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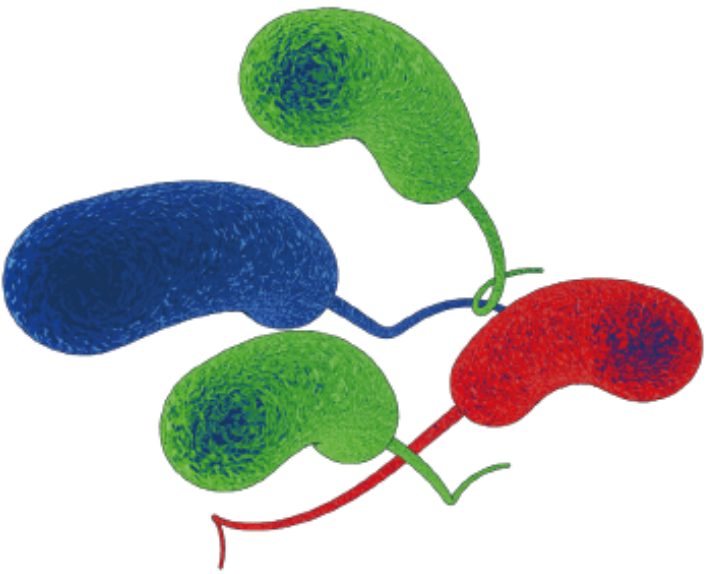

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DEVELOPMENT OF NOVEL VACCINE STRAINS OF *VIBRIO CHOLERAE* AND STUDIES ON THE ROLE OF SEROTYPE IN EPIDEMIC SPREAD OF CHOLERA

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Development of novel vaccine strains of *Vibrio cholerae* and studies on the role of serotype in epidemic spread of cholera

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You and I may not live to see the day, and my name may be forgotten when it comes; but the time will arrive when great outbreaks of cholera will be things of the past; and it is the knowledge of the way in which the disease is propagated which will cause them to disappear.

John Snow 1855

ABSTRACT.

Cholera, caused by bacterium *Vibrio cholerae* O1, is a severe diarrheal disease with an estimated 3-5 million cases and more than 140 000 deaths every year particularly affecting children under 5 years of age.

It can be found all over the world and often causes cholera in places where access to clean water or proper sanitary facilities are limited or compromised. Typically cholera follows in the wake of natural disasters or man-made catastrophes but it is also endemic in many countries including India and Bangladesh.

Today there are two licensed vaccines available on the market in more than 60 countries. Despite the fact that these vaccines are effective they are both expensive and complicated to manufacture and there is scope and motivation for creating a new cheaper and more effective vaccine against cholera.

First, we have shown that it is possible by genetic manipulation to generate a single strain vaccine expressing two phenotypically different phenotypes and shown that the candidate vaccine strains elicit similar immune responses as the current licensed vaccine Dukoral. This is a huge benefit since it will significantly simplify manufacture and reduce production costs.

Further, we have investigated the naturally occurring Inaba serotype mutants and generated a hypothesis as to why O1 serogroup *Vibrio cholerae* maintains a serotype polymorphism. We have conducted a unique study where we could show that selective pressure on the circulating strains in the environment is almost certainly what is driving serotype transition.

Taken together, results from this thesis show how the use of bioinformatics can be used to target genes and even specific amino acids for mutagenesis in order to modify the phenotype of a vaccine strain and understand the unique and fundamental role of serotype with respect to epidemic and endemic cholera.

LIST OF PAPERS

This thesis is based on the following scientific papers, which are referred to in the text by their Roman numerals (I–IV).

Paper I **Construction of Novel Vaccine Strains of *Vibrio cholerae* Co–expressing the Inaba and Ogawa Serotype Antigens.**

Michael Lebens, Stefan L Karlsson, Susanne Källgård, Margareta Blomquist, Annelie Ekman, Erik Nygren, Jan Holmgren.
Vaccine, 2011. 29(43): p. 7505–13.

Paper II **Development of Stable *Vibrio cholerae* O1 Hikojima Type Vaccine Strains Co–expressing the Inaba and Ogawa Lipopolysaccharide Antigens.**

Stefan L Karlsson, Elisabeth Ax, Erik Nygren, Susanne Källgård, Margareta Blomquist, Annelie Ekman, John Benktander, Jan Holmgren, Michael Lebens.
Published in PLoS ONE 2014–09–28.

Paper III **The evolution of O1 *Vibrio cholerae* during annual cholera outbreaks in an endemic setting.**

Stefan L Karlsson, Nicholas Thomson, Ankur Mutreja, Thomas Connor, Dipika Sur, Mohammad Ali, John Clemens, Gordon Dougan, Jan Holmgren, and Michael Lebens.
Submitted.

Paper IV **Non-radom distribution of mutations leading to the Inaba serotype in O1 *Vibrio cholerae* from the El Tor lineage of 7th pandemic.**

Stefan L Karlsson and Michael Lebens
Manuscript.

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Paper not included in the thesis

Alkaline pH is a signal for optimal production and secretion of the heat labile toxin, LT in enterotoxigenic Escherichia coli (ETEC).

Gonzales L, Ali ZB, Nygren E, Wang Z, Karlsson S, Zhu, B. Quiding–Jarbrink, M. Sjoling, A

PLoS One, 2013. 8(9): p. e74069.

CONTENTS

Abbreviations

Ace	Accessory cholera enterotoxin
AI–2	Autoinducer 2
bp	base pair
BSA	Bovine serum albumin
CAI–1	Cholera Autoinducer 1
cAMP	cyclic Adenosine Monophosphate
CFA	Freund′s complete adjuvant
CFR	Case–Fatality Rate
CT	Cholera Toxin
CTA	Cholera toxin A subunit
CTB	Cholera toxin B subunit
dmLT	double mutant LT
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FRT	Flippase Recognition Target
HA	Hemagglutinin A
ID	Intradermal
IFA	Freund′s incomplete adjuvant
Km^R	Kanamycin Resistance
LB	Luria Bertani
LPS	Lipopolysaccharide
LT	Heat labile toxin
MCS	Multi Cloning Region
mmCT	multi-mutant Cholera Toxin
O–PS	O–specific Polysaccharide
ORF	Open Reading Frame
ORT	Oral Rehydration Therapy
PCR	Polymerase Chain Reaction
RS	Repeat Sequence
SAM	S-Adenosyl Methionine
SC	Subcutaneous
SEM	Standard Error of Mean
sIgA	secretory IgA Antibodies
TCP	Toxin Co–regulated Pilus
TLR	Toll–Like Receptor
VPI	Vibrio Pathogenicity Island
Zot	Zonula occludens toxin

1. INTRODUCTION

Cholera is the most severe of the bacterial diarrheal diseases and has affected human populations for centuries; since the beginning of the 19th century it has spread throughout the world. Today there are an estimated 1.4–4.3 million cases of cholera annually world–wide with more than 140 000 deaths with a case–fatality rate (CFR) up to 5% during 2013. These numbers however are likely to be heavily under–estimated due to limitations in surveillance systems, lack of laboratory diagnostics or fear of a negative impact on travel and trade [1]. It is estimated that 2.5 billion people are living with the risk of cholera and other diarrhoeal diseases [1]. Cholera is caused by the bacterium *Vibrio cholerae* and is an acute, often severe diarrheal disease which can be fatal. It is particularly dangerous for young children. The bacterium has been studied ever since it was first isolated in the 19th century [2] but it is only in recent years that tools have developed that allow the characteristics important for their ability to cause cholera and to spread on a pandemic scale to be investigated at a molecular level. The central aim of this thesis was to generate novel strains of *Vibrio cholerae* for inclusion in a new generation of killed whole cell vaccines. In the course of this work however, it has become apparent that the same genes targeted for the development of the vaccine strains may well be of pivotal importance in the ability of the bacteria to spread and cause pandemic disease.

1.1. Cholera

Cholera is a diarrheal disease that has spread throughout the globe and outbreaks often follow in the wake of natural or man–made disasters [3, 4]. In many parts of the world, particularly in areas with poor access to fresh water, cholera is endemic meaning that it resides in the local environment and outbreaks tend to be seasonal. In contrast, epidemic cholera in non–endemic areas is introduced from elsewhere into a population and new outbreaks require a re–introduction from outside [5]. Cholera–like symptoms have been described in records from more than

2000 years ago and cholera–like disease has probably been present in some places in the world since humans started to live in large cities with poor sanitary conditions [6, 7].

1.1.1. The Disease – symptoms, diagnosis and treatment

Cholera is caused by the bacteria *Vibrio cholerae* and is spread via contaminated water and food and poor sanitation. The incubation period can be as short as 2 hours and as long as 5 days [3]. About 75% of all cases are asymptomatic and about 20% of those infected only get mild or moderate symptoms whereas 5% can get severe diarrhoea with purging of up to 2 litres of fluid per hour leading to dehydration and in the worse cases to anuria, acidosis, shock and death [3, 4]. The typical rice water stool of an infected patient is infested with the bacteria and infected individuals can shed up to 10^{13} bacteria per day [8] and continue to shed bacteria for one to two weeks [9]. The severity of cholera is dependent on several different factors such as size of the inoculum, the presence or absence of pre–existing immunity or blood group [8]. *V. cholerae* primarily affects the small intestine where it colonizes and secretes the powerful enterotoxin, cholera toxin (CT) which is largely responsible for the characteristic watery diarrhoea of severe cholera. The disease is commonly diagnosed by isolating *V. cholerae* from the stools of infected individuals and confirmed by agglutination tests with *V. cholerae* specific antisera [10]. About 80% of all cases are successfully treated with oral rehydration therapy (ORT). Severe cases are treated with intravenous hydration and sometimes with antibiotics [4].

Primary measures for prevention include improving sanitary conditions and providing clean water as well as education and encouragement of behavioural changes that reduce the risk of infection [1]. During severe outbreaks vaccination programs have been shown to limit the spread of *V. cholerae* [11-14].

1.1.2. History of cholera

The bacterium responsible for cholera was first discovered and associated with the disease by the Italian scientist Filippo Pacini in 1854. His findings were largely ignored owing to the prevalence of the Miasma Theory at the time and it was not until Louis Pasteur formulated the germ theory in 1862 and Robert Koch could show the correlation between *Vibrio cholerae* and cholera in 1883 that Pacini's finding was finally acknowledged [2, 15-17].

Even if cholera has a long history, it is believed that it was not until the industrial revolution and the introduction of fast travel over large distances (initially in steamships) that cholera could spread and cause worldwide pandemics. Cholera was originally confined to the Indian subcontinent, but since 1816 there have been seven documented pandemics (in which the disease has occurred in populations over a wide area often on different continents) which have killed tens of millions of people. The first pandemic (1816–1826) began in the Ganges river Delta in the region of Bengal [17] and spread across India reaching China, Indonesia and as far as the Caspian Sea. In the five subsequent pandemics (1829–1923) cholera outbreaks were also recorded in Europe and in the Americas [17-19].

These six first pandemics were caused by organisms of the classical biotype whereas the seventh pandemic which started in the early 1960s and is ongoing was marked by the emergence of a new type of organism of the so called El Tor biotype, named after the quarantine station in Sinai, Egypt where it was first isolated in 1905 from six pilgrims returning from Mecca [20, 21].

It is thought that El Tor biotype organisms which were first distinguished due to their haemolytic properties [22], first emerged in Indonesia causing local outbreaks for a long period before rapidly spreading outwards at the beginning of the 1960s, so that by the end of 1962 epidemics had affected South Asia and India [19, 22]. This was the start of the 7th pandemic and by 1970 it had spread to Europe and Africa and was introduced into South America during the 1980s [19, 23]. It is uncertain why the transition from classical to El Tor occurred, but in a matter of a few years classical O1

Vibrio cholerae had disappeared as a cause of pandemic cholera. As will be discussed later, the two biotypes of O1 *Vibrio cholerae* are quite distinct and genomic analysis indicates that they are only distantly related [23].

Since there was some overlap between the classical 6th pandemic and El Tor 7th pandemic at least in Bangladesh (then East Pakistan) several studies have suggested that the differences between the two biotypes might favour the emergence of El Tor organisms. They were suggested to be more adapted to the environment, less virulent causing milder or asymptomatic infections, and giving less protection against future re–infection [19, 24]. However, infection with either El Tor or classical biotype O1 *Vibrio cholerae* can be equally life–threatening [19] and protection against reinfection appears to be equally robust.

1.1.3. The father of modern epidemiology

John Snow is widely acknowledged as the father of modern epidemiology for his work on cholera during the 1850s in England and it was he who established the connection between cholera and contaminated water. During his life–time however, Snow gained more recognition from contemporary physicians for his work on chloroform and other anaesthetics than for his work on cholera.

He published his first hypothesis "On the Mode of Communication of Cholera" in 1849 in which he built his arguments on data published by William Farr during the second outbreak of cholera (1848–1849) in England. He observed that there were more deaths from cholera in the southern districts of London than in all the other districts. He attributed this to poorer water quality due to the fact that their water was taken from the river Thames further downstream were it was more polluted. However Farr, the dominant epidemiologist of the time and an advocate of the Miasma Theory (of disease being caused by bad air) disagreed and stated ′while the effect of water of the districts are apparent, they do not, in this analysis, conceal the effects of elevation′. A modern re–analysis of William Farr's data unsurprisingly supports Snows′ contention that contaminated water sources were the most likely transmission route [25].
Snow however remained undeterred and continued his investigations and the search for evidence that supported the water transmission theory.

During the outbreak in 1854 he created a map of all the cases of cholera. Even if he was not the first to create this kind of map where all the cases were visualized with bars or numbers he was the first to add a Voronoi diagram to a map using the thirteen pumps in the neighbourhood as points with the distance from each pump being calculated based on the time it took to travel to each one on foot [16]. This helped identify the Broad street pump as the most likely source of the outbreak and he managed to convince the committee of health to remove the handle of the pump and thereby prevent further cases.

With the help of Henry Whitehead, who was originally sceptical towards Snow's water based theory, it was possible to identify a possible index case since faecal excrement from an infant with fatal diarrhoea had been disposed of in a cesspool that had started to leak into the fresh water supply of the Broad street pump [16]. Many have argued that removal of the handle was not what stopped the outbreak since it has already started to subside. Whereas others argue that the removal of the handle stopped a second wave since the father of the infant identified as the index case had fallen ill within hours of the removal and the connecting cesspit was again contaminated with faeces containing O1 *V. cholerae* [16].

1.2. *Vibrio cholerae* and cholera disease

Vibrio cholerae is a ubiquitous slightly curved gram negative rod–shaped bacterium with a single flagellum and has more than 200 different serogroups [26, 27]. Although many are pathogenic and can cause outbreaks of diarrhoea, until 1992 only one, serogroup O1, was documented to have caused epidemic cholera. In 1992 a new serogroup, O139, was isolated in India [28] and caused major outbreaks in the following years in the area of the bay of Bengal [29]. However these clones rapidly subsided and no cases of O139 serogroup have been reported in India since 2007 [30] and during 2013 only China reported cases of O139 [1], whereas the O1 serogroup continues to cause seasonal epidemics and has recently caused major epidemics in different parts of the world including Zimbabwe and Haiti [31-33].

1.2.1. Mode of action.

After ingestion, *V. cholerae* colonizes the small intestine, relying on several features including the colonization factor toxin co–regulated pilus (TCP), as well as a battery of secreted proteins including hemagglutins, and other proteases, and it's single flagellum [34, 35]. With the help of the flagellum the bacteria travels from the intestinal lumen into the mucus layer where proteases such as hemagglutin A are secreted which break down mucin and makes it easier for the bacteria to pass through the mucus layer. There, in close proximity to the epithelium it secretes cholera toxin (CT). As the immune system starts to respond, the intestine will start to secrete IgA antibodies both against CT and lipopolysaccharide (LPS). These will block CT's ability to bind to epithelial cells and, as will be discussed later, help clear the bacteria (Figure 1).

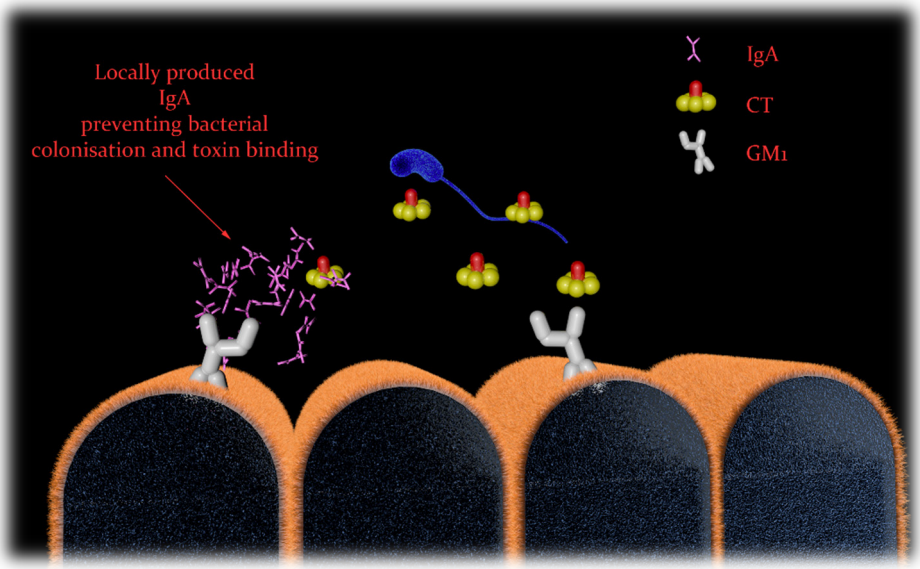

Figure 1 Schematic drawing of V. cholerae close to the epithelial cells in the intestine where it releases its cholera toxin.

1.2.2. O1 Vibrio cholerae biotypes and serotypes

Epidemic cholera strains can be divided into two biotypes, classical and El Tor, both of which have the O1 serotype. The classical biotype caused the first six recorded pandemics and was replaced as the sole agent causing cholera by the El Tor biotype currently causing the 7th pandemic which started in 1962.

Several criteria can be used to differentiate between the two biotypes. El Tor strains are strongly haemolytic, agglutinate with chicken erythrocytes, are resistance to polymyxin B, and generate a positive result in the Voges–Proskauer reaction. The test for haemolytic strains is not conclusive and non–haemolytic El Tor strains have been isolated [36]. The resistance to polymyxin B is likely to be an effect of the gene *msbB* that encodes an acyltransferase that modifies the lipid A in the LPS by incorporation of positively charged groups which reduce the affinity of antimicrobial peptides [37]. A Voges-Proskauer test indicates that the bacteria strains produce 2, 3–butanediol instead of organic acids as their fermentation end product. El Tor strains yield a positive reaction whereas classical strains give a negative reaction [38].

There is also only an 81% homology between El Tor and classical TCP which results in slightly different structures that can be detected by differentiating monoclonal antibodies [39, 40].

Importantly, the patterns of *in vitro* expression of important virulence factors such as CT and TCP require very different growth conditions [41, 42] suggesting that global regulation of virulence in the two biotypes is fundamentally different even if they appear to share the same regulatory genes.

People of blood group O are for unknown reasons at higher risk of severe cholera from *V. cholerae* of the El Tor biotype than those of other blood groups. The prevalence of the blood group O is considerably lower in the Bay of Bengal than in outer parts of the world. Cholera is believed to have resided in the Bengal area over thousands of years and exerted its selective pressure on O group individuals [43]. Since this effect is not seen for classical cholera, the emergence of classical cholera in the 19th century

may have been an exceptional event and the prevalent strains in the Bay of Bengal before that were presumably of the El Tor biotype. The overall risk of infection is not associated with blood group [44] so that the sensitivity of O blood group individuals to El Tor strains is not thought to have contributed to the pandemic spread of the disease in the 19th century.

Of particular interest is the fact that both biotypes have the same O1 serogroup. The O1 serogroup can be divided into two serologically distinguishable serotypes, Ogawa and Inaba named after the family name of those it was first isolated from in Japan [45]. It was found that the Ogawa serotype differs from the Inaba serotype only in the methylation of the terminal sugar of the O–antigen of the surface LPS [46]. This methylation is catalysed by the product of the *wbeT* gene (formerly called *rfbT* [47]) If this gene is inactivated by mutation, truncation, insertion or deletion methylation does not occur resulting in the Inaba phenotype. A third, Hikojima phenotype (named after the quarantine station in northern Kyushu in Japan where such strains were first isolated [45]) is described in the literature as seldom occurring but that expresses both methylated and non–methylated LPS. However, such cultures often prove to be a mixture of different strains or an unstable strain in transition from Ogawa to Inaba [46]. The molecular basis of this phenomenon will be discussed later at greater length.

1.2.3. Lipopolysaccharides biosynthesis

V. cholerae, in common with all gram negative bacteria, has two distinct membranes separated by a periplasmic space. The outer membrane of gram negative bacteria contains LPS which in *V. cholerae* also coats the single flagellum [48]. LPS is a heterogeneous group of large molecules containing a lipid (lipid A) which is an integral part of the outer membrane, and a polysaccharide. The polysaccharide is composed of an inner core linking it covalently to the lipid and an outer core situated between the inner core and the highly variable O–antigen (Figure 2) [49]. LPS is highly immunogenic in animals with antibodies primarily elicited against the polysaccharide and generally directed against the O–antigen.

The difference in the different serogroups of *V. cholerae* is based on differences in the structure of the O–antigen.

Most of our knowledge of LPS biosynthesis comes from studies in other organisms, particularly *Escherichia coli,* but the studies done in *V. cholerae* show that there are many similarities with other gram negative organisms [46].

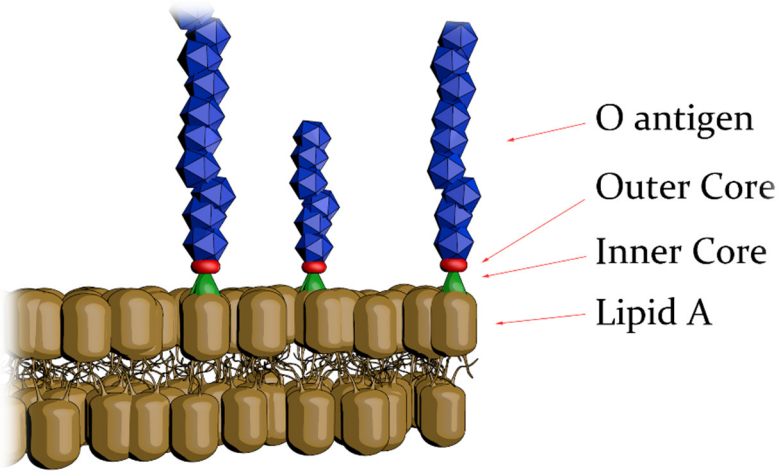

Figure 2 Schematic drawing of the lipopolysaccharide (LPS) three parts; Lipid A, core, and O–antigen.

1.2.3.1. Lipid A

The structure of lipid A and the enzymes catalysing its synthesis and transport are more conserved between different serogroups and even between different species than the core or O–antigen [49]. From studies in *E. coli* it is known that lipid A is responsible for toxicity during infection since it triggers an innate inflammatory response by interaction with host Toll–Like Receptors (TLR). It is therefore also known as endotoxin since it is an integral part of the bacteria as opposed to exotoxins that are produced and secreted [49].

In *E. coli* the first step in the biosynthesis of LPS is the generation of lipid A. This occurs in the cytoplasmic space on the inner surface of the inner

membrane and is mediated by nine enzymes [49]. Very little is known about the biosynthesis and transport of the lipid A in *V. cholerae* since it has not been extensively investigated, however it is postulated on the basis of DNA homology, that the pathway and enzymes are similar to those seen in *E. coli* [46].

1.2.3.2. Core Polysaccharide

The core consists of two parts, the inner part attached to the lipid A and the outer part attached to the O–antigen. The inner core is relatively conserved whereas the outer core shows more structural variation [49]. It is not known whether the core polysaccharide contributes to virulence in *V. cholerae* but in any case there is very little variation between different isolates of *V. cholerae* and indeed the core PS is believed to be shared between serogroups. The high level of conservation of the genes of the biosynthesis pathway suggests that they have been spread by horizontal gene transfer [46]. Thus, perhaps not surprisingly, the core structures of the O1 and O139 serogroups are largely similar [48]. The core polysaccharide is synthesized and sequentially attached to lipid A when the lipid A still facing the cytoplasmic space and before the complex is flipped over in the membrane to end up facing the periplasmic space but still attached to the inner membrane [49].

1.2.3.3. O–antigen Polysaccharide

The O–antigen is the last and third component of the LPS. In O1 *V. cholerae* it is composed of the mannose–derived sugar molecule perosamine. It is synthesised in the cytoplasm by enzymes that are associated with the inner membrane. The assembled O–antigen, a chain of 12–18 perosamine residues [36], is flipped into the periplasmic space and subsequently linked to the outer part of the core. The entire LPS is then transported across the periplasmic space to the inner surface of outer membrane where it is flipped over to face the environment and coat the outer surface of the bacteria [49]. All enzymes required for the synthesis, modification, assembly and transfer of the O–antigen are encoded by the *wbe* gene cluster (former known as *rfb* [47]) [46, 49, 50].

The gene cluster, located on the chromosome 1 of the two circular DNA chromosomes (chromosomes 1 and 2) found in *V. cholerae* [46], contains five regions: perosamine synthesis [51], O–antigen transport [52], tetronate biosynthesis [48, 53], O–antigen modification [54], and other genes that are required [55] the production of LPS in O1 *V. cholerae*, but whose exact function is not known [55].

As already mentioned, the fourth component, responsible for O–antigen modification, consists of a single gene, *wbeT*, encoding a methyl–transferase that methylates the terminal sugar in the LPS [46]. It is not known if the methyltransferase methylates all the perosamine or if it just adds a methyl–group to the terminal sugar. It is also not known when this methylation occurs. It is however known that a functional gene results in a methylation of the terminal perosamine and an Ogawa serotype and a mutated *wbeT* gene results in a non–metyhlated terminal perosamine and an Inaba serotype. Different kinds of mutation and theirs implications have been described in ***Paper IV***.

1.2.3.4. The O139 serogroup

Overall, apart from the difference in serotype, strains of the O139 are very closely related to the El Tor biotype [23] and carry only few minor differences compared to O1 El Tor strains. Indeed it is believed that they arose as a result of the acquisition and substitution of the O1 LPS biosynthetic operon with one encoding O139 O–antigen biosynthesis.

Thus the most important difference is that O139 lacks the O–antigen synthesis genes from O1 and that instead forms not only an unrelated O–antigen, but also a polysaccharide capsule. This means that O139 strains do not agglutinate with antisera raised against O1 antigen and that prior immunity against O1 is not protective against O139 strains [18, 48, 56].

1.2.4. Vibrio cholerae toxins

V. cholerae has been shown to produce three different toxins; cholera toxin (CT), zonula occludens toxin (Zot) [57], and accessory cholera enterotoxin (Ace) [58]. Of these, CT is clearly the most important since it causes the active secretion of water and electrolytes into the gut lumen.

The existence of CT was first postulated by Robert Koch in 1886. He had isolated the bacteria 3 years earlier and believed that the symptoms of cholera were due to a "poison" that was released by the bacteria [59]. However, it was not until De could, more than 70 years later in 1959, demonstrate that a cell–free supernatant from cultures from different *V. cholerae* strains caused accumulation of liquid in rabbit loops similar to "rice–water" stools seen in humans or reddish coloured water [60]. During the 1960s Finkelstein and colleagues isolated and purified the holotoxin CT which they called "choleragen". They also found a variant that could be differentiated on the basis of size and charge and was non–toxic, called "choleragenoid" [61, 62]. Lonnroth and Holmgren were able to demonstrate that the toxin consisted of two proteins; the toxic A subunit and the receptor binding B subunit. They furthermore showed that the holotoxin consisted of a single A subunit (CTA) and five B subunits (CTB) (now called an AB5 toxin) and identified the receptor for the toxin as GM1 ganglioside. CTB forms a pentameric ring with which the CTA is non–covalently associated via the CTA2 part of the molecule from which the enzymatically active CTA1 portion is eventually cleaved [59]. The three–dimensional structure of CT was solved by Merritt and colleagues and largely confirmed the model postulated by Lonnroth and Holmgren [62-64].

Both subunits are expressed from the *ctxAB* operon situated in classical strains at two distinct loci located on each of the two circular DNA chromosomes (chromosomes 1 and 2) and in El Tor (often as tandem repeats) only on Chromosome 1 [65]. The holotoxin is assembled from its subunit components in the periplasmic space of the bacteria and secreted into the growth milieu via a type II secretion system [66, 67].

As mentioned, the CTB pentamer binds to the ganglioside GM1 [68] situated on almost all human cells, especially on the epithelial cells in the small intestine (Figure 1), and CT is endocytosed by the target cell. When

CT reaches the endoplasmic reticulum (ER) the CTA1 dissociates from CTB and enters the cytosol. Here the CTA1 is capable of binding NAD and catalysing the ADP ribosylation of a GTP–binding regulatory protein associated with adenylate cyclase and enhances its activity resulting in abnormally high levels of cyclic AMP (cAMP). A high level of cAMP leads to stimulation of the cells to pump out chloride ions into the intestinal lumen changing the osmotic pressure and resulting in the transfer of water and sodium ions also into the intestinal lumen [69].

The gene of the second toxin, *zot*, is immediately adjacent to the *ctxAB* operon and toxin activity is due to alterations in the structure of epithelial tight junctions in the small intestine [57]. The resulting increased intestinal permeability may give rise to symptoms such as fever, abdominal cramps and/or diarrhoea [58]. The third identified toxin, Ace, is encoded by the gene *ace* located upstream of *zot* and *ctxAB* and is believed to increase the potential difference over the epithelial membrane and may cause diarrhoea [58].

The genes *ace, zot,* and *ctxAB* comprise a "virulence cassette" [58] or the core that is situated together with a repeat sequence (RS) region, called RS2, encoding regulation, replication, and integration functions [70, 71]. It has also been shown that the gene for a colonization factor, core encoded pilin (*cep*), is part of the core [58]. All these components constitute the filamentous bacteriophage designated CTXΦ. In El Tor strains the CTXΦ often is flanked with repetitive RS1 elements. The RS1 element is closely related to the RS2 region. Both RS regions consist of three open reading frames (ORFs) *rstR*, *rstA*, and *rstB* and RS1 also contain an additional ORF named *rstC* [70]. The RS1 elements have site–specific transposase activity and can lead to amplification or deletion of the CTXΦ [58, 72, 73]. The RS2 region of CTXΦ is required for phage DNA replication and site–specific integration and may have a function in repressing the transcription of CTXΦ [71].

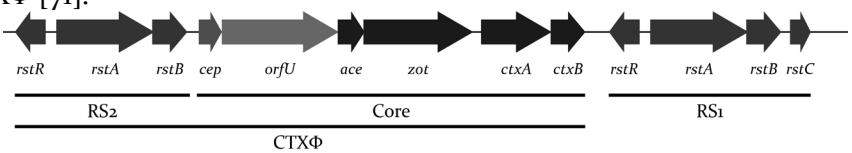

Figure 3 Pictorial representation of the genetic organization of the RS1 and the CTXΦ comprising the RS2 element and the core. Modified from [70, 71, 73].

1.2.5. Regulation of virulence

The regulation and expression of the toxin genes has been extensively studied in both classical and El Tor biotypes. *In vitro* studies in which bacteria of the different biotypes are grown under (the highly different) conditions favourable for toxin expression, has shown that environmental factors such as temperature, pH and bile salts affect the signal pathways involved in the expression and secretion of CT.

It is important to note that the different biotypes of O1 *V. cholerae* do not express virulence genes in the same way. For example, the differences in regulation of CT and TCP expression in the two biotypes result in their expression under completely different conditions *in vitro*, even if the regulatory genes involved appear to be the same.

The transcriptional activator ToxT is responsible of activation of the transcription of several genes among others the *ctxAB* genes encoding CT and *tcpA* that generates TCP essential for colonization of the human small intestine (Figure 4). It also activates its own transcription together with two membrane bound transcription factors ToxR and TcpP. ToxR´s activity is enhanced by the helper protein ToxS and directly induces transcription of *ctxAB*, *toxT* and *ompU* and inhibits transcription of *ompT*. Both *ompU* and *ompT* encode for outer membrane porins involved in osmotic regulation. Transcription of *toxR* is inhibited by adding bile to the growth medium which is surprising since it is believed that bacteria produce a lot of toxin in the intestine where bile is an important constituent. Bile has also been shown to significantly increase the bacteria's motility [42]. One can postulate that bile present in the intestine on the one hand promotes bacterial motility enhancing the ability to move through the mucus layer to the epithelial surface, but on the other hand inhibits the production of ToxR and therefore the production of TCP, known to be required to establish colonization [74]. Premature expression of TCP could immobilize the organisms in the mucus layer without reaching the epithelial cells [75]. At the epithelial surface the concentration of bile may be lower allowing increased transcription of *toxR*. Temperature (30°C in classical and 37°C in El Tor) and low pH induce the transcription of *tcpP* [42, 76] the product of which,

like ToxR, is induced when the helper protein TcpH is bound to TcpP. Transcription of the gene *tcpP* is inhibited by HapR which is a part of the quorum sensing pathway. It is believed that at low cell density the protein LuxO is active and can suppress the gene *hapR*, but at high cell density the LuxO is inactive and the HapR can suppress transcription of *tcpP* and activate transcription of *hapA* that encodes the hemagglutinin A (HA) an extracellular protease that promote detachment from the epithelial cells and facilitates the establishment of new infection sites in the same host or promotes exit of *V. cholerae* from the small intestine and thereby the host (spread) [77-79].

The optimum temperature and pH for production of CT in classical bacteria grown *in vitro* is 30°C and a pH of 6.6 respectively. Whereas El Tor bacteria has an optimum *in vitro* at 30°C and pH of 7.3, however in the intestine lumen the temperature is 37°C and has a slightly alkaline pH [41, 42, 80]. The question arises as to whether the studies done *in vitro* are really applicable *in vivo*.

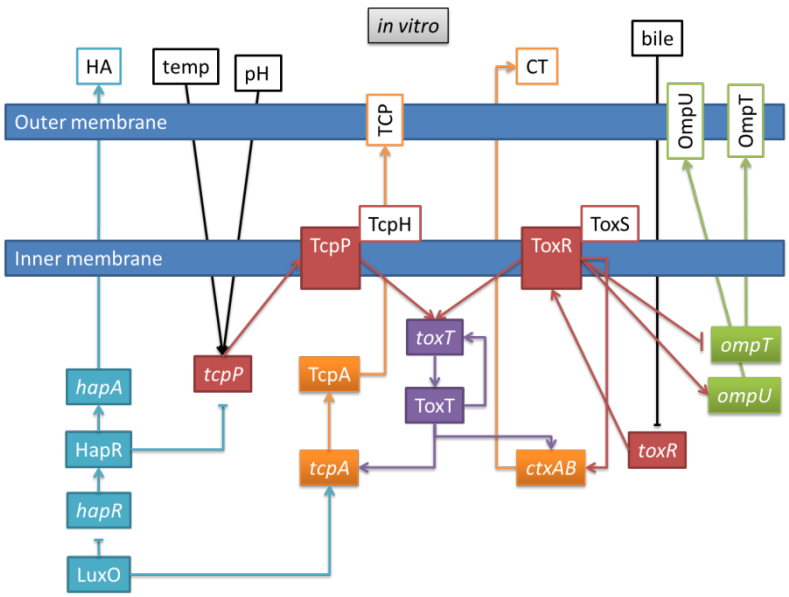

Figure 4 Model of regulation of cholera toxin and TCP in Vibrio cholerae grown in vitro. Modified from [77, 81, 82].

Lee and colleagues has found that the expression pattern of *tcpA* and *ctxA* differ significantly during infection in infant mice versus during growth *in vitro* [74]. They suggest that the expression of *tcpA* is regulated in two temporally and spatially separated events. One early induction while the bacteria are still in the lumen of the upper gastrointestinal tract and one later more pronounced induction occur in bacteria that are retained in the small intestine. They could also show that small amount of TcpA is required at a very early stage of infection for a full induction of *tcpA* transcription. Most interestingly might be the finding that during growth *in vivo ctxA* expression is dependent on the presence of TcpA in contrast to growth *in vitro* and that TcpP does not seemed to be required for induction of *ctxA* and *tcpA* during infection [74], see Figure 5.

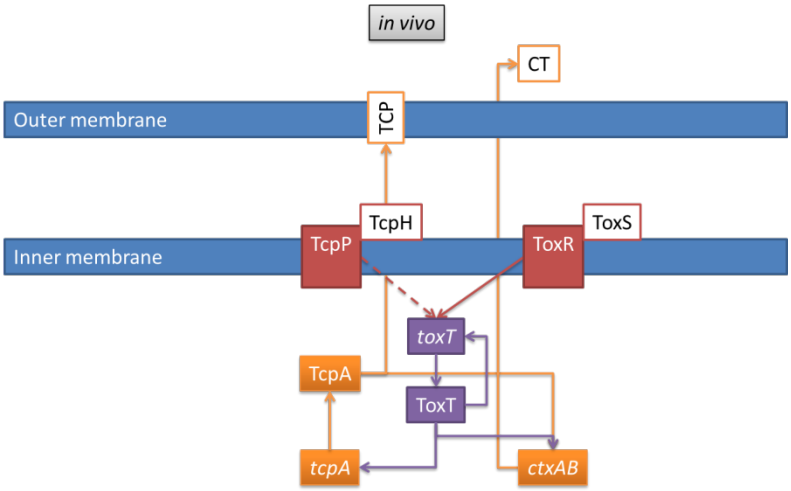

Figure 5 Model of regulation of cholera toxin and TCP in Vibrio cholerae El Tor biotype, grown in vivo. Modified from [74].

1.3. Immunity to cholera

Protective immunity to *V. cholerae* infection in humans is not currently fully understood [83, 84]. It is known from epidemiology studies and challenge studies in healthy volunteers that immunity to cholera is serogroup specific [5, 24, 28, 83, 85] and prior infection of O1 *V. cholerae* does not elicit protection against serogroup O139 [10, 86]. Infection with

V. cholerae of the Inaba serotype protects against both reinfection with Inaba and Ogawa serotype, whereas infection with Ogawa only protects against reinfection of *V. cholerae* of Ogawa serotype [86]. This strongly suggests that protective immunity following both natural infection and vaccination is primarily dependent upon immune responses directed against the LPS. And indeed antibodies formed are mostly directed against the LPS and CT [83, 87-91]. Young children from two to four years of age have the highest prevalence of cholera incidence and prevalence decreases with increasing age presumably due to accumulated immunity [5, 85]. Levels of serum antibodies including vibriocidal antibodies increase with age [85]. However, the presence of vibriocidal and/or agglutinating antibodies does not necessarily correlate with protection [20, 83, 87]. A person after infection can be protected against reinfection even when lacking detectable vibriocidal antibodies [20]. Despite this, vibriocidal antibodies have been used as a measure of immune response and it is widely believed that higher vibriocidal titres increase the level of protection [87-89]. It has been stated for example, that a vibriocidal titre above 160 following natural infection is associated with a 86% lower risk of infection [83].

In 1947 it was reported by Burrows and colleagues that intestinal antibodies (which they called "coproantibody") are responsible for protection against cholera [92] and that after oral vaccination approximately three out of four human volunteers had coproantibodies (secretory IgA antibodies, sIgA [83]) in their faeces [92]. Whether or not these antibodies confer long lasting protection is still a matter of conjecture [84, 93].

One problem with generated antibodies is that they cannot be detected after approximately six to twelve months but protection lasts longer than that [84, 94]. Even if infection leads to a more durable protection (up to 10 years) upon re–exposure than is afforded by vaccination (2 to 3 years) [10, 24, 86, 95] there are no good techniques to quantify protective immunity.

Much indicates the importance of eliciting a memory response that is protective after the initial acute response subsides. One explanation for why vaccines only protects for 2–3 years is that they cannot induce the

same memory response as an infection [83, 94, 96]. However those memory cells that have been found after an infection are against CTB and not of the protective kind against LPS [83]. If memory cells were detected after vaccination it is still not proven if they will be the same as those how are responsible for later protection.

1.4. Vaccines against cholera

Jaime Ferrán, a Spanish physician, is acknowledged as the creator of the first cholera vaccine. He could demonstrate during a cholera outbreak in Spain in 1884 that guinea–pigs that survived an inoculation of live *V. cholerae* were protected from a lethal dose compared to non–vaccinated animals. However the technique gave unsatisfactory results in human with adverse effects due to impure cultures [97]. Some years later, in 1888, Camaleia could show that a suspension of heat–killed *V. cholerae* could generate protection. The technique was further developed by Haffkine in 1892 and Noble in 1964 [97, 98].

The Haffkine vaccination regime consisted of two subcutaneous (SC) inoculations. The first dose was a live attenuated strain (a strain that gives a milder disease) to acclimate the system and a second dose three to five days later with a live hyper–virulent variant strain [99]. The attenuated strain was generated by growing bacteria in media that where continuously aerated with air at a temperature of 39°C until a subcutaneous inoculation only generated local oedema instead of necrosis in guinea–pigs. The hyper-virulent variant was generated by passaging 20 to 30 times in guinea–pigs using parental injection [99]. Haffkine and others had shown that guinea–pigs vaccinated according to this regime had a tolerance to as much as sixteen times the lethal dose of bacteria for non–vaccinated control animals [99, 100].

In 1964 Noble suggested an intradermal (ID) route of vaccination since about ten percent of the humans vaccinated with the subcutaneous route suffered from adverse reactions. The intradermal inoculations given at this time consisted of $3{\cdot}10^8$ heat–killed *V. cholerae* organisms administered in a single dose but could be given in such high does as $8{\cdot}10^8$ without any

adverse reactions in human volunteers [98]. Noble tried also a carbolised (phenol preserved) vaccine of $4{\cdot}10^9$ bacteria of both Ogawa and Inaba bacteria. This preparation had the great advantage over live vaccines that it could be stored for a considerable amount of time without loss of efficacy [97, 99].

It could again be concluded that protection given by the ID route was as good as the SC route but with fewer adverse reactions [97]. It was also suggested that ID cholera vaccines could be combined with other vaccine and administered together rather than separately [97] which is preferable from a cost and time point of view. However, despite the reduced adverse effects of the ID vaccine severe side effects have been reported and even some rare cases of death [101] and so the approach was abandoned.

The scientific community during this time believed in the parental route of immunization and believed that the a mild infection was necessary to stimulate the immune system and a inactivated vaccine that were given by the oral route were most likely to be removed from the body before it could generate this kind of response [20].

It was later discovered that even if a SC vaccination could boost the amount of antibodies in blood, milk and saliva, it could not be assumed that antibodies was produced in the local intestine. This was only likely in humans that has already been primed and that injectable vaccines only worked on those that already had a immunologic response to cholera [8, 102, 103].

This together with the high reactogenicity and the fact that they lacked the ability to interrupt the transmission of *V. cholerae* in communities during outbreaks meant that theses vaccines became were soon obsolete as more effective and safer oral vaccines became available.

The approach of injectable vaccines has not been entirely abandoned as will be discussed later, a purified lipopolysaccharide vaccine, killed whole cell vaccines with various adjuvants, and a polysaccharide–cholera toxin conjugate vaccine [8] are all under development although none are currently in use [5].

1.4.1. Oral vaccines

Attention shifted from parental to oral vaccines when it was discovered that protective immunity was primarily a result of local mucosally secreted antibodies and that the infection, that is most common acquired through ingestion of contaminated water or food, is limited to the gut and is not invasive [5, 20]. Even if Russell reported in 1928 that killed *V. cholerae* administered orally proved to be as effective as SC administration [20] it was not until the beginning of the 1980s that attempts were made to develop an oral cholera vaccine [8].

There are currently two major types of oral cholera vaccines; killed whole cell vaccines and attenuated live vaccines.

Two licensed killed whole cell oral vaccines are available on the market in more than 60 countries. The first, Dukoral, is a monovalent (one serogroup) vaccine against the O1 serogroup and consists of three different strains, representing classical and El Tor biotypes and both serotypes (Ogawa and Inaba). The cells are killed by two different inactivation methods, heat treatment and formalin inactivation. 1 mg of recombinant cholera B–subunit is also added, thus production involves at least 5 different fermentations and downstream processes [104-106].

In an attempt to produce a cheaper and locally manufactured vaccine the Dukoral formulation was modified by omitting the recombinant cholera toxin B–subunit and substituting one of the strains with, as it turned out, a strain producing more toxin. This strain substitution proved counterproductive since traces of toxin caused some of the vaccines to develop diarrhoea. This vaccine, produced in Vietnam, was called ORCVAX. In further development of this vaccine the toxin–producing strain was replaced with its predecessor. Additionally a strain of the new serotype, O139, was included and production of the new bivalent killed whole cell oral vaccine was transferred to India where it is produced under the name Shanchol [105, 106]. Both the Dukoral and Shanchol are administered in two doses 2 weeks apart [105] and both vaccines have proved to be well tolerated and effective in humans [107-110]. Although

Dukoral has been approved by the WHO, it has due to its cost to produce mainly been used as a travellers' vaccine [5].

Many live attenuated strains have been generated in the laboratory by different cultivation methods as described before or by isolation of strains with low pathogenicity but full antigenicity from the environment [20]. More recently strains have been generated by genetic manipulation methods. One of the most extensively tested live oral vaccine is CVD103–HgR. This strain is a derivative of the classical O1 Inaba *V. cholerae* strain 569B where 94% of the *ctxA* gene has been removed and mercury resistance gene has been inserted into the *hlyA* gene thereby inactivating the haemolysin A locus and acting as a selection and a phenotypic marker [5, 8, 111]. The vaccine is administrated in a single dose of $5{\cdot}10^8$ live bacteria [8, 111] and has been proven to be very well tolerated with only few adverse reactions such as diarrhoea, nausea, and vomiting [8, 111, 112]. Due to CVD103–HgR vaccine´s safely profile and the protection observed in human challenge studies it was licensed as a tourist travel vaccine in 1993 as Orochol or Mutacol in Canada [5, 111].

However when the vaccine was tried in an endemic setting it failed to show protection although a tendency was observed in individuals with blood group O [8] and in 2004 the manufacturer ceased the production [5, 111].

Today there are no licenced vaccine based on a live attenuated strain however in 2009 a U.S. manufacturer, PaxVax, got the rights to redevelop CVD 103–HgR [5, 111] under the name PXVX0200 and is currently undergoing phase III trials (ClinicalTrials.gov Identifier: NCT01895855).

A drawback with a live oral vaccine is the risk of spreading bacteria in to the environment and even infecting other people. It has been shown that during a trial with CVD103–HgR vaccine that the strain was shed by 11% of the vaccinees although no transmissions to near household contacts were detected [111].

1.4.2. Vaccine considerations

Even if there are two licensed vaccine on the market, the need for a new vaccine is great. The current vaccines are complicated to manufacture and thereby expensive to produce. They both need at least two doses to give appropriate protection. The upcoming PXVX0200 vaccine along with other live attenuated vaccines is an attempt to elicit effective protection after a single dose. A single dose regime is thought to be more important for interventions in order to interrupt transmission during an ongoing cholera outbreak where rapid efficacy and a high level of conformity are important.

However, it has been shown in several studies that a two dose regime vaccine is very cost effective (if you disregard the cost of the vaccine). Studies on mass vaccination with both Dukoral and Shanchol showed that the cost of vaccination is about 50 U.S. cent per dose [5, 113-118]. In contrast the Shanchol vaccine cost $1.85 which is beyond the acceptable price range for mass vaccination programmes in developing countries [113].

It has been suggested that when vaccinating over half of the population in an area the incidence of cholera can be reduced as much as 93% due to indirect or herd protection [5, 119, 120]. However, this should be considered when designing efficacy vaccine trials since the efficacy will become insignificant if the non–vaccinated control group is part of this herd protected group [83].

Mass vaccination of a population with virtually no risk of cholera such as routine vaccination of travellers is not recommended. A more effective approach is to vaccinate those people travelling to areas with high rate of incidence of cholera [121]. The problem of a two dose regime is always for people to take both doses and studies has showed at approximately 75% of those who take the first dose also take the second [113, 116]. An additional problem with the oral vaccine is the bulkiness and the amount of buffer solution required. Sometimes even water needs to be transported to the vaccines sites. The benefit with Dukoral is that it is

considered stable for one month at 37°C otherwise the need for cold chain can be both logistically difficult and expensive [116].

A new vaccine should be safe, immunogenic, cheap to produce, thermo–stable, easy to administer, and not require the addition of buffer.

1.4.3. Future vaccines

There are several vaccines under development, these include not only killed whole cell vaccines that are the focus of this thesis, but also live attenuated and conjugate vaccines.

CholeraGarde is a live–attenuated *Vibrio cholerae* O1 of the El Tor biotype and Inaba serotype also known as Peru–15 and is derived from a clinical isolate from Peru. The strain is genetically engineered to be non–motile and non–toxinogenic but *ctxB* positive. One dose of $2{\cdot}10^8$ bacteria has been proven safe and immunogenic in adult volunteers in both North America and Bangladesh [122, 123].

V. cholerae 638 vaccine is single dose (10^9 bacteria/dose) oral live attenuated vaccine that is genetically engineered by removing the CTXΦ and introduction of the *Clostridium thermocellum* endoglucanase A gene (*celA*) in the *hapA* gene in an isolated El Tor Ogawa strain from Peru 1991 [124, 125]. Without *hapA* there is no hemagglutinin protease production which is believed to be involved in detaching the bacteria from the epithelial cells. A bacterium that adheres longer presumably gives a more effective immune response as suggested by Finkelstein and colleagues [79]. The *celA* gene is used as a phenotypic marker allowing colonies to be easily detected on agar plates [124]. The *V. cholerae* 638 vaccine has been shown to be safe and immunogenic in healthy volunteers in Cuba [125].

Recently developed vaccine VA 1.4 is a variant of VA 1.3 and is a live attenuated single dose vaccine based on an El Tor Inaba clinical isolate that naturally lacked the CTXΦ. The strain was further genetically modified by insertion of the *ctxB* gene at two loci [126]. In the VA 1.4 vaccine an ampicillin gene linked to one of the inserted *ctxB* genes was mutated by exposure to a germicidal lamp and strains were screened for functional *ctxB* and other attributes identical to VA 1.3. A single dose of

$1.9 \cdot 10^9$ VA 1.4 bacteria has been demonstrated to be well tolerated and immunogenic in adults. Furthermore an additional dose did not improve the observed immune responses seen after a single dose [127].

There have also been some developments of monovalent live attenuated vaccines against the O139 serotype, such as CVD 112, O139-ZJ9693, and VCUSM2 [128-130]. They have all been modified by deleting the ctxΦ and addition of the *ctxB* and proven to elicit immune responses in animal models.

The OSP:TThc vaccine is a conjugated vaccine based on the O–specific polysaccharide (O–PS) of LPS from an O1 El Tor Ogawa strain called X25049 that is conjugated to a recombinant tetanus toxoid heavy chain fragment (TThc). It has been shown to elicit immune responses in mice and in a passive infant mouse protection model showed a protective efficacy of 95% [131].

There have also been improvements in terms of yield and simplicity where the O–PS + core are conjugated with Bovine serum albumin (BSA), necessary steps forward if a cheap and functional conjugated vaccine is to be achieved. If it will work in humans are left to been seen [132].

Transcutaneous immunizations with a synthetic conjugate of Ogawa LPS to bovine serum albumin as a carrier (CHO–BSA) have been proven safe in mice. It has been used as a booster on day 117 after immunization with a live attenuated strain of *V. cholerae* O1 Ogawa named O395–NT to increase serum anti–LPS antibodies. This may be an alternative to prolong protective immunity achieved by only current oral cholera vaccines [133].

A major problem with all the conjugate vaccines is, as already pointed out, that serum immune responses whether measured directly or used in vibriocidal assays or passive protection studies in infant mice do not necessarily reflect protective efficacy. In the absence of functional animal infection models for cholera the only way of testing efficacy will be in phase III clinical trials or human volunteer challenge studies.

1.4.4. Vaccine Adjuvants

Adjuvants, or substances used to improve a vaccine efficacy have been used from the early 1920s [134, 135]. They can be small molecules or proteins that interact with the vaccine and either enhance its presentation to the immune system, control its release over a longer period, or even modify interactions different components of the immune system. Their overall effects will usually be a combination of effects. They are all called adjuvants from the Latin word *adjuvare* meaning "to help" [136].

Addition of an adjuvant to a vaccine can lower the amount of antigen required and/or reduce the number of immunizations. Depending on which adjuvant is chosen it is possible influence the immune system to induce antibody– or cell– mediated responses [135]. In order obtain the desired balance of response for a particular vaccine, a combination of various adjuvant components is not uncommon. One of the best known adjuvants that combines the prolonged release effect of water in oil emulsion with the immune activation properties of *Mycobacterium tuberculosis* is Freund´s complete adjuvant (CFA) [136]. There is not always the need for the *M. tuberculosis* component which can be excluded. In this case the adjuvant is incomplete (Freund´s incomplete adjuvant, IFA). CFA has the ability to elicit Th1 responses whereas IFA tends to elicit a more Th2 biased response. However, neither has been used in human trials since the 1950s due to several safety concerns [135].

The most commonly used adjuvants in humans are the aluminium compounds which have been widely used for more than 80 years [137]. The basic effect is absorption of the antigen to form a depot from which the antigen is slowly released. The higher concentration of antigen makes the uptake by antigen presenting cells more likely [135, 138]. Aluminium compounds can also affect macrophages directly and induce memory responses leading to long lasting protection [139]. Even if aluminium often is used via the parental route it has also been showed to work as an adjuvant via the oral and nasal route in mice [140, 141]. However, a too high dose of the adjuvant may interfere and actually weaken immune responses [142]. Aluminium has also been reported to have potential side effects such as influence on the incidence of Alzheimer's disease [143, 144].

There is a need for additional and safe adjuvants especially for mucosal vaccination. CT is the most powerful mucosal adjuvant known. Some reports also suggest that CTB also works as an adjuvant [135]. It has later been shown that the whole toxin needs to be present for the adjuvanticity. It is likely that initial reports of adjuvant activity were due to contamination with holotoxin [145]. Clearly the problem with CT is that it is far too toxic for use in humans, even if it has been shown tolerated well in mice and act as a powerful adjuvant when administrated orally [146, 147]. Intranasal administrated has been reported to induce inflammation in the brain [148] and a detoxified variant of the closely related heat labile toxin (LT) from *E. coli* has caused some cases of facial paralysis in humans [149]. Another variant of LT, double mutant LT (dmLT), has been extensively tested and shown to be safe and well tolerated during oral administration during a vaccine trial in humans [150] and could be a good further adjuvant for mucosal vaccinations. In this laboratory we have designed a variant of the cholera toxin carrying several mutations , multi-mutant cholera toxin (mmCT), that effectively elicits immune responses comparable with dmLT in mice, but is easier and cheaper to produce (manuscript in preparation, M. Lebens *et al*).

2. AIMS OF THE THESIS

The thesis has two distinct branches; on one side there is the concrete aim of generating a novel vaccine candidate strain that can ultimately replace the multiple strains in the currently licensed killed whole cell vaccines, Dukoral and Shanchol. On the other side and arising from the practical aim developing a novel vaccine was an attempt to use a unique dataset to understand the importance of the O1 serotype and in particular the *wbeT* gene to cholera disease. These branches can be summarized as follows.

A) To develop a new generation of cholera vaccines to replace currently licensed killed whole cell cholera vaccines. The new approach aims to considerably simplify the production of the vaccine and at the same time significantly reduce the cost. The resulting new vaccines should thus be easier, safer and cheaper to manufacture. It should consist of one strain, give rise to protective antibacterial as well as antitoxic immunity. If successful the idea can be extended to other killed whole cell vaccines.

B) To use genomic data to understand the evolution of cholera in an endemic area and to understand the role of serotype in the ecology of *V. cholerae* with respect to epidemic and endemic cholera.

You only need one colony!

3. METHODOLOGICAL CONSIDERATIONS

The different methods described in the included papers will be discussed regarding the choice of methods with less emphasis on details. For more information, please refer to the papers themselves.

3.1. Bacteria strains and culture conditions

Vibrio cholerae strains used in this thesis (Table 1) were all maintained on Luria Bertani (LB) agar plate supplemented when necessary with appropriate antibiotics, more information is found in respective papers.

Strains were stored at –70°C in LB broth supplemented with glycerol (17% final concentration). Strains were grown at 37°C unless otherwise stipulated and liquid cultures were grown in rotary shakers (180 rpm).

Table 1 Presentation of V. cholerae strains used in this thesis.

Wild–type strains	**Description**	**Paper**
VX44945	El Tor, Ogawa	I, II, III, & IV
T19479	El Tor, Inaba	I & II
X25049	El Tor, Ogawa	I & II
Phil6973	El Tor, Inaba	II
N16961	El Tor, Inaba	III
Cario 50	Classical, Ogawa	I & II
Genetically modified strains		
JS1569	*ΔctxA* derivative of classical Inaba strain 569B	I & II
MS1342	Hikojima derivative of JS1569 carrying the pMT–suicide1 plasmid Cm^R	I
MS1356	Ogawa derivative of JS1569 carrying the pMT–suicide1 plasmid, Cm^R	I & II
MS1489	Inaba derivative of X25049	II
MS1568	Hikojima derivative of Phil6973	II
MS1571	Ogawa derivative of Phil6973	II
MS1580	Hikojima derivative of JS1569	II

3.2. Genetic manipulation and modification

The primary aim of the project from the beginning was to design a single strain vaccine with dual expression of Ogawa and Inaba antigen. In ***Paper I*** it is described how a native gene without a functional promoter gives the desired phenotype. Instead of using a commercially available suicide vector we used one that was constructed in the laboratory. It is small (is only 1953 base pair (bp) long) (Figure 6), carries the R6K origin of replication and the origin of transfer *oriT* from the broad host range plasmid RP4 used for transferring the plasmid to other bacteria. Its small size makes it convenient to work with and leaves a relatively small insert when left in the chromosome as in strains MS1342 and MS1356.

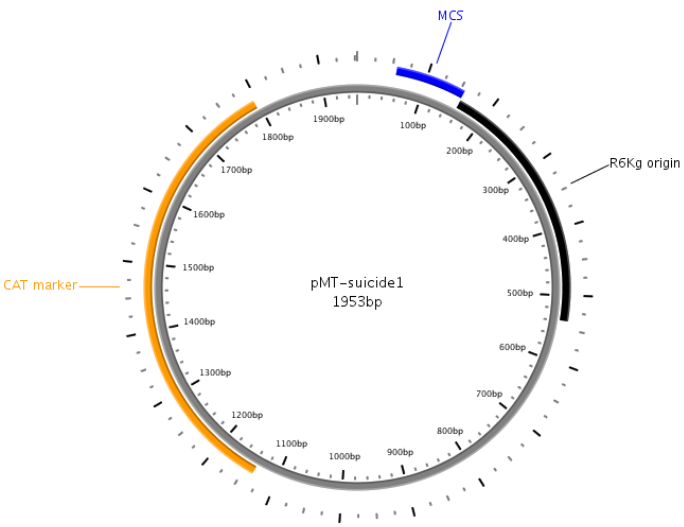

Figure 6 pMT–suicide1 is a R6K –suicide vector constructed in our laboratory carrying a chloramphenicol resistance gene and a multi cloning region (MCS). Figure created using PlasMapper [151].

Although ***Paper I*** demonstrated that it was possible to generate artificial and stable Hikojima strains, these were not suitable for use in a final vaccine formulation due to the presence of an antibiotic resistance gene in the chromosome and the fact that tandem repeats of the *wbeT* gene meant that the chances for deletion by homologous recombination were not insignificant and would result in the loss of the phenotype.

Instead it was decided to modify the *wbeT* gene by random mutation in order to reduce the activity of the gene product so that it would only partially methylate surface LPS resulting in a satisfactory ratio of Ogawa to Inaba antigen. As described in ***Paper II***, this strategy was eventually abandoned in favour of site directed mutagenesis of a single amino acid at a locus shown in naturally occurring Inaba strains to be sensitive to mutation. This required a modification of the suicide plasmid in order to counter-select clones in which the plasmid had been deleted by homologous recombination leaving the mutant *wbeT*. This was achieved by insertion of the saccharase gene (*sacB*) from *Bacillus subtilis* that is lethal when expressed in gram-negative strains in the presence of sucrose resulting in the novel new suicide vector pMT-suicide1-SacB. In order to facilitate selection for the correct clones a kanamycin resistance (Km^R) gene was inserted immediately downstream of the mutant *wbeT* gene. The resistance gene was flanked by tandem flippase recognition target (FRT) sequences that would allow its removal from the chromosome when the gene substitution was complete. The constructed fragment containing the mutant *wbeT* gene and the kanamycin resistance gene was then inserted into the modified suicide vector pMT suicide1-SacB.

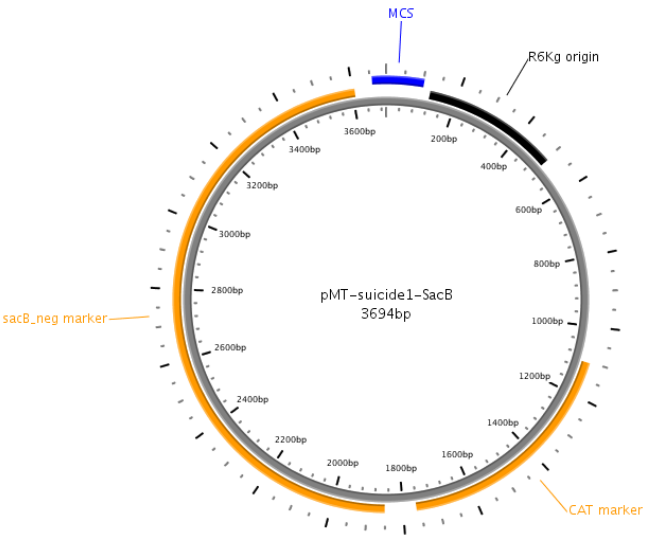

Figure 7 pMT–suicide1–SacB is a R6K –suicide vector constructed in our laboratory carrying a chloramphenicol resistance gene, a MCS and the sacB gene from B. subtilis. Figure created using PlasMapper [151].

The addition of the selection markers *SacB* and Km^R made it possible to easily select colonies with the potential with the desired genotype. The genotype was confirmed by PCR with sequencing. To confirm that the mutation conferred the correct phenotype was done using a serotype-specific agglutination assay.

3.3. Vaccine preparation

When considering the development of a vaccine several aspects need to be considered. Clearly the development of the antigen is of primary importance, but following on from this it must be demonstrated in a suitable model that it can elicit appropriate protective immune responses. This involves at the preclinical level the following considerations: 1) stability of antigen, 2) mode of preparation of the vaccine, 3) choice of animal model, 4) route of administration and 5) analysis of immune responses.

From the outset the new vaccine was proposed to be delivered orally since the formulation is based upon its predecessors Dukoral and Shanchol. These are well tolerated and have few adverse reactions compared to the parental vaccines. They elicit local IgA antibody responses in the intestine that are known to be important in protective immunity. The stability and the ability to store an inactivated vaccine were also aspects the influenced the decision.

Dukoral and Shanchol are made using two different modes of inactivation. The rationale for this is not really valid any longer as we know much more about the antigens that confer protective immunity. It is not necessary for example to use heat killed cells in order to preserve protein antigen on the cell surface, since these do not elicit protective immunity. Thus in a cheap single strain vaccine only one mode of inactivation is preferred. As shown in ***Paper I*** there is no significant difference between heat–killed and formalin–killed preparations in terms of immune responses. Formalin inactivation was chosen due to the fact that it is easier and cheaper to accomplish on a large scale.

3.4. Determination of amount of Ogawa antigen on Hikojima strains

Inhibition ELISA was used in ***Paper I*** to estimate the amount of Ogawa antigen present on the Hikojima strain MS1342. The method was optimised to be able to reduce the variability in the assay. Changes made after ***Paper I*** was published were an increase in number of formalin inactivated cells added in the first step, a reduction in the volume that was transferred and a decrease in the speed of mixing during titration. However, it was still found to be too much variation in the results and the readouts were not sufficiently robust to allow an accurate estimation of the relative amounts of Inaba and Ogawa antigen. An alternative method was therefore developed in which an enzyme-linked immunosorbent assay (ELISA) were constructed where a standard curve was generated using a plate coated with different mixtures of Ogawa and Inaba LPS as described in ***Paper II***. Although this was gave much improved results it required the extraction of LPS from each of the strains rather than using killed whole cells and still gave results with a large degree of variation. The best and most reliable estimate was achieved using the mass spectrometric determination as described in ***Paper II***.

One criticism of the overall approach is that it is difficult to know how the different antigens are distributed on the bacterial surface. Can one be sure that every single bacterium has a mixture of Ogawa and Inaba LPS on its surface? Attempts to determine this with florescent microscopy have been attempted but the absence of specific monoclonal antibodies against Ogawa and Inaba LPS made these experiments inconclusive. However, at the time of printing of this thesis a commercial source of such monoclonal antibodies has become available and renewed efforts will be made to demonstrate the presence of both antigens on the surface of every cell.

3.5. Animal models

In ***Papers I*** and ***II*** three different animal models were used for different purposes. Mice of were used to demonstrate immune response elicited by the vaccine candidates and rabbits were used to generate large volumes of

polyclonal antibodies against Ogawa and Inaba respectively. Lastly, an infant mice model was used to demonstrate protectiveness of serum antibodies generated by the vaccine candidate strains.

3.5.1. Mice

Female inbred Balb/c or outbred CD1 mice form Charles River Laboratories or Taconic was used for all immunizations experiments.

The oral immunizations followed the immunization schedule 1 (Figure 8) except when CD1 mice were used in ***Paper II***. In this case immunization schedule 2 shown in Figure 9 was used.

Immunization schedule 1 is a well tried method for eliciting a mixed antibody response (IgA/IgG/IgM) as well as vibriocidal antibodies, and thus mimics observed immune responses to killed whole cell vaccines in humans. Administration of vaccine on three consecutive days rather than a single administration as in humans is based on common experience in our and other laboratories that while not changing the overall immune responses the divide doses reduce the risk of adverse effects in response to gram–negative whole–cell vaccines in the mice. Furthermore, by giving an immunization on three rather than two consecutive days the intra–group immune responses variability was also significantly reduced.

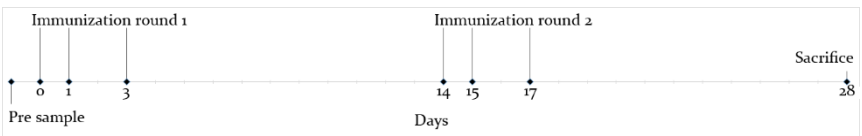

Figure 8 Immunization schedule example 1. Mice were immunized in two rounds 2 weeks apart on three consecutive days. The mice were sacrificed 11 days after the last immunization.

Figure 9 Immunization schedule example 2. Mice were immunized in two or three rounds 2 weeks apart on two consecutive days. The mice were sacrificed 11 days after the last immunization.

Immunization schedule 2 is often used in order to test the effect of an adjuvant. The CD1 experiment in ***Paper II*** was a pooled experiment in which one group received a formulation containing an adjuvant; however data concerning the adjuvant effect is outside the scope of this thesis and is therefore not reported in the presented papers.

Serum antibody titres are often higher after parenteral than oral immunizations. Thus parenteral immunizations were performed in order to further define the specificity of serum vibriocidal and anti–LPS antibodies and demonstrate that the Hikojima strains could indeed induce all types of anti–LPS specificities in a similar manner to the combined Ogawa–Inaba Dukoral vaccine in which the two cell type are mixed together (Figure 10).

Figure 10 Intra parenteral immunization schedule, could be done with two or three immunizations.

3.5.2. Rabbits

Subcutaneous immunizations of purified LPS of either Ogawa or Inaba origin was administered to New Zealand White Rabbits (NZW) from Lidköpings Kaninfarm (Sweden) to generate large quantities of antiserum that could be absorbed to generate specific polyclonal anti–Ogawa and anti–Inaba antibodies (Figure 11).

Figure 11 Subcutaneous immunization schedule for rabbits to generate large amounts of antibodies.

3.5.3. *Infant mice*

Although highly useful for demonstrating immunogenicity, mice are a limited model since *V. cholerae* does not colonize the mouse intestine in the same way as the human intestine and there is no adult mouse infection model that mimics human disease. However it does colonize the intestine on infant mice and causes lethal diarrhoea. Infant mice lack the ability to elicit mature immune responses on challenge. Despite this the infant mouse model is still widely used to test the protective effect of antisera raised in response to candidate cholera vaccines using passive protection in which serum is added to infecting inoculums prior to infecting the mice. It is important to remember in this context that serum antibodies may not reflect true protective efficacy. Which is primarily dependent upon local IgA responses in the gut.

3.6. Assay for determination of Immune responses

In ***Paper I*** and ***II*** both ELISA and vibriocidal essays have been used to determine the magnitude of immune responses elicited in animals immunized with various vaccine candidates. These methods are widely used and are considered to give the best estimations of antibody levels in different blood and tissue samples. The variation in ELISA is always a problem and the method relies on good secondary antibodies. The vibriocidal method is dependent on a reliable and good complement source. The risk for contamination could sometimes be a problem since it is based on observation of bacterial growth. We have improved the method by using an ELISA reader at 600 nm to determine amount of growth as a support in assigning titres.

It can always been discussed what level of antibody titre translates into a protective response in humans, if it can at all. These methods illustrate that the animals reacts to the vaccines and elicit antibodies and compare results with a control that is known through clinical trials to be effective.

3.7. Statistics

Statistically analyses for ***Paper I*** and ***II*** were done using the Prism software system GraphPad 4.03 or 6.04 (Graphpad Software Inc., San Diego, CA, USA). Multi group comparisons were performed using one–way ANOVA with Bonferroni's post–test. Two–tailed Student's t test was used for calculating statistical significance between two groups.

3.8. Predicting phenotype from genotype

The results in ***Paper IV*** and somewhat in ***Paper III*** both depend on a geno-serotype based on the sequenced of the strains compared to a reference strain and previously known Inaba mutations. The likelihood of false positives in the terms of misclassifying an Inaba strain as Ogawa is relatively small. However, a strain with an Ogawa genotype must not necessarily be Ogawa since it can carry additionally mutations. If a mutation is found for the first time it will be hard to know if it confers the Inaba phenotype. We have shown both in ***Paper III*** and ***Paper IV*** that a single amino acid change alters the phenotype. It is may be that some mutations will be found that are phenotypically silent even if they have amino acid changes. Clearly the polymorphisms seen between the classical and El Tor WbeT proteins demonstrate this.

4. Results and Discussion

The results of the included papers will be summarised and discussed in a rather broad context. The results of ***Papers I*** and ***II***, both concern vaccine development and will be considered first. ***Papers III*** and ***IV***, which discuss epidemiology and importance of the O1 serotype and the *wbeT* gene, will then be discussed. For detailed information on all results and a more in–depth discussion of them, please refer to the included papers.

The first aim for this thesis was to investigate the possibility to develop a single strain vaccine strain that could elicit immune responses against both O1 *V. cholerae* serotypes, Ogawa and Inaba. The first strain, MS1342, was constructed and tested in mice. The first thing we could demonstrate was that it didn´t make any difference if we heat or formalin inactivated the strain when it came to elicited antibodies (Figure 12). Despite the slightly higher antibody results of the heat inactivated formulation in these experiments the formalin inactivation method was chosen. The practical difficulties of heat killing bacteria on a large scale compensated for slightly lower immunogenicity. Furthermore, formalin inactivation is already used the preparation of both Shanchol and Dukoral and known to be effective.

It was also shown that the first generated Hikojima strain (MS1342) generated similar antibody responses to Dukoral with extra–ordinary results when looking at the vibriocidal results. It can clearly be seen that strain MS1342 generates similar titres as its parental strain JS1569 (Inaba) but also elicits antibodies specific to Ogawa (Figure 13) which the parental strain does not. The combination of absorption of sera with Inaba or Ogawa antigen with vibriocidal assays could be used to demonstrate specificity in a way that had not been possible before with very clear-cut results.

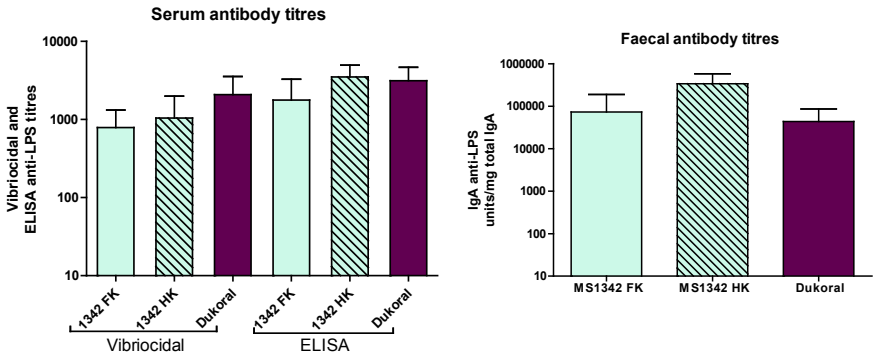

Figure 12 Comparison of immune responses elicited by immunization with Vibrio cholerae strain MS1342 prepared by different methods of inactivation. (Left) Serum vibriocidal antibody titres against Ogawa test bacteria and ELISA IgG+IgM antibody titres against Ogawa LPS; (Right) IgA antibody levels to Ogawa LPS measured by ELISA in faecal extracts collected 10 days after the last immunization. Geometric mean levels and SEM from 5 animals per group are shown. All antibody levels shown are significantly ($p < 0.01$) elevated compared to corresponding samples collected before immunization, whereas post–immunization values do not differ significantly between the different immunization groups.

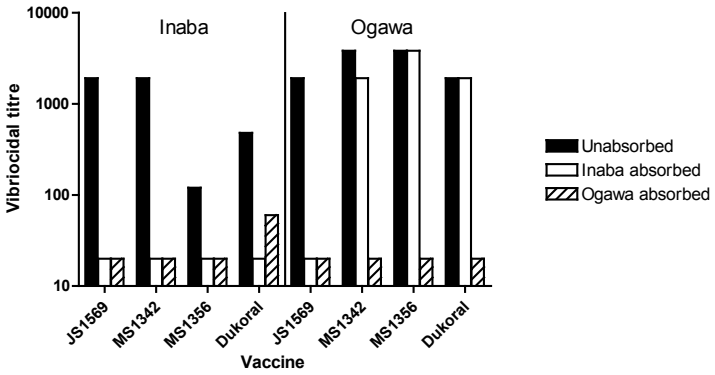

Figure 13 Vibriocidal immune responses elicited by immunization with different vaccine strains of Vibrio cholerae. Vibriocidal antibody titers against Inaba (left panel) and Ogawa (right panel) test organisms in group–wise pooled serum samples (filled bars) and after absorption with formalin–killed cells of the Inaba (open bars) or Ogawa (striped bars) serotype.

A perplexing finding in these experiments was that it was easier to generate Ogawa-specific antibody responses than Inaba specific responses (see Figure 13). This was found to be a function of the immunization route and the mouse strain used, since similar experiments using sera from parenterally immunized C57/Bl6 mice showed high levels of Inaba-specific antibodies (results not shown).

Epidemiological evidence suggests that despite this, Inaba infection (effectively oral immunization) gives rise to cross protection against both Inaba and Ogawa strains. Analysis of historical human sera (Figure 14) demonstrates that in the majority of cases the vibriocidal assays after immunization are similar to those seen in the mice. But we know that these individuals are protected. It seems that there is a difference in the nature of the cross-reactive antibodies elicited by Inaba strains and Ogawa strains. Those produced by Inaba infection are cross protective whereas those produced by infection with Ogawa strains are not.

The later strains, MS1568 and MS1580, were superior to MS1342. As argued in ***Paper II*** these strains carried only one copy of the *wbeT* gene and lacked the antibiotic resistance gene present in MS1342. In addition, MS1568 is also of the El Tor biotype which could be considered a benefit since all cases of epidemic cholera today are of this biotype and tend to give a somewhat stronger immune response (Figure 15). It should be noted that in comparison with Figure 13 this experiment was done with doses based on the same amounts of LPS instead of the same cell density. In these experiments all three vaccine strains and Dukoral showed a tendency to elicit Inaba specific antibodies but still not at levels comparable with levels of Ogawa-specific responses.

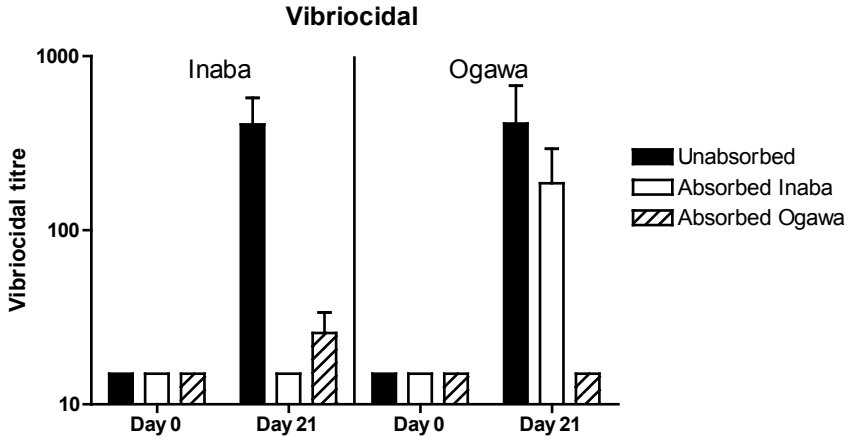

Figure 14 Vibriocidal immune responses elicited by vaccination with Dukoral. Vibriocidal antibody titres against Inaba (left panel) and Ogawa (right panel) test organisms in serum samples (filled bars) and after absorption with formalin–killed cells of the Inaba (open bars) or Ogawa (striped bars) serotype.

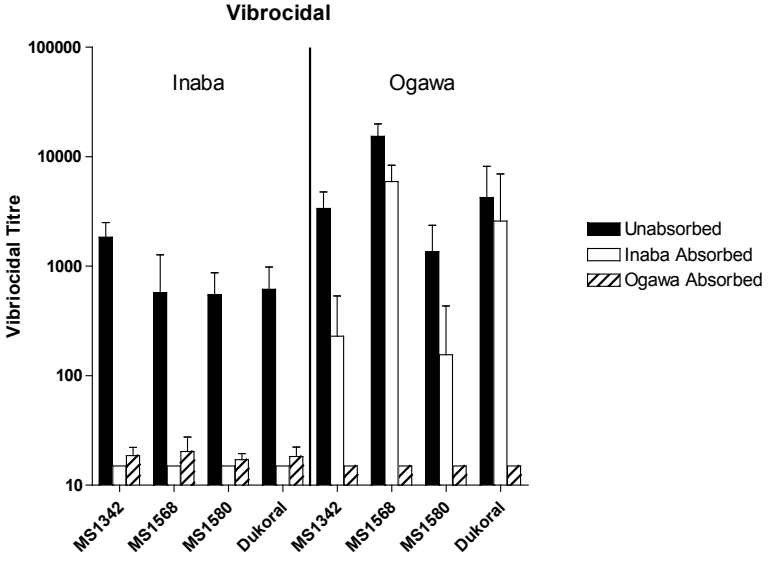

Figure 15 Vibriocidal immune responses elicited by immunization with different vaccine strains of Vibrio cholerae. Vibriocidal antibody titres against Inaba (left panel) and Ogawa (right panel) test organisms in group–wise pooled serum samples (filled bars) and after absorption with formalin–killed cells of the Inaba (open bars) or Ogawa (striped bars) serotype after oral immunization with 8 balb/c mice in each group.

As discussed earlier vibriocidal titres may be a good indicator of protection but a more relevant marker would be whether it can elicit locally produced IgA antibodies in the intestine. As reported in both ***Paper I*** and ***II*** all three vaccine strains were also fully comparable with Dukoral in this respect (Figure 16).

The approach used to generate the MS1568 and MS1580 strains by replacing one amino acid in the *wbeT* gene aroused a curiosity as to the mutations that lead to the Inaba phenotype in naturally occurring strains. In the literature there were many reports that a truncated gene product due to insertions, deletions and single base changes leading to stop codons deletions in the *wbeT* gene were responsible for the Inaba phenotype, beyond this, mutations were not extensively defined. We started to screen our own strain collection and later extended this to the Sanger Institute's database with over 700 strains in order to find which

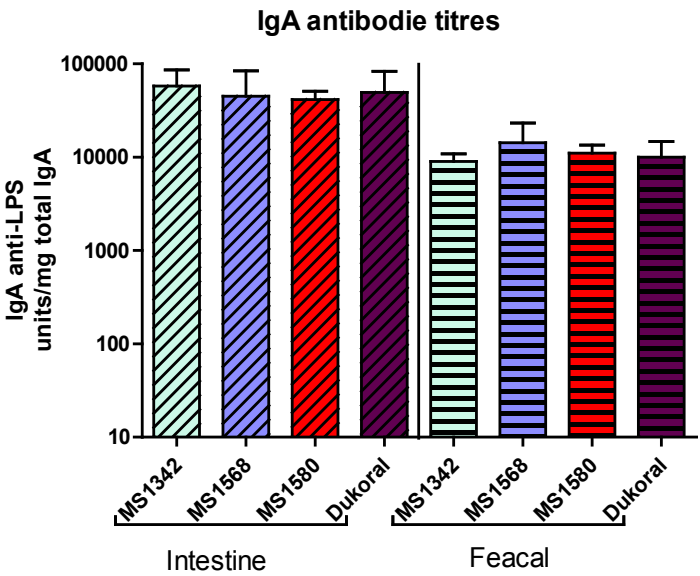

Figure 16 Intestinal–mucosal IgA anti–LPS antibody responses elicited by two rounds of oral immunizations in Balb/c mice two weeks apart with formalin–killed MS1342, MS1568, and MS1580 whole cell vaccines as compared to Dukoral vaccines; immunizations. (Left panel) IgA anti–LPS antibody levels in small intestinal tissue extracts (expressed as units per mg of total IgA measured by ELISA); and (Right panel) the same in faecal extracts. Bars represent geometric mean values ± SEM. Analyses of data by ANOVA showed that post–immunization antibody levels did not differ significantly between any of the immunization groups.

mutations were leading to the Inaba phenotype. All sequences were compared to a reference *wbeT* gene from the El Tor strain VX44945.

The first observation that was made were that all classical strain carried three polymorphisms that didn´t affect the phenotype. First presented by us and then confirmed by Liang and colleagues [152, 153]. Out of all strains analysed there were 276 unique sequences with the phenotype of Inaba serotype. In all these mutants only a small number of the possible mutations that could theoretically lead to the Inaba serotype were actually found. It should be noted that almost all strains analysed have been clinical isolates and therefore the discovery may not necessarily reflect the number of mutations that can be found in the environment. Nonetheless, it was evident from these findings that the mutations leading to the Inaba serotype were non-random. All the strains in question can survive, infect a human host, and cause several diarrhoea. Notably, the same kinds of mutation were found in both classical and El Tor strain as well as in strains isolated on different continents and at different times. This suggested that the same kind of mutations were arising independently (***Paper IV***) but also that classical and El Tor strains were subject to the same selective pressures that were giving rise to the Inaba serotype. In data presented in ***Paper III*** it is again evident that the number of mutants is relatively low and that the mutations that arise are the same as have been isolated previously. Importantly however, it is also evident through the enormous detail of the genetic data, that the mutations in this case had arisen independently from parental strains that were circulating at the time and were not related to Inaba strains with the same mutations that had been cited in previous literature.

The question of why the O1 serotype shows these variants has not previously been addressed. We have shown that once the *wbeT* acquires an inactivation mutation such as an insertion it tends to receive additional mutations and then disappear as cause of severe cholera. We have also shown that there seems to something in the environment that can selectively affect Ogawa or Inaba strains. This is possibly the reason why Hikojima strains are so seldom isolated. In scenarios that favour one

or the other of the two serotypes Ogawa or Inaba, the Hikojima serotype will be selected against.

There is obviously something special with the O1 serotype, since of the over 200 serotypes in *V. cholerae* it is only this one that can cause epidemics of cholera today. When one consider the O139 serotype that in the 1990s caused epidemics it is very similarly to the El Tor biotype but lacks the O1 synthesis operon. It probably caused so many cases of cholera due to no prior immunity to the serogroup which caused all age groups to be infected which enhanced its ability to spread. It also has a capsule that the O1 bacteria lack which probably help it to spread. However, it has since then disappeared as a cause of cholera. What is it that makes O1 serogroup so special and why is the Ogawa and Inaba serotype so important for its survival that the bacteria keeps a gene that seems to so easily acquire mutations?

One possible explanation for the *wbeT* gene to stay intact could be that under normal circumstances the Ogawa serotype has a powerful selective advantage. WbeT appears to be an S-adenosyl methionine (SAM)-dependent methyltransferase (Accession number: TIGR01444) and is responsible for the methylation of the terminal perosamine in the LPS. If WbeT is using SAM as a substrate when methylating every single LPS molecule and a mutation occurs that inhibits the usage of SAM there will be a lot of unused substrate. How this could affect the bacteria is unknown but SAM is known to be a precursor in the biosynthesis pathway to generate auto inducers for quorum sensing such as autoinducer 2 (AI–2) [154]. At high cell density of *V. cholerae* the two autoinducer, AI–2 and cholera autoinducer 1 (CAI–1) is released. The surrounding bacteria will on the presence of these molecules inhibit virulence expression via the *lux* pathway [78, 155]. One effect in the normal case will be an increase in protease expression and the possibility to detach from the epithelial cells and spread to new places or hosts [155]. Interestingly, the strain that expands the most powerfully in the data presented in ***Paper III*** is one in which a mutation oblates methylation activity and yet still gives rise to a full length protein. Similar mutants have been noted elsewhere. It is possible that a residual and as yet

unidentified activity in the WbeT protein gives these strains an advantage over those in which expression of any gene product is destroyed by insertion or other disrupting mutation? The presented data suggests that this could indeed be the case. However, this question requires considerable further work to address. However, could one effect of an inactive WbeT protein lead to a reduced ability to produce AI–2 and therefore a reduced ability to detach from the intestine and spread? One might predict that there will be a difference in the ability of the different mutants to be shed by their hosts.

5. Concluding Remarks

This thesis has not only generated one but three new novel vaccine strains. They all address the problem of killed whole cell cholera vaccines that currently contain several different strains to elicit an effective immune response. By manipulating the three strains to express two serotype determinants simultaneously each one can potentially replace all of strains in the currently licensed vaccines and still elicit the same protective immune response. This is a major step in developing a truly accessible vaccine for this important disease and illustrates how site directed mutagenesis can be used for the rational modification of the phenotype of a vaccine candidate strain of *Vibrio cholerae*.

In broader terms the thesis demonstrates how the use of bioinformatics can be used to target genes and even specific amino acids for mutagenesis in order to modify the phenotype of a vaccine strain. The changes in the strain do not result in any major changes in the genome that could potentially cause problems due to release of recombinant DNA into the environment and yet result in immune responses in immunized mice that are fully comparable with those of the currently licensed oral cholera vaccine Dukoral.

The work of this thesis has also shed light on mechanisms driving a phenomenon that has been known about for many years but has remained poorly understood. With data that is unique in terms of the size of the samples, the time over which the study was conducted and the detailed genomic information obtained. The way in which the data is used makes it clear that there are almost certainly selective pressures on the circulating strains in the environment that are driving serotype transition.

The investigation of naturally occurring mutants has generated a hypothesis as to why *Vibrio cholerae* have two serotypes of the O1 serogroup. Although we cannot say from the data what the selective pressures are, we can postulate that the mutations that arise are a response to a change in conditions favoring one serotype over another and that different types of mutations give rise to differences in fitness as measured by the ability to spread and cause cholera outbreaks.

6. POPULÄRVETENSKAPLIG SAMMANFATTNING

Avhandlingen innehåller i första hand utvecklingen av en ny generation koleravaccin. Detta för att ersätta nuvarande licensierade koleravacciner som kräver storskalig odling av tre till fyra virulenta stammar av *Vibrio cholerae,* vilka sedan behöver avdödas med kemikalie- eller värmebehandling. Denna process är komplicerad vilket gör vaccinerna dyra att tillverka. Den nya generationens vacciner som beskrivs i denna avhandling har modifierats med hjälp av genteknik för att skapa en enda stam av *Vibrio cholerae* med attribut som gör det möjligt att med denna ersätta de olika stammarna i de nuvarande vaccinerna. Med hjälp av olika modifikationer av en gen har tre varianter skapats som alla har visat sig vara fullt jämförbara med det för närvarande licensierade orala koleravaccinet Dukoral i avseendet att stimulera immunsvar i två olika djurmodeller samt påvisandet av potentiellt skydd i en djurmodell.

De nya vaccinerna har även visat sig säkra i djurmodeller vilket har möjliggjort internationella samarbeten för att göra en formulering av dessa som inom kort kommer att testas i en fas 1 studie i människor.

Avhandlingens andra del innehåller en beskrivning av betydelsen för varför vissa gener är viktiga för hur *Vibrio cholerae* orsakar utbrott av kolera. I denna del har kolerastammar från hela världen analyserats. En närmare studie av kolerautbrott i staden Kolkata under en begränsad tid på 5 år har även gjorts. Analysen utfördes på mer än 400 stammar där bland annat hel–genom–sekvensering och stammarnas isolationsplats har spelat en avgörande roll i utformandet av den hypotes som läggs fram.

Även om vi inte kan säga exakt vad som utgör det selektiva trycket, så föreslår vi att de mutationer som uppkommer är ett resultat av ändrade förutsättningar i omgivningen. Därför spelar genen *wbeT* en viktig roll för serotyperna och huruvida stammarna av *Vibrio cholerae* O1 kan orsaka epidemisk kolera eller inte.

7. ACKNOWLEDGEMENT

As all good things always come to an end, my time as a PhD student is no different. Many people have contributed to my work – in the laboratory, through teaching or by simply improving my well–being and I would hereby like to express my gratitude to you.

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An extra big thank you to everyone that should have been mentioned here. You are not forgotten!

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