.30	This study
intIA-1R	TTCAACGCTCGCAACTAGAA	Used with intIA-1F to amplify *intIA* in *V. cholerae*			
recA-F	ATTGAAGGCGAAATGGGCGATAG	Used with recA_R to amplify *recA* in *V. cholerae*	62	0.30	(Marashi et al. 2013)

recA-R	TACACATACAGTTGGATTGCTTGAGG	Used with recA_F to amplify *recA* in *V. cholerae*			
recA-up-F	TTCAGGCGGTCGAGCATAAT	Used with *recA*-up-R to amplify the upstream region of *recA* in *V. cholerae*	65	1.00	This study
recA-up-R	<u>AGATCGTTTT</u>GCATGATGGAGCCTTTACCA				
recA-down-F	<u>GGGCGAATTG</u>CTGATCGCTGAAACATCTTCTG	Used with recA-down-R to amplify the downstream region of *recA* in *V. cholerae*	64	1.00	This study
recA-down-R	GAAGCCGTAGGTGTCGTAGAGT				
aadA7-F	<u>TCCATCATGC</u>AAAACGATCTCAAGAAGATC	Used with aadA7-R to amplify the spectinomycin resistance gene from pOriVn$_{700}$	56	1.00	This study
aadA7-R	<u>CAGCGATCAG</u>CAATTCGCCCTATAGTGAGT	Used with aadA7-F to amplify the spectinomycin resistance gene from pOriVn$_{700}$			

4.2.2 Growth and maintenance of protozoa

The browsing ciliate, *Tetrahymena pyriformis,* was routinely cultured and passaged in 10 mL of peptone yeast-glucose (PYG) medium contained within 25 cm^2 tissue culture flasks with ventilated caps (Sarstedt Inc., Numbrecht, Germany) and incubated in static condition at 23 °C. PYG medium consists of 20 g l^{-1} proteose peptone, 1 g l^{-1} yeast extract and 0.1 M glucose dissolved in 1 L of 0.1x M9 minimal medium (6 g l^{-1} Na$_2$HPO$_4$, 3 g l^{-1} KH$_2$PO$_4$, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH$_4$Cl) . Prior to experiments, 500 µl of *T. pyriformis* was passaged in 20 mL of 0.35x NSS medium (8.8 g L^{-1} NaCl, 0.735 g L^{-1} Na$_2$SO$_4$, 0.04 g L^{-1} NaHCO$_3$, 0.125 g L^{-1} KCl, 0.02 g L^{-1} KBr, 0.935 g L^{-1} MgCl$_2$.6H$_2$O, 0.205 g L^{-1} CaCl$_2$.2H$_2$O, 0.004 g L^{-1} SrCl$_2$.6H$_2$O and 0.004 g L^{-1} H$_3$BO$_3$) (Mårdén et al. 1985) and incubated overnight statically at 23 °C to allow the ciliate to adapt to the nutrient free medium. Subsequently, the ciliates were enumerated microscopically using a haemocytometer. The surface feeding amoeba, *Acanthamoeba castellanii,* was routinely cultured and passaged as above except for incubation at a temperature of 30 °C. For experiments, *A. castellanii* was passaged 3 d prior in 0.1x M9 supplemented with 1% glucose and enumerated microscopically using a haemocytometer. Amoebal cells were then harvested and added into the experimental tissue culture plate for incubation at 23 °C for 1 h to facilitate attachment to the plate surface.

4.2.3 Construction of *V. cholerae* strains

A *V. cholerae* strain that is as an integron-integrase green fluorescent protein (GFP) reporter was created by chitin transforming p4640 (Baharoglu, Bikard & Mazel 2010); purified from *E. coli* β2163), a suicide vector containing an *intIA*::*gfp* transcriptional fusion. Transformation of p4640 allows for recombination of the vector into the chromosome to create the reporter called A1552 *intIA*::*gfp*. To confirm recombination of p4640 into the chromosome, PCR was conducted using oligonucleotides targeting *intIA* and *gfp* (intIA-F/GFP-R) (Table 4.2).

A1552 *intIA*::*gfp* was subjected to an in-frame deletion of *recA*. Using splicing-by-overlap-extension-PCR (SOE-PCR), a DNA fragment with *recA* interrupted with a spectinomycin resistance gene (*aadA7*; sourced from pOriVn$_{700}$) was constructed (Le Roux, Davis & Waldor 2011). Oligonucleotides with overlapping ends were constructed with Genome Compiler (https://designer.genomecompiler.com/app) and then two step SOE-PCR was carried out as described in chapter 2, section 2.2.6. The upstream and downstream regions of *recA* and, *aadA7* were amplified using oligonucleotides with annealing temperatures and extension times for

each oligonucleotide set listed in Table 4.2. The gel purified PCR construct was chitin transformed into A1552 *intIA*::*gfp* to create A1552 *intIA*::*gfp, ΔrecA*. PCR confirmations was carried out on each transformant colony using oligonucleotides (Table 4.2) that bind upstream and downstream in the deleted region and compared to the wild type. Homologous recombination at the target site was confirmed when no amplification of the target region was observed.

4.2.4 Co-incubation experiments with protozoa

Co-incubation assays were conducted in a 24-well microtitre plate containing 1 mL of co-culture per well (Nunclon Delta surface, Thermo Fisher Scientific, Denmark) as previously described (Espinoza-Vergara et al. 2019; Noorian et al. 2017). *T. pyriformis* and *A. castellanii* were enumerated and adjusted to 10^3 cells ml^{-1} and 10^4 cells ml^{-1}, respectively. Then the adjusted bacterial cells (strains of *V. cholerae* and *E. coli*) were added into wells containing protozoal cells (10^8 cells ml^{-1} for co-incubations with *T. pyriformis* and 10^7 cells ml^{-1} for co-incubations with *A. castellanii*) and incubated at 23 °C in static conditions for 4 h.

In LGT co-incubation experiments using pKC01$^+$ (see section 2.2.3 in chapter 2 for genetic architecture of this artificial gene cassette), chitin-competent *V. cholerae* cells were used and were prepared using commercially available chitin flakes as previously described (see details section 2.2.4 in chapter 2). Briefly, following overnight static incubation of *V. cholerae* cells with chitin flakes (see detail in chapter 2, section 2.2.4), 500 µl of supernatant above the settled chitin flakes was carefully aspirated and discarded. 500 µl of fresh 0.35x NSS was added and the tube rigorously vortexed for 5 min to detach the chitin-competent *V. cholerae* cells from the chitin flakes. Chitin flakes were left to settle for 2-3 min before the supernatant was carefully aspirated. From multiple tubes, approximately 5 mL of supernatant was collected and concentrated by centrifugation to achieve an OD$_{600}$ of 1.0 (equivalent to 10^9 cells ml^{-1}). pKC01$^+$ was provided as both free DNA (5 µg) and within *E. coli* WM3064 (10^8 cells ml^{-1} for co-incubations with *T. pyriformis* and 10^7 cells ml^{-1} for co-incubations with *A. castellanii*) in co-cultures of *V. cholerae* A1552 and protozoa and incubated for 4 h.

In the case of surface attached *A. castellanii*, the well was carefully washed three times with sterile culture media (0.1x M9 containing 1% glucose) before an equal volume of culture media containing 1% Triton-X was added to lyse the amoeba and release the internalised bacterial cells. In the case of *T. pyriformis,* co-cultures were aspirated from the plate and gently collected

by centrifugation at 400 x g for 10 min and the supernatant carefully aspirated and discarded. The pellet containing *T. pyriformis* cells was gently washed 3 times with sterile 0.35x NSS and resuspended in an equal volume of 0.35x NSS containing 1% Triton-X to lyse the protozoa and release the internalised bacteria. Appropriate dilutions of lysates were plated onto agar plates containing kanamycin. Transformation frequencies were measured by dividing the number of kanamycin resistant CFUs with the total number of CFUs (Marvig & Blokesch 2010). To confirm that the transformed strains were *V. cholerae* containing the integrated donor gene cassette, PCR reactions using *V. cholerae* species-specific primers (ompW_F/ompW_R) and kanamycin specific primers (KanF/KanR) (Table 4.2) were conducted on 10 random colonies.

4.2.5 Confocal laser scanning (CLSM) microscopy

To spatially visualise GFP expression in the integron-integrase *V. cholerae* A1552 reporter strains (*intIA*::*gfp* and *intIA*::*gfp* Δ*recA*) during co-incubation with the protozoa, confocal microscopy (Nikon A1 confocal laser scanning microscope) at the UTS Microbial Imaging Facility was used. For CLSM of co-culture containing *T. pyriformis* and *V. cholerae*, the samples were collected after 4 h incubation, fixed and mounted on a glass slide. The lipophilic dye, FM4-64 FX (Invitrogen, Carlsbad CA) and DAPI (diamidino-2-phenylindole, ThermoFisher Scientific, USA) were used to stain the membrane and nucleus, respectively following the manufacturer's instructions. To fix samples for microscopy, 200 µl of co-cultures containing bacteria and ciliates were collected by centrifugation (400 × g, 10 min) in an Eppendorf tube (1.5 mL), then resuspended in PBS containing 2 % paraformaldehyde (pH 7.2-7.4) and incubated for 10 min at room temperature (RT), Subsequently, the fixed cells were washed three times by centrifugation (400 × g, 10 min) and resuspended in PBS. Ten µl of the fixed sample was placed on a glass slide and dried at RT for 15 min. After drying, 10 µl of VECTASHIELD HardSet Antifade Mounting Medium (Vector Laboratories, USA) was added and a coverslip (1.5 mm thickness, Neuvitro, USA) was placed on the sample and incubated in the dark at RT overnight to allow the mounting media to set. Mounted samples were used for CLSM analysis immediately after overnight incubation or stored at 4 °C for future analysis. The co-culture containing *A. castellanii* and *V. cholerae* were directly subjected to live imaging in CellCarrier 96 - Ultra Microplates (PerkinElmer, UK) after 4 h of co-incubation. Images were analysed using Fiji (Schindelin et al. 2012) and Imaris v9.2 (Bitplane, USA) analysis software.

4.2.6 GFP fluorescence quantification

During co-incubation with the protozoa, protozoal cells were collected by centrifugation at 400 x g for 10 min and the supernatant carefully aspirated and discarded. The pellet containing *T. pyriformis* cells was gently washed 3 times with sterile 0.35× NSS and resuspended in an equal volume of 0.35x NSS containing 1% Triton-X to lyse the protozoa and release the internalised bacteria. GFP fluorescence was quantified as previously described (Kicka et al. 2014; Schlimme et al. 1997). Briefly, 200 µl of resuspended cells was transferred into a 96 well microtitre plate (Corning® 96 Well Black Polystyrene Microplate flat bottom clear) for measuring GFP fluorescence at 488 nm excitation and 530 nm emission wavelengths using a multimode microplate reader (Spark microplate reader, TECAN, Switzerland). The cell densities in each well of the microtitre plate containing co-cultures were determined at 600 nm. Background fluorescence of *V. cholerae* and protozoa was determined using co-cultures containing the wild type strain with *T. pyriformis,* and with *A. castellanii* respectively, which do not contain the *gfp* reporters and subtracted. The GFP expression units in figure are the ratio of fluorescence intensity to cell density divided by 10,000.

4.2.7 Reactive oxygen species (ROS) quantification and quenching experiments

T. pyriformis and *A. castellanii* cells were separately co-incubated with *V. cholerae* in order to measure ROS production within the food vacuoles of protozoa. Following 4 h incubation, the protozoan cells were treated with 1% Triton-X to release the ROS which resides inside the food vacuoles and centrifuged at maximum speed for 2 min to collect the supernatant containing the ROS. ROS were measured using the dye 2`,7`-dichlorofluorescin diacetate (DCF-DA) according to a previously published method (Ye et al. 2019; Zhang et al. 2015). In brief, the collected fluids were stained with 10 µM DCF-DA (Sigma, USA) and incubated at 37 °C for 30 min. To quantify fluorescence, 200 µl samples were transferred into 96 well microtitre plate (Corning® 96 Well Black Polystyrene Microplate flat bottom clear) and fluorescence was measured using a multimode microplate reader (Spark microplate reader, TECAN, Switzerland) with 485 nm excitation and 525 nm emission wavelengths.

In order to determine the effects of host derived reactive oxygen species (ROS) (produced inside the food vacuoles of protozoa) on *V. cholerae* integron-integrase expression and transformation, *T. pyriformis* and *A. castellanii* cells were treated with the commonly used global ROS quencher, thiourea (Fang 2013; Wang et al. 2020). Briefly, 100 mM concentration of thiourea (Sigma, USA) (pH 7.0) was added to treat the protozoan cells before experiments

were conducted. The fluorescence of DCF-DA was finally calculated as fluorescence intensity divided by 10,000.

4.2.8 Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was prepared from co-incubation assays with *T. pyriformis* and *A. castellanii* in 24-well plates with or without addition of ROS quencher. Protozoal food vacuoles containing bacterial cells were collected as described above and RNA was extracted by lysozyme digestion followed by using the Aurum Total RNA mini kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry (NanoDrop ND-1000; NanoDrop Technologies). Complementary DNA (cDNA) was prepared from 400 ng RNA from each sample by iScript Reverse Transcription (Bio-Rad, Hercules, CA, USA) following the kit instructions. Quantitative reverse-transcriptase PCR (qRT-PCR) was done using a SSOADV Universal SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) by QuantStudio 6 Flex Real-Time PCR System using the specific primers intIA-1F/intIA-1R targeting integrase (*intIA*) gene (Table 4.2). The expression was determined relative to the expression of the control gene *gyrA (*GyrA F/GyrA R*)* (Table 4.2) using the comparative Ct ($\Delta\Delta$Ct) method of RT-PCR.

4.2.9 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California, USA (www.graphpad.com). Two-tailed student's t-tests were used to compare means between experimental samples and controls. For experiments with multiple samples, one-way ANOVAs were used with Tukey's or Dunnett's Multiple Comparison Test post-hoc comparisons of means.

4.3 Results

4.3.1 The *V. cholerae* integron-integrase is expressed inside protozoal food vacuoles

To determine whether the *V. cholerae* integron-integrase is expressed within the food vacuoles of protozoa, a reporter strain containing a transcriptional fusion of *intIA* with *gfp* was independently co-incubated with the suspension feeding *T. pyriformis* and the surface feeding protozoa *A. castellanii*. Following 4 h of co-incubation, the co-cultures were subjected to confocal laser scanning (CLSM) microscopy. *V. cholerae* cells expressing GFP were observed inside the protozoa but not outside in the surrounding medium (Figure 4.1A and 4.1B). Specifically, GFP fluorescent cells were observed inside the food vacuoles (FV) of both protozoa (Figure 4.1A and 4.1B, second panel row). In addition to the food vacuoles, GFP fluorescent cells were also observed inside the contractile vacuoles (CV) of *A. castellanii* (Figure 4.1B, second panel row, yellow dotted circle).

In order to quantify the level of integron-integrase induction, the intensity of bacterial GFP fluorescence was measured using a spectrofluorometer at 4 h co-incubation. The results showed that GFP fluorescence was significantly higher (2280 fold in *T. pyriformis* and 2573 fold in *A. castellanii*) in the presence of both protozoa, while no GFP fluorescence was detected in the control medium (Figure 4.2A and 4.2B). To confirm that *intIA* is induced inside the protozoa, we extracted total RNA from the co-incubations with the results showing that after 4 hrs predation by both protozoa, the relative expression of *intIA* in wild type (WT) was 29.20 fold (in *T. pyriformis*) and 5.0 fold (in *A. castellanii*) higher compared to the no protozoa controls (Figure 4.3).

A.

	Transmitted light	GFP and DAPI	FM 4-64fx and DAPI	Merge
intlA::GFP (-RS)				
FV				
intlA::GFP Δ*recA*				
intlA::GFP (+RS)				
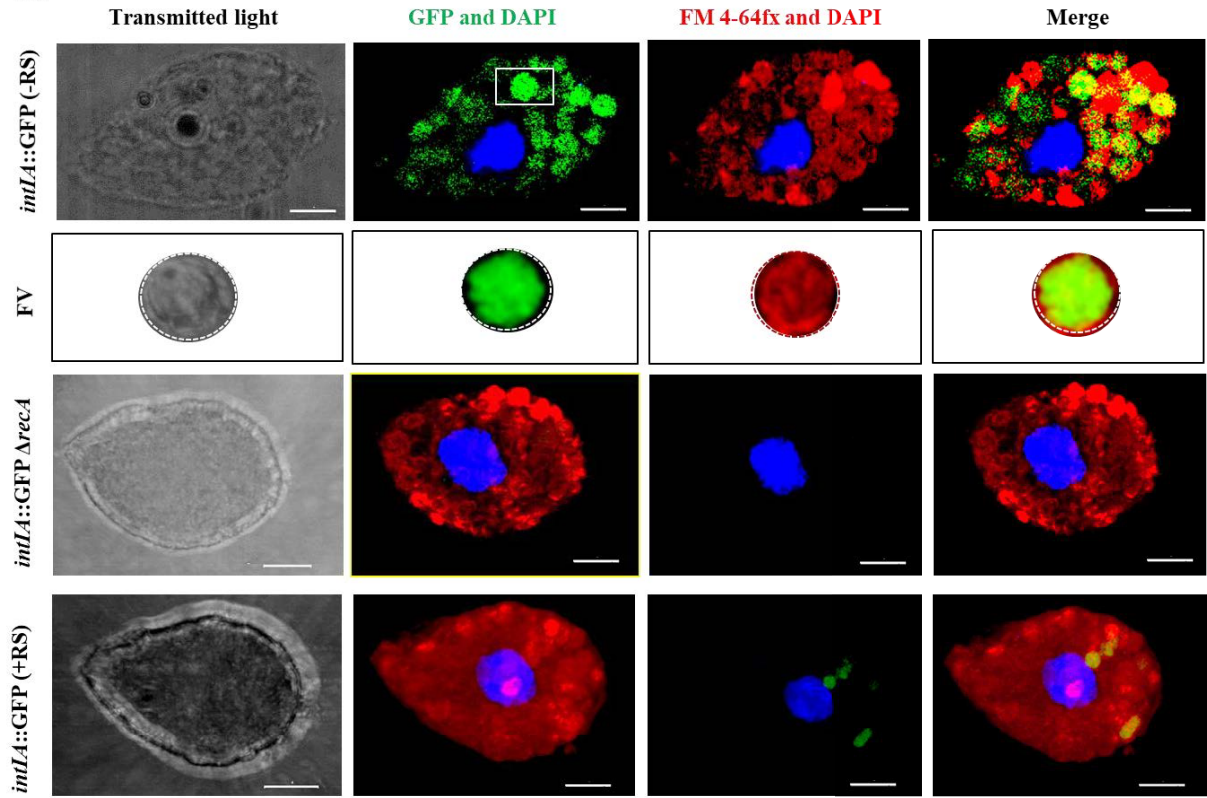
B.
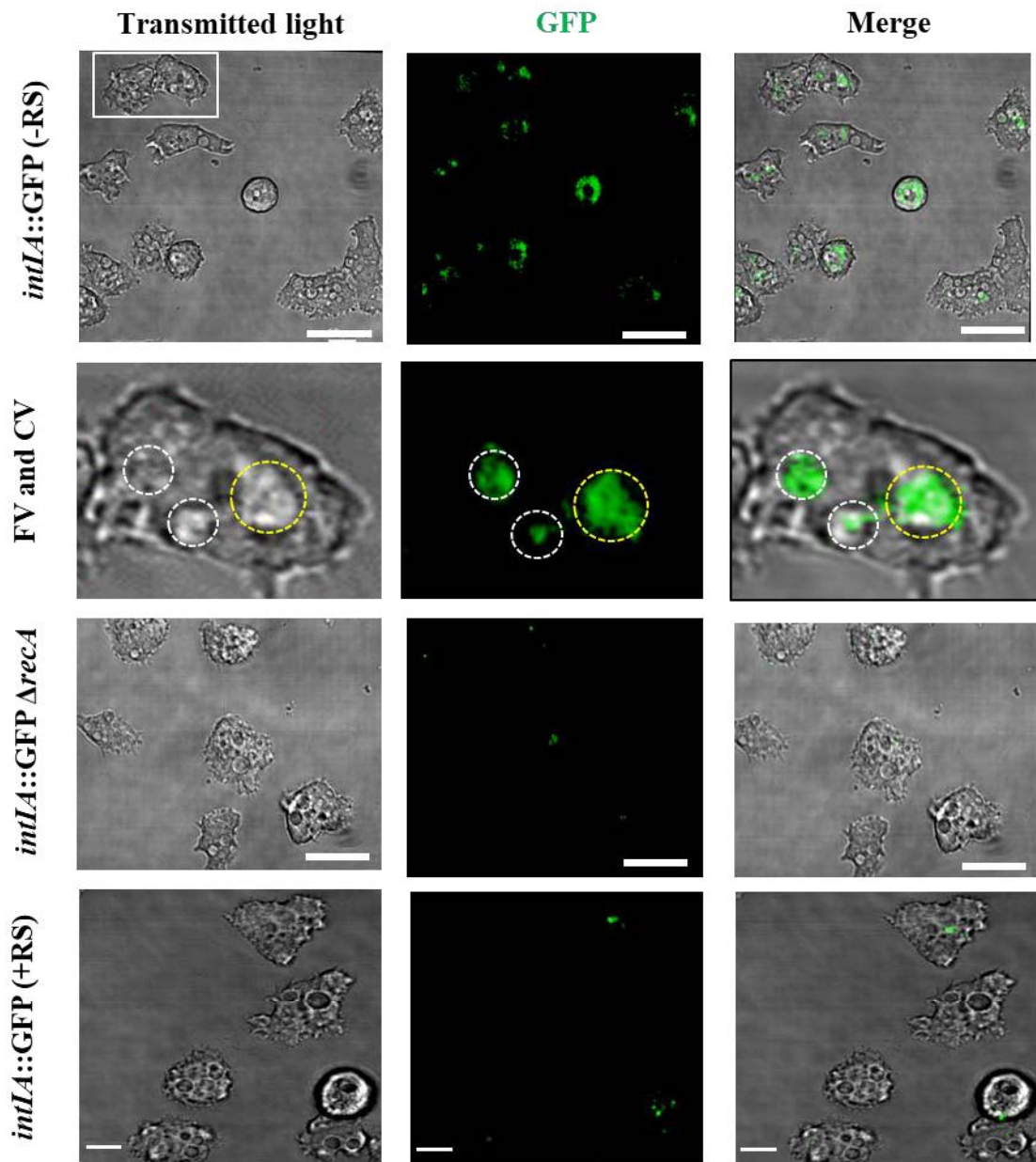
Figure 4.1: Fluorescence confocal microscopy of co-incubations of *V. cholerae* with, *T. pyriformis* (A) and *A. castellanii* (B). The *V. cholerae* integron-integrase reporter strain (*intIA*::*gfp*) contains a transcriptional fusion of *intIA* and *gfp*. A. Columns of panels show transmitted light containing *T. pyriformis*, signal from green fluorescence (GFP) and DAPI, signal from FM4-64FX and DAPI and all fluorescence channels (GFP + FM4-64FX + DAPI, labelled Merge) respectively. Scale bar 10 µM and 100× magnification was used. B. Columns of panels show transmitted light containing amoebae, signal from GFP and merge. White and yellow dashed circles show food and contractile vacuoles respectively. Scale bar 10 µM and 40× magnification was used.
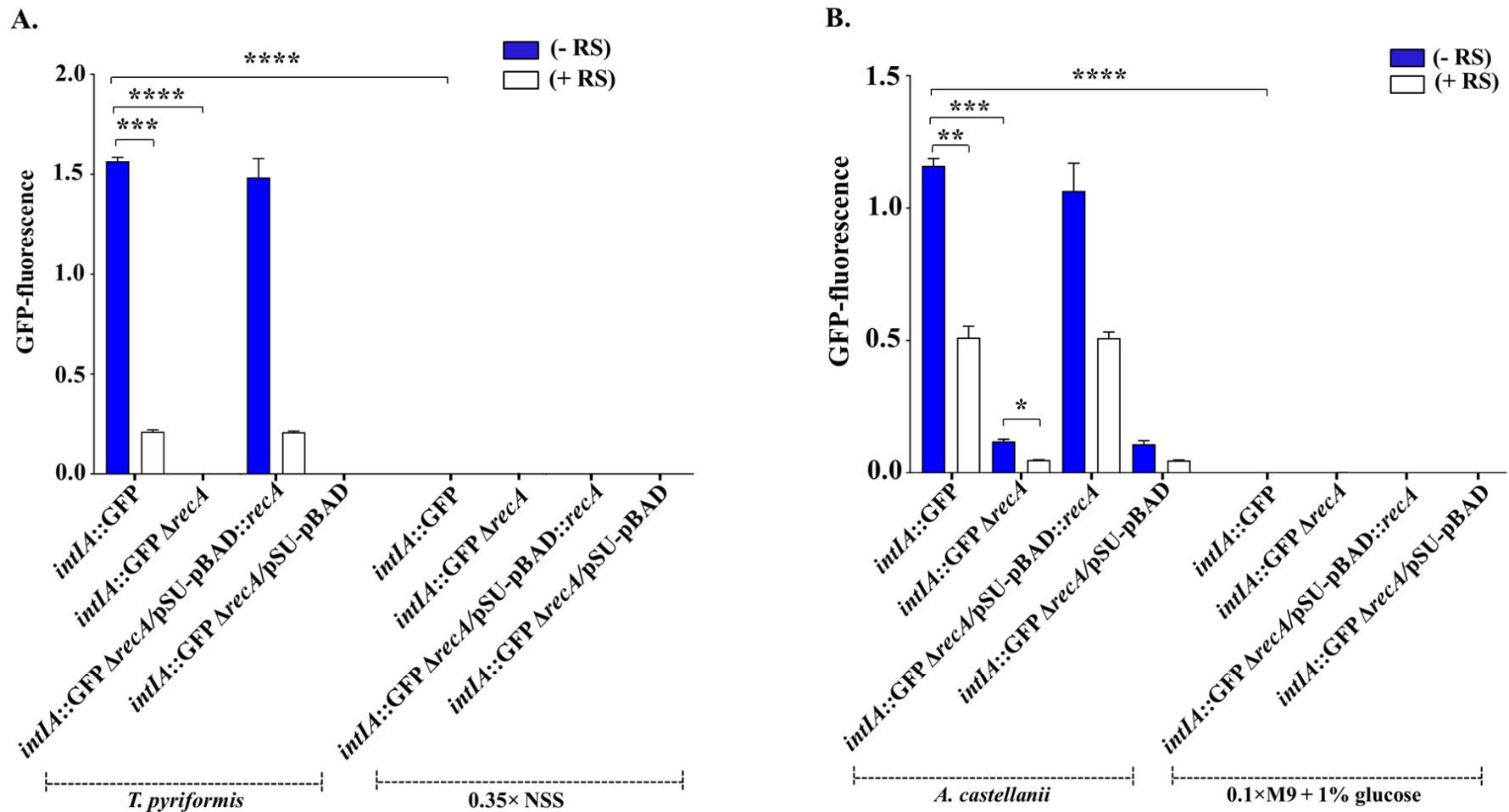
Figure 4.2: Fluorescence spectrometry of co-incubations of *V. cholerae* strains with *T. pyriformis* (A) and *A. castellanii* (B). The *V. cholerae intIA*::*gfp* strain contains a transcriptional fusion of *intIA* with *gfp*. Cells external to the protozoa were removed and GFP fluorescence was measured from internalised bacterial cells recovered from purified protozoa after 4 h incubation. +RS indicates the addition of 100 mM of the ROS quencher thiourea to the co-incubation. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA and Tukey's multiple comparisons test (**$p<0.001$, ***$p<0.0001$, ****$p<0.00001$).
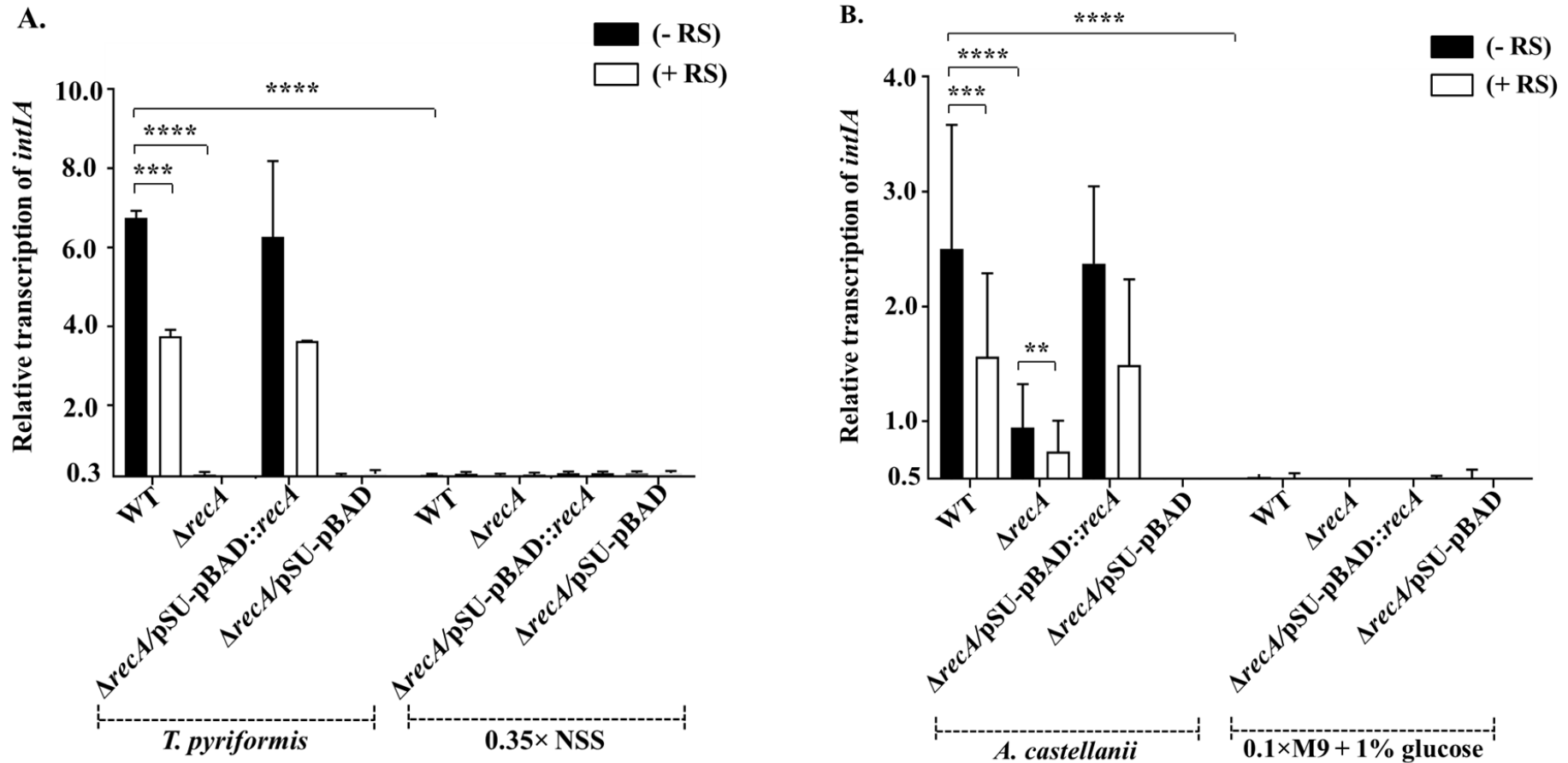
Figure 4.3: qRT-PCR of *V. cholerae intIA* following 4 h of co-incubation with *T. pyriformis* (A) and *A. castellanii* (B). Cells external to the protozoa were removed and total RNA was extracted from purified protozoa. +RS indicates the addition of 100 mM of the ROS quencher thiourea to the co-incubation. *intIA* transcription levels were normalized to the *gyrA* by using the comparative Ct (ΔΔCt) method. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA and Tukey's multiple comparisons test (**p<0.001, ***p<0.0001, ****p<0.00001).

4.3.2 Enhanced gene cassette transfer and integration in *V. cholerae* occurs within the food vacuoles of protozoa

Given that the *V. cholerae* integrase is induced in the presence of protozoa and more specifically, within the food vacuoles (also CV in *A. castellanii*), a gene cassette transformation assay was conducted to determine if transfer was enhanced inside the protozoa. The artificial gene cassette pKC01$^+$ (5 μg) was provided as purified DNA in co-incubations of *V. cholerae* and protozoa and, in no protozoa controls. Unsurprisingly, only *V. cholerae* wild-type cells that were pre-grown on chitin produced pKC01$^+$ transformants in both the protozoal co-incubations and media controls (Figure 4.4A and 4.4C). However, in the presence of *T. pyriformis* and *A. castellanii*, transformation efficiency was 405-fold and 18-fold higher respectively compared to the non-protozoal treatments (Figure 4.4A and 4.4C). For all treatments, no transformants were obtained when the integron-integrase mutant (Δ*intIA*) was used and complementation restored the phenotype. To confirm that transformant colonies were *V. cholerae* and contained of pKC01$^+$, 10 colonies were randomly picked from each protozoal treatment and subjected to genomic DNA extraction and PCR for the *V. cholerae* species-specific *ompW* and the kanamycin resistance genes (Supplementary Figure S4.1).

4.3.3 The *V. cholerae* T6SS is required for integron-integrase mediated HGT between bacterial cells inside protozoa

To determine whether the protozoal digestion enhances uptake and integration of pKC01$^+$ contained within another bacterial cell, we used *E. coli* WM3064 carrying pKC01$^+$ as a donor and conducted the gene cassette transfer transformation assay. The results showed that *V. cholerae* cells pre-grown on chitin within the protozoa are capable of uptake and integration of pKC01$^+$ from within *E. coli* WM3064 at a transformation frequency of 7.73×10^{-05} (in co-incubation with *T. pyriformis*) and 2.06×10^{-05} (in co-incubation with *A. castellanii*) whereas, no transformation of pKC01$^+$ was detected in the media controls (Figure 4.4B and 4.4D). As expected, no transformants were detected in the non-chitin grown wild-type and chitin grown Δ*intIA* mutant in all treatments. Complementation of the Δ*intIA* mutant resulted in restoration of cassette transformation back to wild-type levels (Figure 4.4B and 4.4D). To address whether *V. cholerae* utilises its T6SS to kill and release DNA from *E. coli* WM3064 to make it accessible for HGT, a double *hcp* mutant incapable of producing T6SS (Δ*hcp1*, Δ*hcp 2*) was tested. No transformants could be detected in both the protozoa and media controls when chitin-

induced *V. cholerae* was used (Figure 4.4B and 4.4D). To confirm that the T6SS mutant was capable of transformation, 1 µg of pKC01$^+$ was added to wild-type *V. cholerae* and the double *hcp* mutant in a standard chitin transformation assay (Marvig & Blokesch 2010) and no significant difference in transformation efficiency between the strains was observed (Supplementary Figure S4.2). Following complementation, transformation efficiency of the Δ*hcp1*, Δ*hcp 2* double mutant was restored to wild-type levels (Figure 4.4B and 4.4D). To confirm that transformant colonies were *V. cholerae* and contained of pKC01$^+$, 10 colonies were randomly picked from each protozoal treatment and subjected to genomic DNA extraction and PCR for the *V. cholerae* species-specific *ompW* and the kanamycin resistance genes (Supplementary Figure S4.1).

A.

Transformation frequency

Legend:
- *T. pyriformis* (- RS)
- *T. pyriformis* (+ RS)
- 0.35× NSS (- RS)
- 0.35× NSS (+ RS)

X-axis categories:
WT (+ chitin), WT (- chitin), Δ*intIA* (+ chitin), Δ*intIA*/pSU-pBAD::*intIA* (+ chitin), Δ*intIA*/pSU-pBAD (+ chitin), Δ*recA* (+ chitin), Δ*recA*/pSU-pBAD::*recA* (+ chitin), Δ*recA*/pSU-pBAD (+ chitin)

B.

Transformation frequency

Legend:
- *T. pyriformis* (- RS)
- *T. pyriformis* (+ RS)
- 0.35× NSS (- RS)
- 0.35× NSS (+ RS)

X-axis categories:
WT (+ chitin), WT (- chitin), Δ*intIA* (+ chitin), Δ*intIA*/pSU-pBAD::*intIA* (+ chitin), Δ*intIA*/pSU-pBAD (+ chitin), Δ*recA* (+ chitin), Δ*recA*/pSU-pBAD::*recA* (+ chitin), Δ*recA*/pSU-pBAD (+ chitin), Δ*hcp*1 Δ*hcp*2 (+ chitin), Δ*hcp*1 Δ*hcp*2/pSU-pBAD::*hcp*1 (+ chitin), Δ*hcp*1 Δ*hcp*2/pSU-pBAD (+ chitin)

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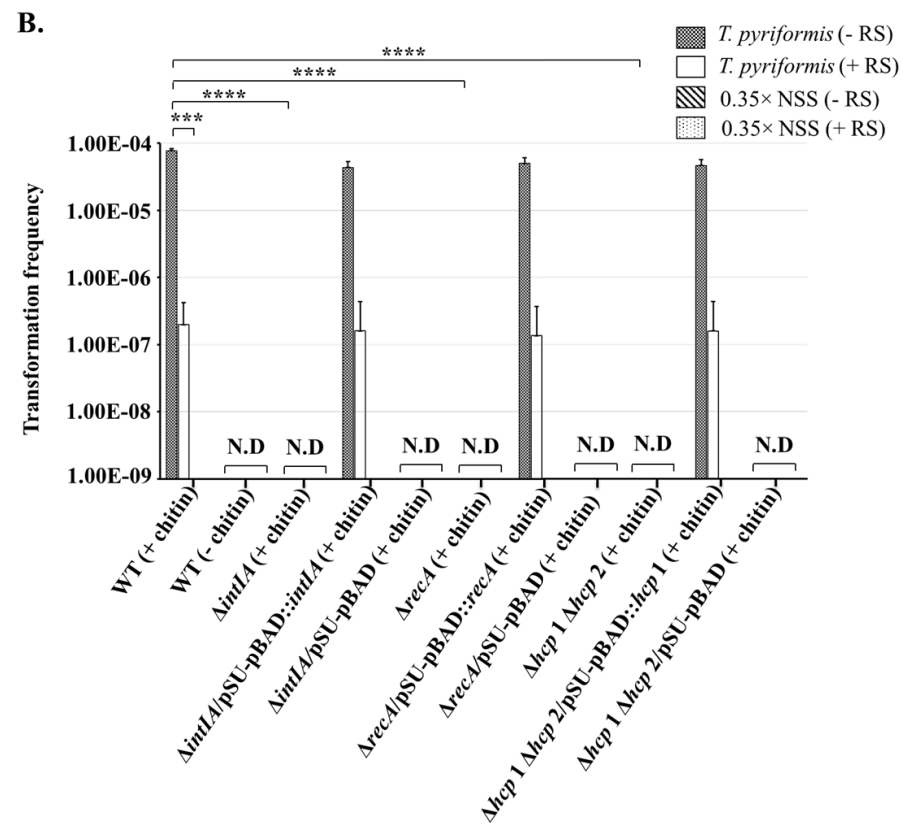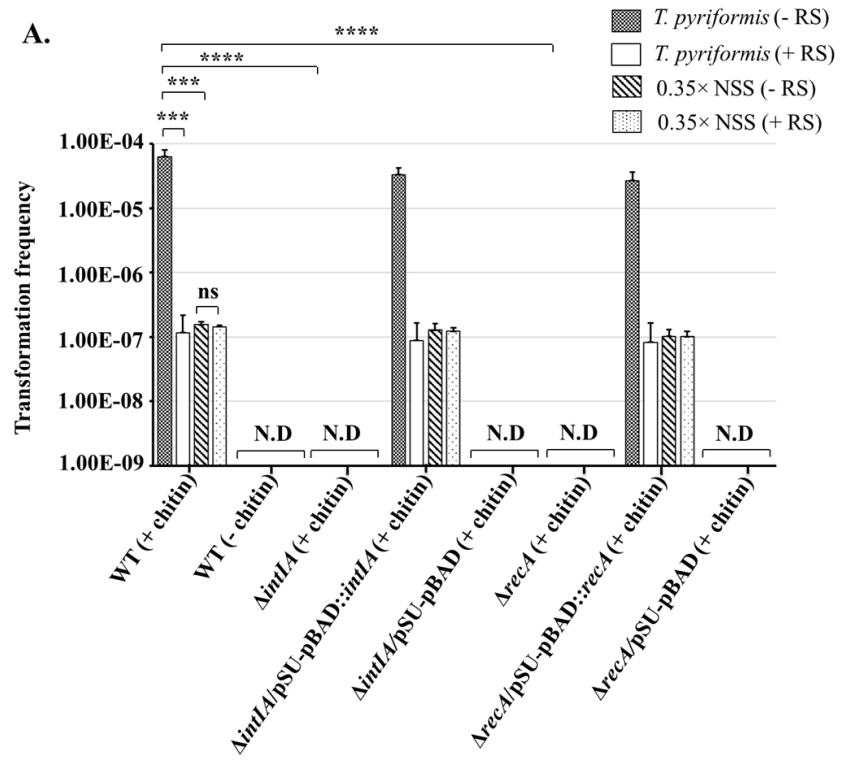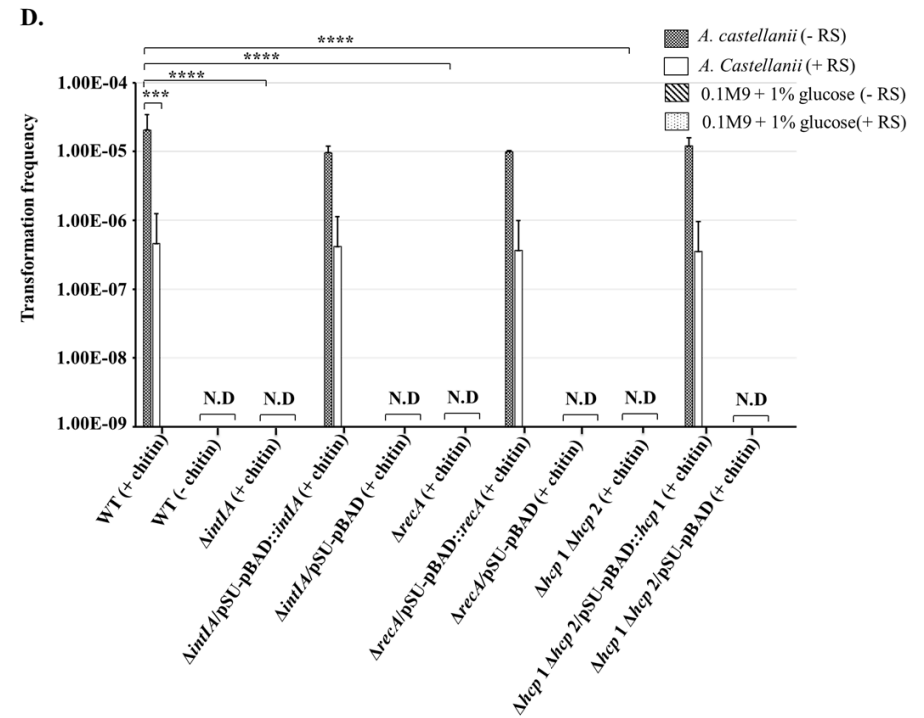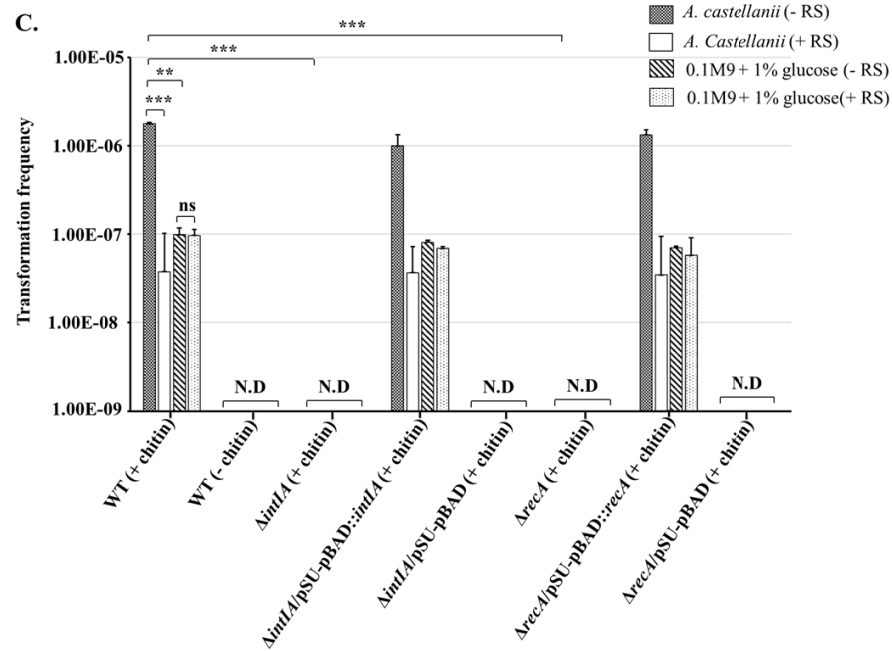
Figure 4.4: Frequency of gene cassette integration in *V. cholerae* strains co-incubated with protozoa. In co-incubations with protozoa, *V. cholerae* must be pre-grown on chitin for successful transfer of the artificial gene cassette pKC01[+] when provided as either free DNA (A and C) or when contained within *E. coli* WM3064 (B and D). N.D. indicates non-detectable transformants. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA and Tukey's multiple comparisons test (**$p < 0.001$, ***$p < 0.0001$, ****$p < 0.00001$).

4.3.4 The SOS response is required for *intIA* expression in *V. cholerae* within the food vacuoles of protozoa

The integron-integrase (*intIA*) gene is regulated by the SOS response, a global response to DNA damage that involves an interaction between the RecA recombinational repair protein and the repressor protein LexA (Cambray et al. 2011; Guerin et al. 2009). To determine whether *intIA* was capable of induction within protozoa in the absence of *recA*, the gene was knocked out in the integron-integrase reporter strain and independently co-incubated with *T. pyriformis* and *A. castellanii* and subjected to CLSM, spectrometry and the pKC01$^+$ transformation assay. The CLSM results showed that no GFP fluorescent cells were observed in the *recA* mutant background within the *T. pyriformis* and outside in the surrounding media (Figure 4.1A) and spectrometry showed no fluorescence (Figure 4.2A). However, some GFP fluorescent cells were observed within the amoebae (Figure 4.1B) and spectrometry showed fluorescence but with a 9.8-fold reduction compared to the wild-type (Figure 4.2B). Using RT-qPCR, no transcript of *intIA* in the *recA* mutant was detected compared to the wild-type in co-incubation with *T. pyriformis* treatment (Figure 4.3A). Some *intIA* transcript was detected in the *recA* mutant (2.6 fold lower than WT) in co-incubation with *A. castellanii* (Figure 4.3B). The transformation frequency result showed non-detectable transformation of pKC01$^+$ (either free DNA or within from *E. coli* WM3064) in the *recA* mutant strain in both the protozoal treatments or media controls. Complementation showed the restoration of the *recA* mutant to wild-type levels in all assays (Figure 4.2, 4.3 and 4.4).

4.3.5 ROS is involved in SOS response induction of *intIA* and HGT in *V. cholerae* within the food vacuoles of protozoa

As protozoal food vacuoles are commonly filled with ROS following internalization of bacteria, it was hypothesized that ROS may be inducing the bacterial SOS response leading to *intIA* transcription and subsequent pKC01$^+$ integration. To test this, thiourea, an ROS quencher (Fang 2013; Wang et al. 2020) was added to the co-incubation experiments containing the integron-integrase reporter strain. The CLSM result showed that, addition of thiourea resulted in a substantial decrease in GFP fluorescence intensity within both protozoa (Figure 4.1A and 4.1B). Spectrometry showed that addition of thiourea resulted in a 7.4 fold and 2.2 fold reduction of GFP fluorescence in co-incubation with *T. pyriformis* and *A. castellanii* respectively, compared to the no thiourea treatments (Figure 4.2A and 4.2B). RT-qPCR

showed thiourea treatment caused a 1.8 fold and 1.6 fold drop in *intIA* transcription in the wild-type compared to the thiourea treated *T. pyriformis* and *A. castellanii* co-incubations, respectively (Figure 4.3A and 4.3B).

Thiourea lowered the pKC01$^+$ transformation in chitin-induced wild-type *V. cholerae* by 535 fold and 47 fold, within *T. pyriformis* and *A. castellanii* respectively and although transformation was detected in the media controls, thiourea had no impact on transformation levels (Figure 4.4A and 4.4C). As expected, no transformation was detected in the Δ*intIA* and Δ*recA* strains. When pKC01 was provided within *E. coli* WM3064, thiourea was found to reduce transformation frequency by 389 fold and 45 fold in *T. pyriformis* and *A. castellanii,* respectively (Figure 4.4B and 4.4D). Complementation of mutants resulted in restoration of transformation phenotype to wild-types level (Figure 4.4A-4.4D).

To determine the level of host derived ROS production during digestion of prey cells, the protozoan cells (*T. pyriformis* and *A. castellanii*) were co-incubated independently with *V. cholerae* WT with or without treatment of ROS quencher, thiourea. After 4 h of feeding, protozoal cells were purified as described above and lysed with 1% Triton X stained to release the intracellular ROS. Then ROS reporter dye, DC-FDA (2`,7`-Dichlorofluorescin Diacetate) was added and DCFDA fluorescence measured using spectrometry. The result showed that in the addition of ROS quencher thiourea, intracellular ROS level was ~3 fold lower in both *T. pyriformis* and *A. castellanii* (Figure 4.5A and 4.5B). Although some DCF-DA fluorescence was observed in the media controls, DCF-DA-positive fluorescence in the presence of *T. pyriformis* (17-fold higher) and *A. castellanii* (18-fold higher) was much higher (Figure 4.5).
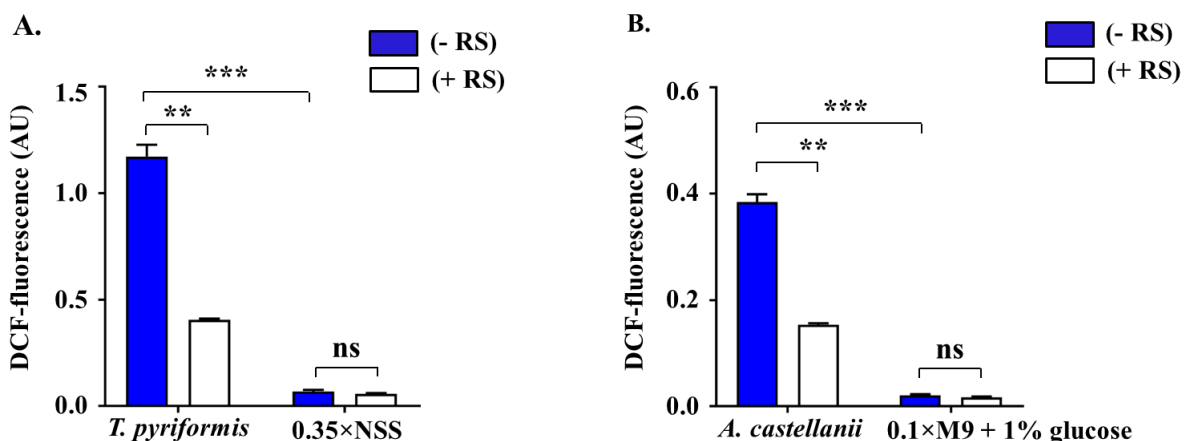
Figure 4.5: ROS production inside the ciliate *T pyriformis* (A) and amoebae *A. castellanii* (B). *V. cholerae* WT was co-incubated with both protozoa separately and level of internal ROS was measured using the fluorescent dye DCF-DA and fluorescence spectrometry. +RS indicates the addition of 100 mM of the ROS quencher thiourea to the co-incubation. Fluorescence was normalized to media controls

containing wild-type *V. cholerae*. Error bars represent the standard deviations of three independent experiments. Significance was calculated using one-way ANOVA and Tukey's multiple comparisons test (**$p<0.001$).

4.4 Discussion

In this study, a series of investigations were conducted to determine whether the *V. cholerae* integron-integrase was induced within the food vacuoles of ciliates *T. pyriformis* and amoebae *A. castellanii*. Confocal microscopy showed that a *V. cholerae intIA-gfp* reporter produced fluorescent cells within the food vacuoles of *T. pyriformis* and *A. castellanii* and, in the contractile vacuoles of *A. castellanii*. Increased expression of IntIA was confirmed by fluorescence spectrometry in the *intIA-gfp* reporter and increased transcription of *intIA* was confirmed with RT-qPCR when *V. cholerae* was co-incubated with the protozoa. As no fluorescent cells were observed in the surrounding medium but observed in the protozoal digestive vacuole of both protozoa, it can be deduced that this environment is responsible for inducing the *V. cholerae intIA*. In *A. castellanii*, fluorescent cells were also observed in the contractile vacuole indicating induction of *intIA* however, *V. cholerae* enters the contractile vacuole through fusion of the food vacuole (Van der Henst et al. 2016) and so it is possible that *intIA* induction occurs in the food vacuole and/or that components of the food vacuole are deposited in the contractile vacuole that facilitate continued induction. More *intIA* transcription was observed in the *T. pyriformis* co-incubation compared to *A. castellanii* which may be explained by the difference in feeding rates with the browsing *T. pyriformis* ciliate capable of feeding on a higher number of bacterial cells (Fenchel 1980; Iriberri et al. 1995) compared to the surface feeder *A. castellanii* (Heaton et al. 2001; Rogerson, Hannah & Gothe 1996).

Expression of the integron-integrase generates genetic diversity in the cassette array through integration of new cassettes, deletion and/or rearrangement of existing gene cassettes in the array (Guerin et al. 2009). Therefore, it was expected that protozoal digestion would foster enhanced rate of integration of the artificial cassette pKC01[+]. No kanamycin resistant transformants were detected in the presence of both protozoa and respective media controls when non-chitin cultured *V. cholerae* cells were provided as a recipient however, pKC01[+] integration was significantly higher in the presence of *T. pyriformis* and *A. castellanii* compared to the media controls containing *V. cholerae* cells made competent through growth on chitin. This suggests that *V. cholerae* competency is a pre-requisite for successful cassette integration and that integration is significantly enhanced by predation of protozoa. It is worth noting that the *V. cholerae* is naturally competent during biofilm formation on chitin surfaces which is its

preferred mode of growth in the environment (Rawlings, Ruiz & Colwell 2007; Tamplin et al. 1990). Due to the indiscriminate feeding behaviour of protozoa, *V. cholerae* likely finds itself within the food vacuole with diverse strains and species providing an ample opportunity for genetic exchange. Indeed, *V. cholerae* was able to source pKC01$^+$ from *E. coli* within the food vacuoles of protozoa. A prior study has shown that *V. cholerae* utilises T6SS to kill neighbouring cells and release DNA to make it accessible for LGT (Borgeaud et al. 2015). Here, chitin grown *V. cholerae* lacking T6SS mediated killing function (Δ*hcp*1, Δ*hcp*2) were unable to access pKC01$^+$ from *E. coli* but were still competent when pKC01$^+$ was provided as free DNA suggesting that *V. cholerae* T6SS is involved in killing of *E. coli* inside the food vacuoles of protozoa to release pKC01$^+$ and make it accessible for uptake and subsequent integrase-mediated integration.

Given that *intIA* transcription is induced in the food vacuole of protozoa, it was hypothesised that enhanced cassette integration was due to an SOS response driven by the conditions of the food vacuole. Deletion of *recA*, which is required for SOS induction, resulted in no fluorescent cells or *intIA* transcript as determined by RT-qPCR in *T. pyriformis* suggesting that *intIA* expression and enhanced rate of gene cassette transformation and integration in *V. cholerae* is linked to the bacterial SOS response. A similar result was observed in *A. castellanii* however, some detection of fluorescent cells and *intIA* transcript in the Δ*recA* mutant was detected within *A. castellanii* although, this was not sufficient to produce transformants in the cassette integration assay. This is an interesting result and indicates that another pathway(s) is involved in *intIA* induction in addition to the SOS response in *A. castellanii*. A previous study has reported that the carbon catabolite repression system, requiring the cyclic AMP (cAMP) receptor protein (CRP), regulates *V. cholerae intIA* expression and gene cassette recombination (Baharoglu, Krin & Mazel 2012) so this could be an ideal pathway to delete in future studies with *A. castellanii*. ROS generation within the food vacuoles of protozoa is considered a key feature of killing prey within the phagosome (Nusse 2011) causing DNA damage and mutagenesis (Baharoglu & Mazel 2011; Colin et al. 2014; Srinivas et al. 2019) and so this was hypothesised as the trigger for the SOS response. In this study, high levels of ROS were detected in the protozoa during co-incubation with *V. cholerae* with addition of the ROS quencher, thiourea, capable of reversing the effects of ROS generation. Thiourea decreased fluorescence in the *intIA*::*gfp* reporter in the co-incubation assays and *intIA* transcript in the RT-qPCR assay. It also reduced cassette integration in the co-incubation assay but not in the media controls. These data indicate that *intIA* induction and enhanced cassette integration in

the presence of protozoa is due to ROS within the food vacuoles of protozoa through induction of the SOS response.

Altogether, this study provides evidence that the food vacuoles of protozoa are an important niche where *V. cholerae* can generate genetic diversity through SOS-induced LGT.

4.5 Supplementary information
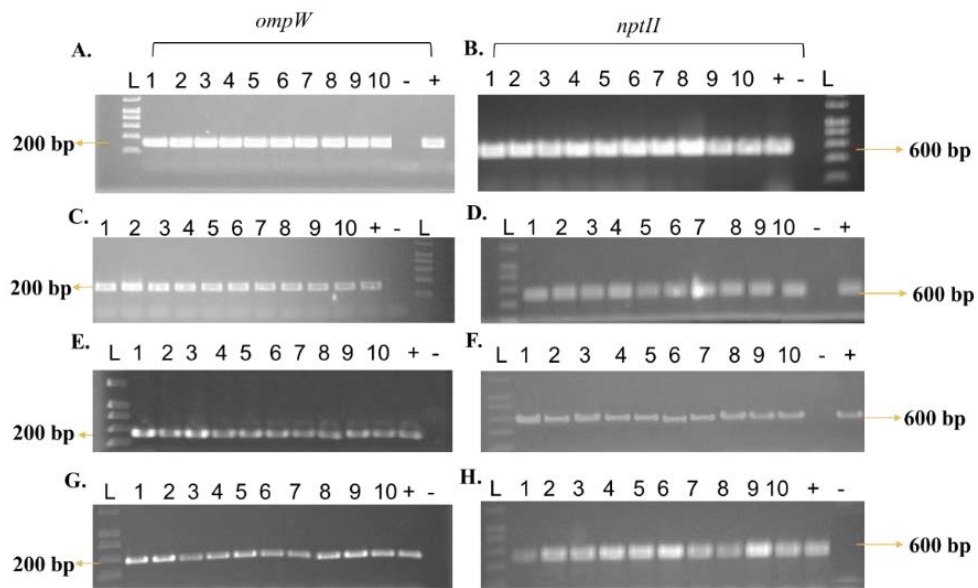
Supplementary Figure S4.1. PCR amplification of *ompW* (200 bp) and pKC01+ containing kanamycin resistance gene (*nptII;* 600 bp) from *V. cholerae* transformants. Panel A-B and C-D represents transformants when free pKCO1+ DNA was added and when pKC01+ was provided from within *E. coli* WM3064 respectively in the presence of *T. pyriformis*. Panel E-F and G-H represents transformants when free pKCO1+ DNA was added and when pKC01+ was provided from within *E. coli* WM3064 respectively in the presence of *A. castellanii.* L, + and - indicates 10 kb ladder; positive control (A1552 gDNA) and negative control (dH$_2$O).
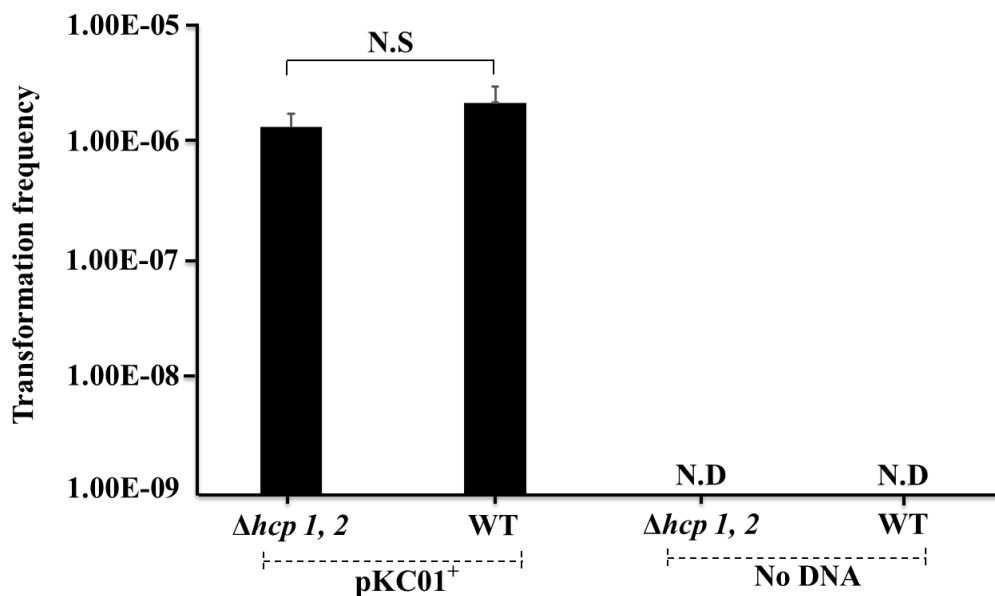
Supplementary Figure S4.2. Frequency of gene cassette integration in *V. cholerae* strains during growth on chitin. No significant (p<0.005) differences in transformation frequency was found between the WT and Δ*hcp1,*Δ*hcp2* strain unable to make T6SS using 1µg of pKC01+. N.D. indicates non-detectable transformants. Error bars represent the standard deviations of three independent experiments.

Chapter 5 : General discussion and future work

5.1 Short thesis summary

The overall goal of this thesis was to investigate the role of the *Vibrio cholerae* integron/gene cassette system in biofilm formation (chapter 3) and resistance to protozoa (chapter 4). To achieve this goal, synthetic gene cassettes were designed and created to characterise and quantify gene cassette transfer dynamics (chapter 2). Overall, this thesis has contributed new knowledge and tools to the broader scientific *V. cholerae* research community. First, a comprehensive literature review emphasizing the known mechanisms utilized by *V. cholerae* to survive and persist in the aquatic environment and the role of the integron/gene cassette system was generated identifying knowledge gaps which set up the specific aims of this thesis (chapter 1). Second, through synthesis of artificial circular and linear gene cassettes, insight into the dynamics of cassettes integration and other changes (i.e. duplication of gene cassette) in the cassette array of the *V. cholerae* chromosomal integron (chapter 2) was investigated. Additionally, chapter 2 proposes a new tool for *V. cholerae* researchers for complementation of mutations by easily integrating DNA at the *attI* site of the integron. Chapter 3 identified gene cassette candidates involved in *V. cholerae* biofilm formation, a key mechanism for environmental survival and adaptation. This chapter elucidated that the insertion of an artificial gene cassette into *attI* and two different *attC* sites caused changes in the surface properties and biofilm formation of *V. cholerae* that is most likely due to enhanced transcription of downstream gene cassettes driven by the artificial gene cassettes containing an internal promoter(s). Fourth, two bacteriovorist protozoan models including the ciliate *Tetrahymena pyriformis* and the amoeba *Acanthamoeba castellanii* were used to investigate the cassette transfer dynamics in *V. cholerae* providing fresh insight into how protozoa drive the evolution of *V. cholerae* through integron mediated lateral gene transfer (LGT) (chapter 4). This chapter showed that following internalization and packaging of *V. cholerae* into the food vacuoles (also called phagosomes) of both protozoa, intracellular ROS induces the SOS response leading to enhanced integron-integrase expression and gene cassette recombination. In addition, chapter 4 showed that co-localisation of different species such as *V. cholerae* and *E. coli* in the same phagosome can facilitate LGT between different strains and species. It was shown that *V. cholerae* utilizes its T6SS to kill and release artificial gene cassette DNA from *E. coli* to make it accessible for uptake and subsequent integron-integrase mediated integration.

In this final discussion chapter (chapter 5), two main points broad questions drawn from all the data chapters will be discussed. These are: i) Are gene cassettes important in driving surface property variation in *V. cholerae*? and ii) Are protozoal food vacuoles an important site for bacterial evolution?

5.2 Are gene cassettes important in driving surface property variation in *V. cholerae*?

As highlighted in chapter 1 (section 1.4.5.6), the integron-gene cassette system is well known for its contribution to the acquisition by bacteria of antibiotic resistance genes (Hall et al. 1996). Unlike multi-resistant integrons, which carry small gene cassette arrays of between 0 and 8 cassettes (Partridge et al. 2009), chromosomal integrons in *Vibrio* species contain large cassette arrays (>100 cassettes) mostly encoding proteins of unknown or uncharacterised function (Cambray, Guerout & Mazel 2010; Mazel 2006). Chapter 3 showed that insertion of an artificial gene cassette at three distinct positions in the cassette array impacted surface properties and biofilm formation probably from enhanced transcription of cassettes downstream of artificial cassette insertion. This is remarkable in the sense that all three insertion points, which were not targeted, impacted surface properties and biofilm formation. This raises the question as to whether a substantial proportion of gene cassettes in the *V. cholerae* integron cassette array have a role in modifying surface properties and biofilm formation. It has been previously observed that multiple strains with deletion of gene cassettes in *V. rotiferianus* DAT722 impacted surface properties and/or biofilm formation (Rapa et al. 2013) and it was proposed that at least a proportion of cassette are involved in this process. This is likely the case in *V. cholerae* and needs further investigation. How surface properties are being modified by gene cassette products is unclear, but it has been suggested that they may be modifying sugars that make up the polysaccharide structure which in turn affect its physical properties (Rapa et al. 2013). The integron is dynamic and it has been proposed that the cassette array is a bank of novel proteins waiting to be shuffled in front of the P_c promoter to allow transcription when novelty is required (Guerin et al. 2009). In support of this is the fact that the integron-integrase is induced by the SOS response that is activated under high stress conditions (Guerin et al. 2009).

The biological consequences of modifying surface properties are significant because they can affect bacterial-host interactions (Post et al. 2012), virulence (Chen et al. 2010), resistance to bacteriophage (Scholl, Adhya & Merril 2005), evasion of human immune cells (Pier et al. 2001), resistance to protozoal predation (Sun, Kjelleberg & McDougald 2013) and resistance to antimicrobial peptides (Westman et al. 2008). For example, other bacteria have proven to be masters in modifying their surface properties to evade host immune systems such as *Neisseria gonorrhoeae* (Song et al. 2000; van Vliet et al. 2009; Wetzler et al. 1992) and *Streptococcus pneumoniae* (Davis et al. 2008; Gosink et al. 2000). In these organisms, lysis of bacterial cells due to the action of immune cells during infection provides opportunities for LGT with recombination of DNA leading to genetic diversity that modifies surface properties and allowing immune system evasion. Here, it is proposed that perhaps the integron provides this same capability to *V. cholerae* and possibly, other *Vibrio* species through recombination of existing gene cassettes or acquisition of novel gene cassettes. As chapter 4 has shown the *V. cholerae* integron-integrase is substantially induced in the food vacuole of protozoa, it might be worthwhile to investigate the genetic changes that occur in the cassette array following co-incubation and determining the effect on surface properties and biofilm formation and resistance to protozoal digestion. A first step might be to use metagenomics on *V. cholerae* DNA extracted from within protozoa and comparing the changes in the cassette array such as rearrangements and deletions. Duplication of gene cassettes is another variation that can occur as observed during the integration of artificial gene cassettes experiments (chapter 2). Long-read Nanopore sequencing would be worthwhile as it would help read through the *attC* repeats to look at cassette organisation and there are techniques for targeting the specific DNA regions, in this case the integron region, so that more sequencing data could be obtained rather than filtering from the entire metagenome (Gilpatrick et al. 2019; Gilpatrick et al. 2020). For example, metagenomic DNA could be collected from co-culture containing *V. cholerae* and protozoa, then the molecular scissor Cas9 (RNA guided endonuclease) could be used to cleave the target site (e.g. integron containing gene cassette array) of *V. cholerae* chromosomal DNA and ligating Nanopore sequencing adapters directly to those sites, a method termed 'nanopore Cas9 Targeted-Sequencing' (nCATS). Additionally, enrichment of the target DNA can be achieved efficiently through utilization of biotinylated probes that target the integron region at high resolution (Dapprich et al. 2016; Hill et al. 2019; Welcher, Torres & Ward 1986). Sequencing of the enriched integron region would give more information on the variation in the cassettes array. Once these genetic changes have been identified, how they affect surface properties and survivability in the presence of protozoa can be determined using the molecular

microbiology techniques in chapter 3 (e.g. deletion of gene cassettes or overexpression). A significant limitation is the lack of an assay to accurately characterise changes to surface properties. Whilst the Congo red assay used in chapter 3 is useful, it doesn't differentiate between changes to the polysaccharide or protein on the bacteria cell surface and overall, is rather crude. Therefore, outer membrane protein analyses such as sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 2D-gel electrophoresis, Maldi-TOF/TOF and LC-MS/MS (Aldubyan et al. 2017; Rapa et al. 2013) could be conducted as well as engaging with a polysaccharide chemist to use NMR, mass spectrometry, fourier transform infrared (FT-IR) spectra techniques (Rapa et al. 2013; RuiDian et al. 2010) to characterise changes to surface polysaccharide.

5.3 Are protozoal food vacuoles an important site for bacterial evolution?

Cholera is a water-borne disease caused by the *V. cholerae*. This pathogen spends most of the time outside the human host in the aquatic environment causing periodical outbreaks of this disease (List et al. 2018). Therefore, understanding the environmental lifestyle of this pathogen is important that might provide enhanced level of knowledge on evolutionary development of pathogenic traits and mechanisms through which aquatic bacteria converted to pathogenic. Aquatic predators are recognized as a constant threat for *V. cholerae* that limits the survival of this pathogen. Protozoa take up bacterial prey into their food vacuoles that becomes filled with several toxic components including reactive oxygen species (ROS) for digestion however, ROS is also a potent inducer of the bacterial SOS response which is known to create genetic diversity through mutations, recombination and LGT (Baharoglu & Mazel 2011; Rodríguez-Rosado et al. 2018). Genetic diversity is important when bacteria are subject to selection pressure such as protozoal predation and due to the overlap of protozoal survival factors with virulence factors, studies have reported that protozoal predators contribute to the emergence of pathogens (Van der Henst et al. 2018). In this thesis (chapter 4), it was shown that protozoal predation by *T. pyriformis* and *A. castellanii* induces the SOS response which enhances genetic diversity, specifically increased transcription of the integron-integrase. This provides a solid foundation for the selection of strains that can better survive predation. It has been reported that other amoebae species such as *Dictyostelium discoideum* and ciliates *Colpodia* spp. and *Glaucom* spp. can also package and release food vacuoles containing live bacterial cells (Berk et al. 2008; Brandl et al. 2005; Faulkner et al. 2008; Gourabathini et al. 2008; Hojo et al. 2012; Koubar et al. 2011; Paquet & Charette 2016; Raghu Nadhanan & Thomas 2014; Rehfuss, Parker & Brandl 2011; Trigui et al. 2016) therefore, it would be interesting to investigate

whether other species of protozoa can induce the integron-integrase and enhance gene cassette recombination within their food vacuoles.

The SOS response is recognized to play a major role in the generation and spread of antibiotic resistance and other clinically relevant genes in bacteria and, is an important factor in the evolution of pathogenic bacteria (Baharoglu & Mazel 2014; Zgur Bertok & Podlesek 2020). Antibiotic resistance development and dissemination among bacterial population is a major concern for public health (Levy & Marshall 2004). It has been reported that several genetic elements are associated in this spread however, the class 1 integron is especially important with resistance genes commonly embedded within gene cassettes (Stokes & Hall 1989b). Similar to the chromosomal *V. cholerae* integron, integration of gene cassettes into the class 1 integron is mediated by the activity of the class 1 integrase, which is regulated by the SOS response (Guerin et al. 2009; Lacotte, Ploy & Raherison 2017). As class 1 integrons are highly prevalent in many clinically important bacteria including the Enterobacteriaceae (Ahmed, Nakano & Shimamoto 2005; Guerra et al. 2006; Rao et al. 2006), it would be interesting to test whether the class 1 integron-integrase is induced within the food vacuoles of protozoa and whether this enhances acquisition and spread of antibiotic resistance genes cassettes.

Additionally, the bacterial SOS response can induce the activity of other mobile genetic elements such as genomic islands (GIs) (Juhas et al. 2009). Studies have shown that the SOS response enhances the transfer of GIs in many bacteria (Hacker & Kaper 2000; Lindsay et al. 1998; Maiques et al. 2006) such as SaPIbov1, a pathogenicity GI of *Staphylococcus aureus* (Úbeda et al. 2005). SOS also regulates the production of CTXφ in *V. cholerae* which encodes cholera toxin, a principal virulence factor of this pathogen (Quinones, Kimsey & Waldor 2005) which, is critical in the evolution of toxigenic *V. cholerae* leading to the emergence of new pathogenic strains (Davis & Waldor 2003). Also, SOS triggers the production of phage encoding shiga-toxin (Stx) in pathogenic *E. coli* (Nassar et al. 2013). As a result, it would be exciting to test whether movement of other mobile genetic elements is enhanced during co-incubation with protozoa and whether this is due to the SOS response. As it has recently been shown that *V. cholerae* and other bacteria are released into the environment as ejected food vacuoles (EFVs) by protozoa and that these packaged cells are more infectious *in vivo* by better surviving transit through animal guts (Espinoza-Vergara et al. 2019), it may be interesting to investigate whether EFVs provide enhanced opportunity for "testing" the fitness of genetic variants as they pass through animal hosts. For example, metagenomic DNA sequencing of *V.*

cholerae in EFVs could be used to determine the level of genetic variation and then passage through a selection pressure (e.g. mouse animal model) could be done to identify specific variants associated with fitness advantages to the selective pressure. Furthermore, it would be expected that the level of genetic variation within EFVs would be higher than cells cultured in media without protozoa.

The human gut microbiota is considered as a complex ecosystem where diverse species of bacteria, viruses (mostly bacteriophages), fungi, parasites/protozoa exist and interact and compete with each other (Filyk & Osborne 2016). While *V. cholerae* cause millions of cholera cases each year globally they are usually not the only pathogen within the gut of the infected. Intestinal parasites are also common in developing areas when cholera is endemic. For example, a study conducted by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) reported that at least 15 % of patients were infected with parasites among 361 cholera patients (Harris et al. 2009). Similar reports have found in India, Nepal, Peru, Latin Americas and other developing countries, where they reported that a high rate of mixed infection with amoeba parasites such as *Giardia lamblia, Cryptosporidium* sp and *Entamoeba histolytica* and, *V. cholerae* in young children that results in severe diarrhoea and recurrent infection (Berendes et al. 2019; Mukherjee et al. 2009; Mukherjee et al. 2014; Tamang et al. 2005; Zerpa & Huicho 1995). Additionally, studies have noted that gut amoeba parasite such as *E. histolytica* interacts with enterobacteria within the human gut and consume bacteria by phagocytosis (Guillén 2019; Iyer et al. 2019; Shaulov & Ankri 2020; Verma et al. 2012). Therefore, it is speculated that such interaction between *V. cholerae* and other bacterial pathogens and protozoa inside the human gut may play an important role in evolution of bacteria through LGT. In particular, the dissemination of antibiotic resistance genes may be enhanced in people with gut parasites. A study conducted in Mozambique showed a link between parasitic pathogen carriage and antimicrobial resistance genes (ARGs) detection in stool samples of young children gut conferring resistance to a first-line drugs (e.g. tetracyclines, β-lactams, fluoroquinolones and aminoglycosides) for multiple infections, including enteric infections (Berendes et al. 2019). Although highly speculative, parasites may enhance ARG transfer in the gut microbiome and would be an exciting future avenue of research.

5.4 Summary of future directions

In summary, future work should focus on using protozoa to creating diversity in the gene cassette array following co-incubation with the protozoa and elucidating their effect on surface properties and biofilm formation and resistance to protozoal digestion. Regarding LGT in *V. cholerae* within the protozoa, future work should focus on whether other protozoal species can induce the integron-integrase and enhance gene cassette recombination within their food vacuoles and, impact the activation and movement of other mobile genetic elements such as the class 1 integrons, genomic islands and lysogenic bacteriophage. In addition, metagenomic sequencing of *V. cholerae* in EFVs would help identify genetic variants generated from passage through protozoa and then test their fitness to various selection pressures. Finally, the co-existence of bacteria and parasites (e.g. amoeba *E. histolytica*) in the human gut and the phagocytosis of bacteria by parasites provides an ideal opportunity to investigate whether parasitic infections enhances transfer of antibiotic resistance and virulence genes in bacteria in the gut environment.

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Appendices

Appendix 2.1. List of unique and paralogous gene cassettes of *V. cholerae* A1552 and N16961 integron. Cassette were grouped if they shared equal or greater than an 80% nucleotide identity.

N16961	A1552	Casstte type	N16961	A1552	Casstte type
VCH 1	Absent	Paralogous	VCH 44	VCH 44	Paralogous
VCH 2	Absent	Paralogous	VCH 45	Absent	Unique
VCH 3	VCH 3	Unique	VCH 46	VCH 46	Paralogous
VCH 4	VCH 4	Unique	VCH 47	VCH 47	Paralogous
VCH 5	VCH 5	Unique	VCH 48	VCH 48	Unique
VCH 6	VCH 6	Paralogous	VCH 49	VCH 49	Unique
VCH 7	VCH 7	Paralogous	VCH 50	VCH 50	Paralogous
VCH 8	VCH 8	Unique	VCH 51	VCH 51	Paralogous
VCH 9	VCH 9	Unique	VCH 52	VCH 52	Paralogous
VCH 10	VCH 10	Unique	VCH 53	VCH 53	Unique
VCH 11	VCH 11	Paralogous	VCH 54	VCH 54	Paralogous
VCH 12	VCH 12	Paralogous	VCH 55	VCH 55	Unique
VCH 13	VCH 13	Paralogous	VCH 56	VCH 56	Unique
VCH 14	VCH 14	Paralogous	VCH 57	VCH 57	Unique
VCH 15	VCH 15	Unique	VCH 58	VCH 58	Paralogous
VCH 16	VCH 16	Unique	VCH 59	VCH 59	Unique
VCH 17	VCH 17	Unique	VCH 60	VCH 60	Paralogous
VCH 18	VCH 18	Unique	VCH 61	VCH 61	Unique
VCH 19	VCH 19	Paralogous	VCH 62	VCH 62	Unique
VCH 20	VCH 20	Unique	VCH 63	VCH 63	Paralogous
VCH 21	VCH 21	Paralogous	VCH 64	VCH 64	Unique
VCH 22	VCH 22	Paralogous	VCH 65	VCH 65	Paralogous
VCH 23	VCH 23	Paralogous	VCH 66	VCH 66	Paralogous
VCH 24	VCH 24	Paralogous	VCH 67	VCH 67	Unique
VCH 25	VCH 25	Paralogous	VCH 68	VCH 68	Unique
VCH 26	VCH 26	Unique	VCH 69	VCH 69	Unique
VCH 27	VCH 27	Unique	VCH 70	VCH 70	Paralogous
VCH 28	VCH 28	Paralogous	VCH 71	VCH 71	Paralogous
VCH 29	VCH 29	Paralogous	VCH 72	VCH 72	Unique
VCH 30	VCH 30	Paralogous	VCH 73	VCH 73	Paralogous
VCH 31	VCH 31	Paralogous	VCH 74	VCH 74	Unique
VCH 32	VCH 32	Paralogous	VCH 75	VCH 75	Paralogous
VCH 33	VCH 33	Paralogous	VCH 76	VCH 76	Paralogous
VCH 34	VCH 34	Paralogous	VCH 77	VCH 77	Unique
VCH 35	VCH 35	Unique	VCH 78	VCH 78	Unique
VCH 36	VCH 36	Unique	VCH 79	VCH 79	Paralogous

VCH 37	VCH 37	Unique	VCH 80	VCH 80	Paralogous
VCH 38	VCH 38	Unique	VCH 81	VCH 81	Unique
VCH 39	VCH 39	Paralogous	VCH 82	VCH 82	Unique
VCH 40	VCH 40	Paralogous	VCH 83	VCH 83	Paralogous
VCH 41	VCH 41	Paralogous	VCH 84	VCH 84	Paralogous
VCH 42	VCH 42	Paralogous	VCH 85	VCH 85	Paralogous
VCH 43	VCH 43	Unique	VCH 86	VCH 86	Unique
VCH 87	VCH 87	Paralogous	VCH 133	VCH 133	Paralogous
VCH 88	VCH 88	Unique	VCH 134	VCH 134	Paralogous
VCH 89	VCH 89	Unique	VCH 135	VCH 135	Unique
VCH 90	VCH 90	Paralogous	VCH 136	VCH 136	Unique
VCH 91	VCH 91	Unique	VCH 137	VCH 137	Unique
VCH 92	VCH 92	Paralogous	VCH 138	VCH 138	Unique
VCH 93	VCH 93	Paralogous	VCH 139	VCH 139	Unique
VCH 94	VCH 94	Unique	VCH 140	VCH 140	Paralogous
VCH 95	VCH 95	Unique	VCH 141	VCH 141	Unique
VCH 96	VCH 96	Paralogous	VCH 142	VCH 142	Unique
VCH 97	VCH 97	Unique	VCH 143	VCH 143	Unique
VCH 98	VCH 98	Paralogous	VCH 144	VCH 144	Paralogous
VCH 99	VCH 99	Paralogous	VCH 145	VCH 145	Paralogous
VCH 100	VCH 100	Paralogous	VCH 146	VCH 146	Unique
VCH 101	VCH 101	Unique	VCH 147	VCH 147	Paralogous
VCH 102	VCH 102	Unique	VCH 148	VCH 148	Paralogous
VCH 103	VCH 103	Paralogous	VCH 149	VCH 149	Paralogous
VCH 104	VCH 104	Unique	VCH 150	VCH 150	Paralogous
VCH 105	VCH 105	Paralogous	VCH 151	VCH 151	Paralogous
VCH 106	VCH 106	Paralogous	VCH 152	VCH 152	Paralogous
VCH 107	VCH 107	Paralogous	VCH 153	VCH 153	Unique
VCH 108	VCH 108	Paralogous	VCH 154	VCH 154	Unique
VCH 109	VCH 109	Unique	VCH 155	VCH 155	Unique
VCH 110	VCH 110	Paralogous	VCH 156	VCH 156	Paralogous
VCH 111	VCH 111	Unique	VCH 157	VCH 157	Unique
VCH 112	VCH 112	Paralogous	VCH 158	VCH 158	Paralogous
VCH 113	VCH 113	Unique	VCH 159	VCH 159	Unique
VCH 114	VCH 114	Unique	VCH 160	VCH 160	Paralogous
VCH 115	VCH 115	Paralogous	VCH 161	VCH 161	Unique
VCH 116	VCH 116	Paralogous	VCH 162	VCH 162	Paralogous
VCH 117	VCH 117	Paralogous	VCH 163	VCH 163	Unique
VCH 118	VCH 118	Paralogous	VCH 164	VCH 164	Unique
VCH 119	VCH 119	Paralogous	VCH 165	VCH 165	Unique
VCH 120	VCH 120	Paralogous	VCH 166	VCH 166	Unique
VCH 121	VCH 121	Unique	VCH 167	VCH 167	Unique

VCH 122	VCH 122	Unique	VCH 168	VCH 168	Unique
VCH 123	VCH 123	Paralogous	VCH 169	VCH 169	Paralogous
VCH 124	VCH 124	Paralogous	VCH 170	VCH 170	Unique
VCH 125	VCH 125	Paralogous	VCH 171	VCH 171	Unique
VCH 126	VCH 126	Paralogous	VCH 172	VCH 172	Unique
VCH 127	VCH 127	Unique	VCH 173	VCH 173	Unique
VCH 128	VCH 128	Unique	VCH 174	VCH 174	Paralogous
VCH 129	VCH 129	Paralogous	VCH 175	VCH 175	Unique
VCH 130	VCH 130	Paralogous	VCH 176	VCH 176	Paralogous
VCH 131	VCH 131	Unique	VCH 177	VCH 177	Paralogous
VCH 132	VCH 132	Unique	VCH 178	VCH 178	Paralogous

Appendix 2.2. List of paralogous gene cassettes in both *V. cholerae* A1552 and N16961 integron. Cassette were grouped if they shared equal or greater than an 80% nucleotide identity.

Paralogous set	Cassette	Paralogous set	Cassette	Paralogous set	Cassette
P1	VCH 14	P3	VCH 90	P14	VCH 19
	VCH 123		VCH 169		VCH 80
	VCH 75		VCH 58	P15	VCH 51
	VCH 125		VCH 63		VCH 176
	VCH 59	P4	VCH 23	P16	VCH 22
	VCH 103		VCH 130		VCH 108
	VCH 72		VCH 98	P17	VCH 124
	VCH 110		VCH 52		VCH 177
	VCH 161	P5	VCH 93	P18	VCH 129
	VCH 13		VCH 158		VCH 156
	VCH 25		VCH 50	P19	VCH 92
	VCH 174		VCH 178		VCH 160
	VCH 96	P6	VCH 116	P20	VCH 12
	VCH 140		VCH 134		VCH 150
	VCH 147		VCH 60	P21	VCH 71
	VCH 99		VCH 105		VCH 73
	VCH 11	P7	VCH 21	P22	VCH 41
	VCH 87		VCH 151		VCH 106
	VCH 6		VCH 152	P23	VCH 165
	VCH 39		VCH 117	P24	VCH 100
	VCH 54	P8	VCH 47		VCH 144
	VCH 79		VCH 118	P25	VCH 32
	VCH 34		VCH 133		VCH 149
	VCH 85	P9	VCH 30	P26	VCH 112
P2	VCH 28		VCH 44		VCH 120
	VCH 33		VCH 148	P27	VCH 76
	VCH 70	P10	VCH 7		VCH 170
	VCH 107		VCH 46		
	VCH 115		VCH 31		
	VCH 83	P11	VCH 40		
	VCH 42	P12	VCH 126		
	VCH 84		VCH 24		
	VCH 145		VCH 162		
	VCH 29	P13	VCH 66		
	VCH 119				

Appendix 2.3. PCR cartography of chitin transformants carrying the integrated circular (pKC01$^+$ and pKC01$^-$) and linear (gc_kan) gene cassettes.

Donor DNA	Transformants	IntIA-VCH 3 600 bp	VCH 3-10 4296 bp	VCH 10-20 7616 bp	VCH 20-27 4810 bp	VCH 26-38 7760 bp	VCH 38-49 7681 bp	VCH 48-57 6557 bp	VCH 57-64 4611 bp	VCH 64-69 7731 bp	VCH 69-82 8219 bp	VCH 81-91 7986 bp	VCH 91-102 6964 bp	VCH 101-114 8078 bp	VCH 113-122 5746 bp	VCH 121-128 5802 bp	VCH 127-135 6323 bp	VCH 135-146 8756 bp	VCH 146-159 8939 bp	VCH 159-168 6732 bp	VCH 167-175 7036 bp	VCH 175-IS3 transposase 2786 bp
pKC01 +	1					pKC01*																
	2	pKC01*																				
	3					pKC01*																
	4	~1500				pKC01*																
	5	pKC01*																				
	6					pKC01*																
	7	pKC01*																				
	8					pKC01*																
	10	pKC01*																				
pKC01 -	1								pKC01$^-$													
	2	pKC01$^-$																				
	3	pKC01$^-$																				
	4	pKC01$^-$																				
	5	pKC01$^-$																				
	6								pKC01$^-$													
	7								pKC01$^-$													
	8	~1500							pKC01$^-$													
	9								pKC01$^-$													
	10	pKC01$^-$																				
gc_kan	1	1500 bp																				
	2	1500 bp																				
	3	1500 bp																				
	4	1500 bp																				
	5	1500 bp																				
	6	1500 bp																				
	7	1500 bp																				
	8	1500 bp																				
	9	1500 bp																				
	10	1500 bp																				

Insertion site of circular (pKC01) and linear gene cassette (gc_kan) into the integron platform of V. cholerae A1552

■ (grey) Presence of target amplicon compared to wild strain that indicates no changes occurred in the target cassette

■ (yellow) Absence of target amplicon that indicates changes in the target cassette due to either insertion of donor cassette and deletion or movement of existing cassettes across

■ (blue) Larger amplicon length compared to the wild type strain

Appendix 2.4. Assembly statistics of pKC01$^+$ chitin transformant genomes.

Genome	Contigs	Size	Depth	Circular?
Transformant 1	2	3,015,076	1.0x	Yes
		1,077,123	1.0x	Yes
Transformant 2	2	3,015,176	1.0x	No
		1,077,123	1.0x	Yes
Transformant 3	2	3,015,091	1.0x	Yes
		1,077,123	0.97x	Yes
Transformant 4	2	3,015,090	1.0x	Yes
		1,078,001	1.0x	Yes
Transformant 6	2	3,015,140	1.0x	Yes
		1,077,251	1.0x	No
Transformant 10	4	3,014,748	1.0x	Yes
		981,682	0.93x	Yes
		55,709	0.40x	No
		29,834	0.40x	No

Appendix 3.1. Putative conserved domains, sub-cellular location and functional annotation of deleted gene cassettes products

Gene cassette	BlastX				PSORTb	UniProt
	Putative conserved domains	E-value	Score	Identity	**Sub-cellular localization**	**Feature key**
VCH3	No putative conserved domain	1.63-18	189	50%	Cytoplasmic membrane	Transmembrane
VCH4	No putative conserved domain	8.0e-65	201	100%	Unknown	Unknown
VCH5	No putative conserved domain	4.6e-42	359	100%	Unknown	Unknown
VCH36	No putative conserved domain	8.0e-159	448	100%	Cytoplasmic	Unknown
VCH37	Ribonuclease toxin, BrnT, of type II toxin-antitoxin system	9e-55	175	100%	Unknown	Unknown
	CopG transcriptional regulator	5.0e-42	141	98.77%	Unknown	Unknown
VCH38	No putative conserved domain	2.0e-167	473	100%	Unknown	Unknown
VCH61	Transcriptional regulator, contains Arc/MetJ-type RHH (ribbon-helix-helix) DNA-binding domain	5.0e-103	404	100%	Unknown	Unknown
VCH62	No putative conserved domain	5.0e-120	348	100%	Cytoplasmic	Unknown
VCH63	No putative conserved domain	6.0e-54	172	100%	Cytoplasmic membrane	Unknown

Appendix 3.2. Sub-cellular location of deleted gene cassettes products

Gene cassette	PSORTb (Score)				
	Cytoplasmic	Cytoplasmic membrane	Periplasmic	Outer membrane	Extracellular
VCH3	0.04	**9.82**	0.12	0.01	0.01
VCH4	2.0	2.0	2.0	2.0	2.0
VCH5	2.0	2.0	2.0	2.0	2.0
VCH36	**8.96**	2.0	2.0	2.0	2.0
VCH37	2.0	2.0	2.0	2.0	2.0
VCH38	2.0	2.0	2.0	2.0	2.0
VCH61	2.0	2.0	2.0	2.0	2.0
VCH62	**8.96**	0.51	0.26	0.01	0.26
VCH63	0.04	**9.82**	0.12	0.01	0.01