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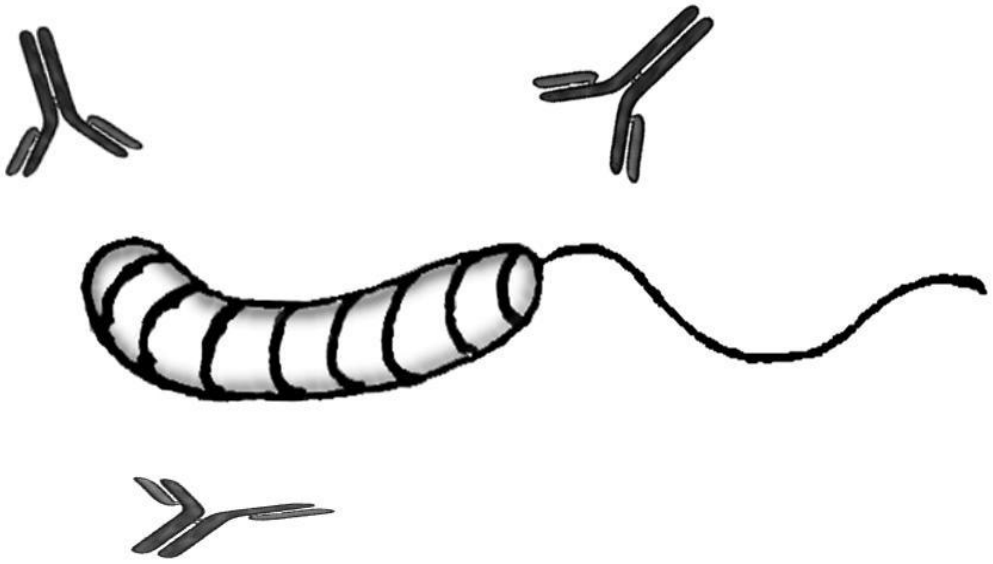

Mixed serotype expression in a novel vaccine strain of Vibrio cholerae

Master of Science Thesis in the Master Degree Program Biotechnology

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Göteborg, Sweden, 2010

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Cover:
An illustration of the slightly curved rod, *Vibrio cholerae,* with three antibodies.
Stefan Karlsson.

[tryckeriets namn]

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Abstract

Background

Cholera is a severe disease that exists all over the world but mostly in developing countries. Currently available oral vaccines are effective but are complicated to make and therefore relatively expensive. The vaccine comes in liquid form and requires both a cold chain for distribution and two doses for full protection. If a more effective and cheaper vaccine that requires only a single dose for full protection could be developed in a dry formulation, it would have a potentially profound effect in developing countries. This study is a part of a larger project that aims to generate a single vaccine strain.

Results

During the limited time available in this study neither of the two methods, *In vitro* transposition and *red/gam* recombination system, used for introducing mutations into the genome of *Vibrio cholerae* was successfully developed. However the genes are cloned and standard suicide vector mutagenesis can proceed in further studies.

It has been demonstrated that when a *wbeT*-gene from an Ogawa strain are inserted *in trans* in an Inaba strain, and the levels of expression are limited, the result is a culture that expresses both Ogawa and Inaba serotypes. Immunisation studies are ongoing.

Conclusions

There is interest in developing the *red/gam* recombination system for *Vibrio cholerae*, since it is more straightforward and simpler than the traditional methods. However additional studies and more time are needed to investigate why the recombination system did not work and how to make it functional in *Vibrio cholerae*.

The results indicate the described approach to make mixed serotype strains is valid but more studies are also needed to determine if the culture that expresses both serotypes consists of cells that express both serotypes or if it is a mixture of cells each expressing a single serotype. Strains for the experiment are available and with FACS it is easily demonstrated.

Keywords

Vibrio Cholerae, Ogawa, Inaba, Hikojima, *In vitro* transposition, Agglutination test, *red/gam* recombination system.

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1. BACKGROUND

Cholera is a disease caused and named by the rod shaped, slightly curved and gram negative bacteria, *Vibrio cholerae* [1]. The cbacteria can be found in the entire world, but cause disease in places where there is poor access to fresh water, such as development countries, refugees' camps or area of disasters. [2] Cholera has throughout history caused epidemics and has brought death and devastation on the effected populations. There are records describing illnesses that are similar to cholera from 460 BC and 130 AD[3, 4]. The first pandemic in modern times started in 1817 in south-east Asia [3]. The 7th pandemic wave started in 1961 in Indonesia and is still ongoing but is now rather classified as an epidemic in many countries[2, 3]. The bacteria were first isolated by the Italian scientist Filippo Pacini in 1854 [5]. However it took 82 years before the scientific community and the public accepted that the cause of cholera was a microorganism rather than toxic particles [6]. Even though the treatment is relatively simple the mortality rate is about 2%, 2´272 deceased out of 131´943 infected humans during 2005 [7]. During last year, August 2008 – May 2009, almost 100´000 suspected cases of cholera where observed in Zimbabwe and of those about 4% (4276) were fatal [8]. However it is estimated that less than 10 % of all cases are reported to WHO and therefore the impact of the disease is underestimated[2].

1.1 THE DISEASE

The main symptom of epidemic cholera is acute severe diarrhoea. The incubation period varies from as short as 2 hours to up to 5 days and therefore enhances the risk of an explosive expansion of the disease [1, 2]. The bacteria are spread via contaminated food and poor hygiene, and affect the small intestine through secretion of the enterotoxin, cholera toxin. The mucosal cells that are affected in the small intestine start to produce abnormally high levels of cAMP and this leads to stimulation of the cells to pump out chloride ions into the intestine [9]. The change in osmotic pressure leads to transfer of water and sodium ions into the intestine. To compensate the loss in the cells, the electrolytes are replaced from the blood flow with severe effects such as dehydration, anuria, acidosis, and shock. Cholera is therefore one of the few diseases that can kill a healthy adult in as little as two hours from the onset of symptoms [1]. The usual method of treatment is to compensate for fluid losses and for serious symptoms antibiotics are administered [10]. Severe symptoms that go untreated can have a mortality rate of 50% [1, 2].

It has been shown in several studies that the rate of infection is season dependent and comes in either one or two peaks during a year [11, 12]. There is no good explanation for this although theories involving shifts in water temperature and heavy rain are considered. [11]

1.2 VIBRIO CHOLERAE

Vibrio cholerae is most common in surface waters, both in marine and freshwater environments but thrives in brackish waters, coastlines and estuaries. These are the most common reservoirs for the bacteria[1, 2]. There are little more than 100 different serogroups of *Vibrio cholerae*, however there are only two known to cause epidemics, the O1 and O139, where the O1 serogroup is responsible for > 98% of all cases [9]. The O1 has three different serotypes named Ogawa, Inaba, and Hikojima[13]. All three can have the biotype "Classical" or "El Tor". It is believed that the six first pandemics in modern time was caused be the classical biotype and the ongoing 7th pandemic by the El Tor biotype [14]. The serotype O139 emerged in 1993 and has subsided as a major cause of epidemic cholerae and is only present in South-East Asia [9]. It is

unclear if it will spread to other regions, therefore it is the O1 serotype that is the continued focus for the development of a new generation of oral cholera vaccine.

Since *Vibrio cholerae* is a gram negative bacteria the cell wall consist of lipopolysaccharides (LPS). The LPS work as antigens and the presence of different sugar antigens (A, B, and C) on the bacterial surface determines which serotype is present [15]. All three serotypes carry the A antigen whereas Inaba strains express antigen B and Ogawa strains the C antigen. Both B and C antigen are present on Hikojima strains[1, 16]. The Hikojima serotype is considered to be unstable and rarely found in nature [17].

The presence of the A or B antigen on the cell surface is regulated by a single gene, the *wbeT* gene which is also called *RfbT* [18]. This gene is located on the 1st chromosome out of two but only exists in one copy and is 841 bp long. Inactivation of the *wbeT* gene by mutation such as an insertion, deletion or truncation of the gene will result in the B antigen and thereby an Inaba serotype. [1, 16]

1.3 THE VACCINE

There is a functional oral cholera vaccine that protects against severe disease, reduces the number of infections and increases the survival rate. This vaccine called DUKORAL® produced by SBL Vaccin AB is administrated orally and contains whole killed cells of *V. cholerae* O1 –bacteria, $25x10^9$ cells of 3 different strains and 1mg of the non-toxic B subunit of cholera toxin (CTB)[19]. The strains are Inaba El Tor formalin killed, Inaba classical heat killed, Ogawa classical formalin killed, and Ogawa classical heat killed. The vaccine is administrated orally two times two weeks apart and since the vaccine is not stable in acidic environment no food or drink, that will increase stomach acids, is allowed one hour before and one hour after the vaccination[19]. The vaccine will induce production of antibodies in the small intestine against both the bacteria and its toxin. The antibodies reduce the bacteria's and the toxin's ability to colonize in the small intestine and thereby a decrease the risk for symptoms [9]. One vaccination, 2x1 dose, costs today for a consumer in Sweden approximately 400 SEK, ~40€ [20].

1.4 THE AIM

Vaccines available today include both serotype, Ogawa and Inaba, but when manufacturing the vaccine it requires four different batches, one batch for each strain and killing method. The aim of this study is to express both serotypes in the same strain and thereby reducing the number of batches from four to one since we believe that only one type of killing is enough[1]. This study is part of a larger project to create an inexpensive and more efficient and powerful vaccine against *Vibrio cholerae*.

[1] Not proven in this thesis.

2. METHODS

Methods and materials used in this study are presented in this chapter.

2.1 MATERIALS

The bacteria strains used in this study are shown in Table 1 and plasmids in Table 2.

Table 1, Bacteria strains used in this study.

Name	Type	Biotype	Serotype	Genotype	Reference
Xl1-blue	*Escherichia coli*	-	-	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)].	Stratagene
JS1569	*Vibrio cholerae* O1	classical	Inaba	ΔCTA	[21]
Phil6973	*Vibrio cholerae* O1	El Tor	Inaba	wt	Clinical isolate Institution's strain collection
Vx44945	*Vibrio cholerae* O1	El Tor	Ogawa	wt	Clinical isolate Institution's strain collection

Table 2, Plasmids used in this study.

Plasmid	Description	Derived from	Reference
pBluescript SK+	Cloning vector		Stratagene
pBluescript-*espEΔ*-pTnkan		pBluescript SK+	This study
pBluescript-*espE*-pTnKan		pBluescript SK+	This study
pBluescript-*thyA*-pTnKan		pBluescript SK+	This study
pBluescript-*wbeT*-pTnKan		pBluescript SK+	This study
pkD46	Carrier of the *red/gam* genes from phage λ		[22]
pkD46 *tac/lac*	ara-promotor substituted with lac-promotor	pkD46	This study
pML-CTB::OVA	Carrier of *tac/lacIq* cassette		[23]
pML-*wbeT*	*wbeT*-gene for expression analysis	pML-CTB	This study
pTnKan-1-sce	Tn5 mini transposon with the kanamycin resistance gene		[22]

2.1.1 THE GENES

In this study there are three genes of interest in the *Vibrio cholerae's* genome. They are the *wbeT*, *epsE*, and *thyA* genes.

wbeT, 1.0 kb, is believed to regulate serotype. The wild type is Ogawa and a mutation such as deletion, truncation or insert in the gene will result in an Inaba serotype [13].

epsE, 1.6 kb, is a gene in the secretion pathway that regulates the release of cholerae toxin into the medium. An effect of a non-functional *epsE* gene is that the produced cholera toxin is located into the periplasma of the bacteria instead of released it to the medium [24]. This is a wanted effect in the new vaccine candidate.

A deletion in the *thyA* gene, 1.035 kb, will affect the bacteria to need thymine in the medium for survival [25] which then can be used as a selection marker.

2.1.2 RECEIPT

Agarose-gel, 1%

Agarose	1.25 g
0.5xTBE buffer	125 ml

- Dissolve the agarose in TBE buffer using the microwave.
- When it is dissolved, cool it down to around 40^{o}C and pour the liquid into a mould.

Agarose-plate

Agarose	1.25 g
LB medium	100 ml

Ampicillin

LB or Agar	x ml
Amp [100 mg/ml]	x µl

Glucose 50%

Glucose	50 g
H_2O deionised	100 ml

- Sterilize by filtration.

IPTG

LB	1 ml
IPTG 100	10 µl

Kanamycin

LB or Agar	x ml
Km 50	x µl

Luria Bertani (LB), pH 7.5

Contents:

Bactotryptone (pancreatic digest of casein)	1%
Yeast extract	0.5%
NaCl	1% 0.17 M

Preparation:

Bactotryptone (Difco)	10 g
Yeast extract	5 g
NaCl	10 g

- Dissolve in about 800 ml of deionised H_2O.
- Adjust pH to 7.5 with NaOH.
- Add more water to final volume 1000 ml.
- Autoclave.

PBS, pH 7.2-7.4 (0.01 M phosphate, 0.15 M NaCl)

Contents:

2.3 mM NaH_2PO_4

7.7 mM Na_2HPO_4

150 mM NaCl

Preparation:

NaH_2PO_4	274 mg
Na_2HPO_4	1.09 g
NaCl	8.5 g
H_2O deionised	900 ml

Adjust pH to 7.2-7.4

Add water to final volume 1000 ml.

5xTBE buffer

Tris	54 g
Boric acid	27.5 g
EDTA 0.5M pH 8.0	20 ml

- Boric acid somewhat intractably soluble, dissolve it first in 500 ml of water while stirring. Do not use ice cold water.
- Need not to be autoclaved.

0.5xTBE buffer

5xTBE buffer	100 ml
H_2O deionised	900 ml

2.2 DNA MANIPULATION

Several methods for DNA manipulation were used in this study. This chapter gives a short introduction to the methods used and standard operation protocol.

2.2.1 DNA EXTRACTION FOR PCR AMPLIFICATION

DNA extraction for PCR amplification from cells was done by picking colonies from a freshly grown agar plate and suspending them in 200 µl deionised water in a 1.5 ml eppendorf tube. With a needle the lid was punctured once, to let out vapours. The tube was boiled in water for 5 minutes and centrifuged for 3 minutes at 16'000g. Decant the supernatant into a new eppendorf tube and discard the pellet.

2.2.2 PCR

Polymerase chain reaction, PCR, was used to amplify DNA both from genomic material and plasmids. The primers used for amplification of the different genes as referred to in the text are shown in Table 3. The standard mixtures and operation program for these reactions were:

Reaction mixture

x µl	Sample, with a final concentration of ~0.3 ng/50 µl
5 µl	x10 PCR buffer

1 µl	x50 dNTP:s
1.5 µl	Primer mixture
42-x µl	H_20
0.5 µl	Enzyme
50 µl	Total

Primer mixture contains 10 pmol/µl of each primer. 1.5 µl primer mixture in 50 µl reaction in other words 15pmol of each primer in each reaction.

Amplification program

1. 94°C 5.0 minutes
2. 65°C 1.0 minutes
3. 72°C 1.5 minutes
4. 94°C 0.5 minutes
5. 65°C 0.5 minutes
6. 72°C 1.5 minutes

} x29

7. 72°C 8.0 minutes

The time length of point 3 and 6 corresponds to the DNA molecule length 1kbp ≈ 1 minute.

Table 3, PCR primers used in this study.

Name	Sequence (length)	Use
***epsE* 1**	CGCCTTTTTTATGGCGTCCCTTGGC (25)	Cloning
***epsE* 2**	CGATGACCGCCGCCATTCACCTG (23)	Cloning
***epsE* re 1**	TGGCCGAACCGGTATCCACGAGC (23)	Reverse PCR
***epsE* re 2**	CCCCCACCGGTTTCGACGAGCG (22)	Reverse PCR
litmus 1	GCAGCACATCCCCCTTTCGCCAG (23)	Amplify TnKan-fragment
litmus 2	GGGCCTCTTCGCTATTACGCCAG (23)	Amplify TnKan-fragment
Taclac 1	CCCGAGCTCGAATTCTGTTTCCTGGTGGAAATTG (34)	Amplify *tac/lac*Iq cassette
Taclac 2	CCCGGTCTCGATTTGATCCCGAACGCCAGCAAGACG (36)	Amplify the *tac/lac*Iq cassette
***thyA* 1**	GATGGTTTGGTCTTACAAGCGCGG (24)	Cloning
***thyA* 2**	GGCAGCATTCTTGCCCTAACCGC (23)	Cloning
***thyA* re 1**	CCCCGGTACCTAATTGGCGAAAATCCG (27)	Reverse PCR
***thyA* re 2**	CCCGGTACCCTGCAGGATTTGGAAACTTGG (30)	Reverse PCR
***wbeT* 1**	CTGCATCTGCAAGTTGATTCTGTATG (26)	Cloning
***wbeT* 2**	ATAGTGAACTCTTCGGAAATGTCTG (25)	Cloning
***wbeT* 1 EcoRI**	CCCGGTCTCGAATTCCTGCATCTGCAAGTTGATTCTGTATG (41)	Expression analysis
***wbeT* 2 HindIII**	CCCGGTCTCAAGCTTATAGTGAACTCTTCGGAAATGTCTG (40)	Expression analysis

2.2.3 LIGATION

Ligation is a common step in cloning where two double stranded DNA molecules are joined together, either with compatible sticky ends or blunt ends.

Reaction mixture

x µl	DNA no 1 [~0.5 µg]
y µl	DNA no 2 [~0.5 µg]
4 µl	x10 Ligase buffer
36-x-y µl	Water
40 µl	Total

Divide this 40 µl into two times 20 µl and add 1 µl of T4 DNA ligase to one of the two 20 µl samples and keep the other as control. Incubate at room temperature in water bath over night.

2.2.4 DELETION WITH REVERSE PCR

The gene of interest is cloned into a plasmid. The designed primers were used to amplify part of the gene and the whole plasmid. The result will be a linear DNA with the plasmid flanked by fragments from the gene.

Reaction mixture

x µl	Sample, with a final concentration of ~0.2 ng/50 µl
5 µl	x10 PCR buffer
1 µl	x50 dNTP:s
1.5 µl	Primer mixture
42-x µl	H20
0.5 µl	Enzyme
50 µl	Total

Amplification program

1.	94°C	5.0 minutes	
2.	65°C	1.0 minutes	
3.	72°C	3.5 minutes	
4.	94°C	0.5 minutes	} x29
5.	65°C	0.5 minutes	
6.	72°C	3.5 minutes + 2 seconds each cycle	
7.	72°C	8.0 minutes	

The genes *thyA* and *epsE* that were amplified with their cloning primers, see Table 3, by PCR from Vx44945 Ogawa was blunt ended and ligated into the Eco32I digested plasmid pBluescript. Reversed PCR was used to generate a deletion in the gene and the fragment was blunt ended. The kanamycin resistance gene, from the plasmid pTnKan-1-sce, TnKan, was used to ligate the plasmid together again, see Figure 1, and electroporated into XL-1 with kanamycin as selection marker. Plasmids from colonies that showed kanamycin resistance was purified and amplified by PCR. These constructs was then electroporated into JS1569 with pkD46 *tac/lac*.

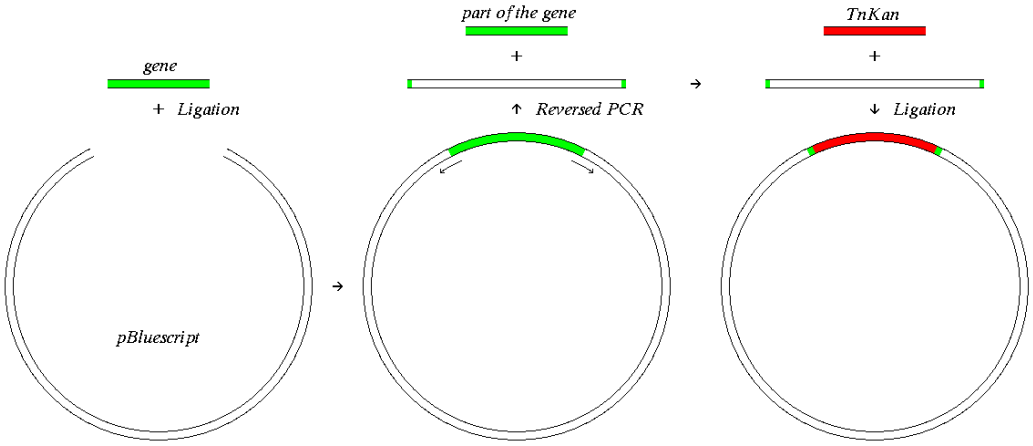

Figure 1, Cloning with reversed PCR.

2.2.5 BLUNT END REPAIR

Blunt end repair is used to make incompatible DNA ends blunt for ligation and to repair the ends of PCR fragments.

Fermentas T4 DNA polymerase was used together with dNTP:s to blunt end repair DNA fragments.

Reaction mixture

x µl	Sample, ~1 µg
1 µl	x10 Tango buffer (Fermentas)
1 µl	x50 dNTP:s
17.5-x µl	H_2O
0.5 µl	T4 polymerase (Fermentas)
20 µl	Total

The reaction was incubated at 37°C for 25 minutes. The enzyme was then inactivated at 65°C for 15 minutes.

2.2.6 RESTRICTION WITH ENDONUCLEASES

A lot of different restriction enzymes were used, see Table 4 below, for cloning and analysis of recombinant plasmids following cloning. The standard incubation time was two hours at 37°C. Enzymes are from Fermentas if nothing else is indicated.

For cloning the following reaction mixture was used:

Reaction mixture, one enzyme

x µl	DNA, 15 ng-1 µg
1 µl	x10 EcoRI buffer
0.5 µl	EcoRI, ~5units
8.5-x µl	H_2O
10 µl	Total

Reaction mixture, two enzymes

x µl	DNA, 15 ng-1 µg
1 µl	x10 Eam1105I buffer
0.5 µl	Eam1105I, ~5 units
0.5 µl	BglII, ~5 units
8.5-x µl	H_2O
10 µl	Total

When restriction analyses were performed the amount of enzymes used was decreased. With n number of recombinant plasmid samples after purification the reaction mixture was:

Reaction mixture, one enzyme		**Reaction mixture, two enzymes**	
8n-1-(n+1) µl	Water	8n-(n+1)-2 µl	Water
n+1 µl	Buffer O	n+1 µl	Buffer R
1 µl	NotI	1 µl	PstI
10(n+1) µl	Total	1 µl	HindIII
		10(n+1) µl	Total

8 µl of reaction mixture was mixed with 2 µl of sample and incubated at 37°C for two hours. Buffer used were those provided by Fermentas. Buffers for double digests were recommended by Fermentas in their double digest tool (http://fermentas.com/en/tools/doubledigest/).

Table 4, Enzymes used in this study and theirs sequences.

Enzyme	Sequences
BamHI	5'...G^G A T C C...3' 3'...C C T A G^G...5'
BglII	5'...A^G A T C T...3' 3'...T C T A G^A...5'
BveI	5'...A C C T G C $(N)_4$^...3' 3'...T G G A C G $(N)_8$^...5'
Eam1105I	5'...G A C N N N^N N G T C...3' 3'...C T G N N^N N N C A G...5'
Eco31I	5'...G G T C T C $(N)_1$^...3' 3'...C C A G A G $(N)_5$^...5'
Eco32I	5'...G A T^A T C...3' 3'...C T A^T A G...5'
EcoRI	5'...G^A A T T C...3' 3'...C T T A A^G...5'
HindIII	5'...A^A G C T T...3' 3'...T T C G A^A...5'
KpnI	5'...G G T A C^C...3' 3'...C^C A T G G...5'
NotI	5'...G C^G G C C G C...3' 3'...C G C C G G^C G...5'
PstI	5'...C T G C A^G...3' 3'...G^A C G T C...5'
PvuII	5'...C A G^C T G...3' 3'...G T C^G A C...5'
SacI	5'...G A G C T^C...3' 3'...C^T C G A G...5'
XhoI	5'...C^T C G A G...3' 3'...G A G C T^C...5'

2.3 SEQUENCING

The pML-*wbeT* plasmids were sent off for sequencing to Eurofins in sample sizes of 16 µl with a concentration of 54-62 ng/µl. Both *wbeT* cloning primers, see Table 3, was included, 50 µl of each primer with a concentration of 2 ng/µl.

2.4 TRANSFORMATION

All bacterial strains were transformed by electroporation. Cells of *Vibrio cholerae* were made competent for electroporation by growing a starter culture of 20 ml LB-medium overnight at 37°C. 5 ml of the starter was used to inoculate a prewarmed flask containing 500 ml LB. The culture did grow with shaking at 37°C until OD600 reached 0.5-0.7. The flask was chilled on ice for 15 minutes and then centrifuged at 7'000g for 5 minutes at 4°C. The pellet was resuspended and washed twice in 500 ml ice-cold solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and immediately centrifuged under the same conditions as before. The pellet was resuspended in 1 ml of an ice cold solution of 1.5 ml 1 mM$CaCl_2$ and 1 mM $MgCl_2$, 1.0 ml 50% glycerol, and 0.5 ml H_2O. This final volume of 1 ml was divided into 50 µl aliquots and was stored at -80°C.

Selection markers such as ampicillin and kanamycin were added when needed. When the temperature sensitive plasmid, pKD46 or pKD46 *tac/lac*, was present a lower temperature was used, 30°C instead of 37°C, and IPTG as the inducer for the *red/gam* system was added.

50 µl of competent cells were on ice mixed with 1 µl of sample, plasmids or DNA fragments, and transferred to a cuvette designed for electroporation. The electroporation was conducted with the system parameters of 2.0 or 2.5 voltage, 25 µFO capacitance, and 200 Ω resistance. 2.0 V was used for *Vibrio cholerae* and 2.5 V for *E coli*.

Immediately after electroporation 1 ml of LB-medium was added and the sample was transferred to a falcon tube and was incubated for 90 minutes at 30°C with 180 rpm shaking. After 90 minutes the sample was concentrated from 1 ml to about 150 µl and plated onto a LB-plate with suitable selection marker.

2.5 BLUE WHITE SCREENING

Blue white screening is a common and well based method to easy determine if a colony of bacteria is carrying a plasmid with or without an insert. The recombinant plasmid of the type pBluescript was electroporated into Xl-1 and was plated out and incubated in presence of x-gal.

This method was used to screening for constructs, one of the cloned genes (*wbeT, epsE* or *thyA*) with the transposon, that was inserted into pBluescript. A white colony with kanamycin and ampincillin resistance was positive candidates.

2.6 EXPRESSION ANALYSIS

The *wbeT* gene was extracted by PCR-primers "*WbeT* EcoRI & HindIII", Table 3, from Vx44945 Ogawa and where cut with Eco31I. The expression vector pML-CTB was digested with EcoRI and HindIII and dephosphorylated and then religated with the *wbeT*-gene. This new vector, pML-*wbeT*, was electroporated into JS1569 to see if the Ogawa serotype resurfaced in the Inaba serotype strain.

2.7 *RED/GAM* SYSTEM IN *VIBRIO CHOLERAE*

The *red/gam* system is a method that works in *E. coli*, but in *V. cholerae* it is untested. In a prior study [22]) they used a single temperature sensitive plasmid, pkD46 Figure 2 (Created using PlasMapper [26]), to carry all the genes for the recombination. The *red/gam* system allows a linear double stranded DNA that is transformed into *E. coli* to be inserted into the genome at a certain place. However to use this pKD46 in *Vibrio cholerae* the promoter need to be changed due to the fact that *Vibrio cholerae* lack possibility to take up arabinose that works as the inducer for the genes on the pkD46.

The taclac primers, see Table 3, where designed and the *lacIq/tac* promotor were amplified from pML-CTB by PCR. The PCR product was digested with SacI and Eco31I and the pkD46 was digested with SacI and BspMI (BveI) and dephosphorylated. The plasmids cannot reliagate with it self after dephosphorylation and due to the design of the restriction sites the *lacIq/tac* promoter fragment can only be ligated to the plasmid in the correct orientation. This new plasmid called pkD46lac,Figure 3 (Created using PlasMapper [26]), was electroporated into *Vibrio cholerae* JS1569. Restriction analyses were performed of purified plasmid with at first AhdI (Eam1105I) and BamHI and second with AhdI and BglII. The product was run on a 1% agarose gel to compare the fragment sizes.

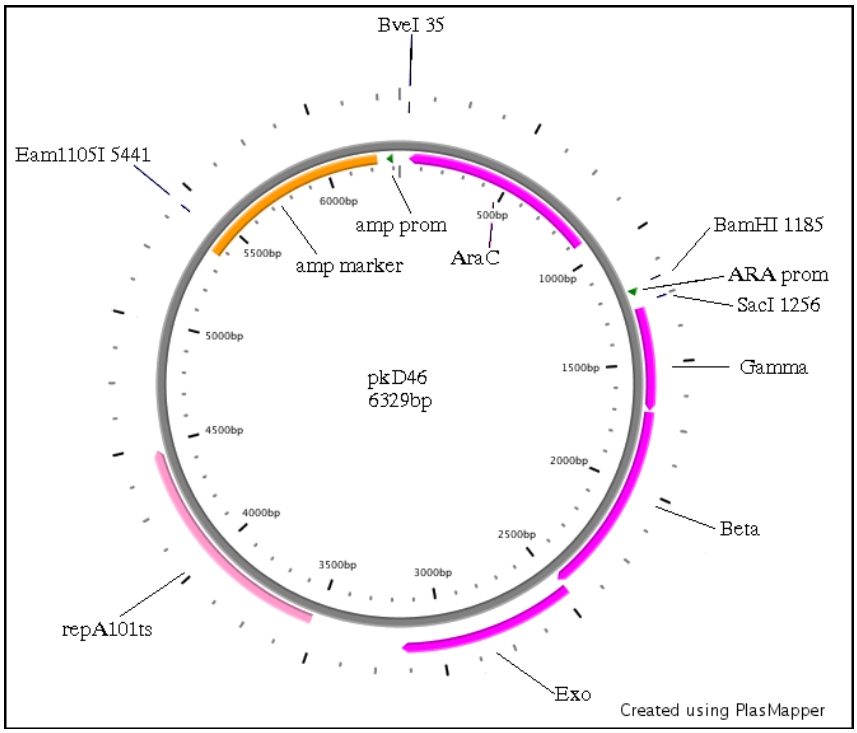

Figure 2, the unmodifed pkD46 plasmid.

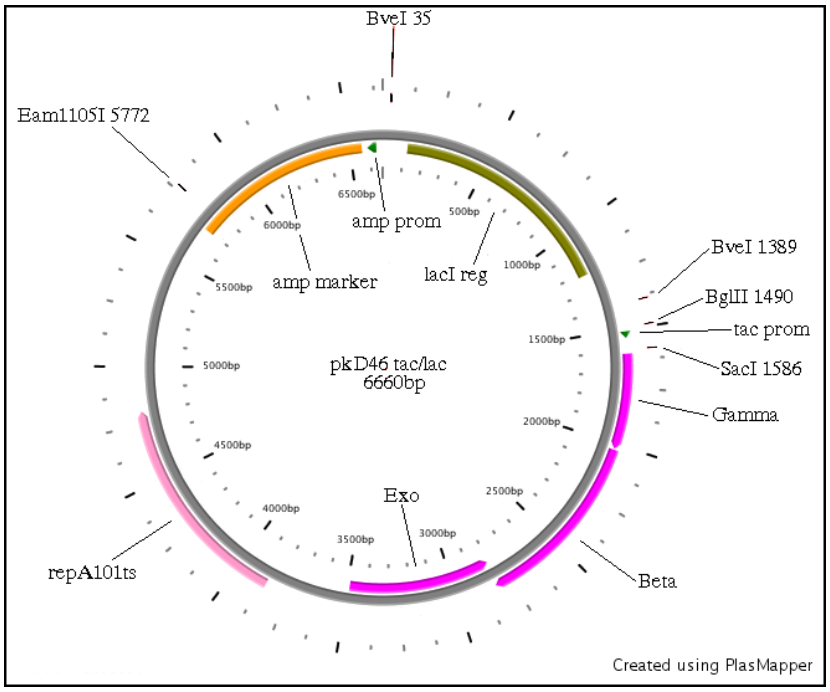

Figure 3, the modified version.

2.8 *IN VITRO* TRANSPOSITION

The genes *wbeT*, *epsE*, and *thyA* extracted by PCR from V.C Vx44945 Ogawa using primers for cloning, see Table 3, and a kanamycin resistance gene carrying transposes were extracted by PCR from the plasmid pTnKan-1-sce using litmus primers, see Table 3. The extracted fragment, see Table 5, were used in the transposes reaction kit bought from EPICENTRE® Biotechnologies.

This kit randomly inserted the kanamycin resistance into to the genes.

Table 5,Genes used in this study and theirs sizes.

Fragment	Size [kb]	Concentration [pmoles/µg]
TnKan	1.2	1.25
wbeT	1.0	1.52
thyA	1.035	1.47
epsE	1.626	0.935

Reaction mixture

x µl	DNA, 0.2 µg
y µl	Transposon, 0.2 µg
1 µl	EzTn5 10x Reaction Buffer
8-x-y µl	Sterile water
1 µl	EzTn5 Transposase
10 µl	Total

The reaction mixture was incubated for 2 hours at 37°C. To stop the reaction 1 µl EzTn5 10x Stop Solution was added and incubated for 10 minutes at 70°C.

2.9 CLONING *WBET* GENE

The plasmid pBluescript was digested with Eco32I and the amplified *wbeT* gene from Vx44945 Ogawa was blunt ended. These two products were then ligated and electroporated into Xl-1.

After plasmid purification the plasmid was cut with EcoR32I which generated a cut in the middle of the *wbeT*-gene. The kanamycin resistance gene with the transposes was amplified using the litmus primers, see Table 3, and the template plasmid pTnKan-1-sce. The primer sequences on the fragment were removed by digest with PvuII. The digested plasmid with *wbeT* and the TnKan fragment was ligated together and electroporated into XL-1 with selection for kanamycin.

Plasmids were purifed from colonies with kanamycin resistance and PCR with the *wbeT* primers was used to amplify the construct and the construct was electroporated into JS1569 with pkD46 *tac/lac*.

2.10 SERA ABSORPTION

Since the subunits B and C, present on the surface on either Ogawa or Inaba see chapter 1.2, are two antigens that is present on the surface of the *V. cholerae* it is possible to determine with antibodies which serotype a certain population have.

Polyclonal antisera from sacrificed rabbits were absorbed to remove cross reactive antibodies with bacteria of the opposite serotype.

1.5 ml cell suspension of formalin killed bacteria was centrifuged at 7000 rpm for 7 minutes. The pellet was washed with 2 ml Fys-buffer and then resuspended in 0.5 ml of the rabbit sera. The suspension was turned end over end for one hour and centrifuged at 7000 rpm for 7 minutes. The supernatant was stored at -18°C

The rabbit sera 1395† LPS 596B where absorbed with Ogawa cells and the sera 1562† LPS 34 Ogawa where absorbed with Inaba cells.

2.11 AGGLUTINATION TEST

An agglutination test is used to see if cells exposed to antibodies will stick together and form aggregates. For this 10 µl cell suspension was used and mixed with 10 µl antiserum on a microscope glass slide. 10 µl cell suspension and 10 µl Fys-buffer was used as a negative control for spontaneous agglutination. For positive control strains with known serotype was used. In Figure 4 a glass slide with a test strain, that agglutinates with both Ogawa and Inaba sera, and it's positive and negative controls.

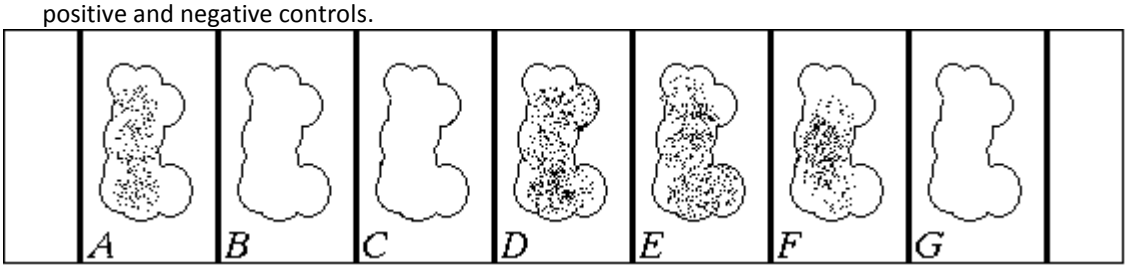

Figure 4, a glass slide for agglutination test. (A) Ogawa + Ogawa sera, positive control. (B) Ogawa + Inaba sera, negative control. (C) Inaba + Ogawa sera, negative control. (D) Inaba + Inaba sera, positive control. (E) Strain + Ogawa sera. (F) Strain + Inaba sera. (G) Strain + fys buffer, negative control.

2.12 STRAIN COLLECTION

Each generated strain during this study was stored for future studies.

From a plate, cells were scraped off and dissolved in 2 ml LB medium, 1 ml 50% glycerol was added and culture of 3 ml was equally divided into two marked glass tubes and stored at -80^{o}C

3. RESULTS

The aim of this study was to make one strain express both Ogawa and Inaba antigens simultaneously by modulating the expression of the *wbeT* preferably located in the chromosome. The *red/gam* system was used for incorporation of the gene into the genome and the *In vitro* transposition to produce the construct needed for the *red/gam* system to work. To investigate if it was possible to convert an Inaba strain back to Ogawa an expression analyses was performed.

3.1 *IN VITRO* TRANSPOSITION

As described before the *In vitro* transposition generates a construct with the gene of interest and a random inserted selection marker and transposes. The construct is then used in the *red/gam* system for incorporation into the genome. After the reaction the constructs were analysed on a 1% agar gel, Figure 5, and it can be seen that the reaction has worked. When electroporation was performed with the different constructs into JS1569 with pkD46 *tac/lacIq* none of the controlled colonies carried the correct fragment. Different methods of cleaning and amplification of the construct was performed to achieve a higher concentration good enough for electroporation. Methods used was for example gel extraction followed by PCR and cloning the construct into pBluescript and then elctroporate into Xl-1, check with blue white screening and selection for kanamycin resistance. However none of the methods used resulted in a higher concentration of the construct. No result shown.

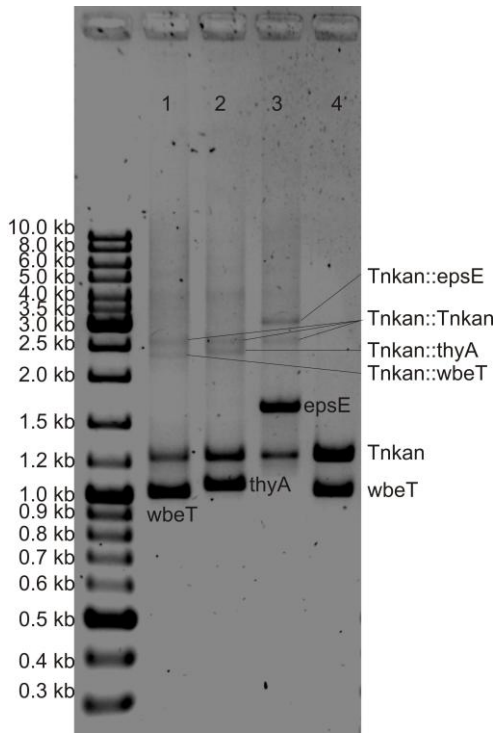

Figure 5, 1% agarose gel (inverted colors) of the in vitro transposition.[1]*wbeT* and TnKan. [2] *thyA* and TnKan. [3] *epsE* and TnKan. [4] Control with *wbeT* and TnKan. Note the thin bands with the transposon inserted into the genes and the lack of bands in the control.

3.2 RECOMBINATION

Since the *In vitro* transposition did not generate enough product for electroporation, traditional cloning was used instead. The *wbeT* gene was cloned as described in chapter 2.9 and *epsE* and *thyA* as described in chapter 2.2.4. The constructs were amplified by PCR and then electroprated into JS1569 with pkD46 *tac/lac* and with selection for kanamycin resistance. Since no colony showed resistance against kanamycin a test, of competence, chapter 3.3, of the JS1569 pkD46 *tac/lac* was performed. Electroporation with the new optimised parameters were done with the constructs made by both cloning and *In vitro* transposition, but none of the tested clones had the right construct. No result shown.

3.3 ELECTRO COMPETENCE

The test showed that the cells were not very competent when using 2.5 V during electroporation. So the test was extended to test competence at different voltages. The result of the test shown in Table 6 and the conclusion is that the JS1569 with pkD46 *tac/lac* are more competent when using 2.0 volt than 2.5 volt during electropration. Due to this test all electroporation with JS1569 was from now on done at 2.0 V.

Table 6, Results for electro competence analysis.

Voltage	Number of colonies
2.0	42
2.1	24
2.2	8
2.3	10

3.4 EXPRESSION ANALYSIS

The aim with this part was to check the functionality of the *wbeT* gene from VX44945 Ogawa and to demonstrate that a mixed or fully Ogawa serotype was achieved when the gene was introduced into an Inaba strain. The pML-*wbeT* plasmid was generated with the amplified *wbeT*-gene and inserted into the inducible plasmid pML-CTB::OVA. This plasmid was then electroporated into JS1569 and agglutination tests was performed. The same colonies were also induced with IPTG and analysed with an agglutination test. The plasmids were purified and with restriction analysis it could be shown that in all cases the gene had been inserted into the plasmid. These recombined plasmids were sent off for sequencing with the *wbeT* 1 and 2 primers which allowed the entire sequence to be determined in both orientations. All three clones contained the correct sequence. As shown in Table 7 both induced and none- induced cells have both serotypes. Since this was a positive result it was decided to use pML-*wbeT* colony three, none- induced, for an immunisation assay in mice. Bacteria used in this assay were formalin killed by a co-worker and an agglutination test was performed to control the serotype of the culture.

After some primary results from other immunisation assays it was concluded to change the strain of the vaccine candidate from JS1569 to Phil6973, since the strain Phil6973 gave somewhat higher antibody concentration against Vibrio cholerae LPS in ELISAs. Due to this the pML-*wbeT* plasmid was electroporated into Phil6973 and tested for agglutination. Strains that contantd the functionally *wbeT* gene in either genome or plasmid did agglutinate with the Ogawa antibody whereas strains with truncated *wbeT* gene only agglutinated with the Inaba antibody. A strain

that agglutinates with both antibodies has both Ogawa and Inaba LPS present on its surface and thereby has a Hikojima phenotype. All strains were tested against both Inaba and Ogawa antibodies and against Fys-buffer, as a control for self agglutination. Results shown in Table 7.

Table 7, Agglutination test. '+' means agglutination visible and '-'means no visible effect.
*** Fk stands for formalin killed.**

Name	Strain	Induced	Ogawa antibody	Inaba antibody	Fys-buffer
Ogawa	Vx44945	-	+	-	-
Inaba	JS1569	-	-	+	-
Inaba	Phil6973	-	-	+	-
pML-*wbeT* col 3	JS1569	no	+	+	-
pML-*wbeT* col 4	JS1569	no	+	+	-
pML-*wbeT* col 5	JS1569	no	+	+	-
pML-*wbeT* col 3	JS1569	yes	+	+	-
pML-*wbeT* col 4	JS1569	yes	+	+	-
pML-*wbeT* col 5	JS1569	yes	+	(+)	-
pML-*wbeT* col 3 Fk*	JS1569	no	+	+	-
pML-*wbeT* col 3	Phil6973	no	++	++	-
pML-*wbeT* col 4	Phil6973	no	++	(+)	-
pML-*wbeT* col 5	Phil6973	no	++	+	-

4. DISCUSSION

The main aim of this study was to make a *Vibrio cholerae* strain that expresses a mixture of Ogawa and Inaba serotype determinants on its surface. It was proposed to achieve this by mutagenesis of a candidate vaccine strain using the phage lamba-derived *red/gam* recombination system from *E. coli* adapting it for use in *V. cholerae.* A secondary aim was to establish a generalized method for mutagenesis in *V. cholerae* based on a combination of *In vitro* transposition and the *red/gam* recombination system that has been used previously in *E. Coli* [22].

***In vitro* transposition and r*ed/gam* system**

In vitro transposition was attempted using a purified recombinant transposase and a modified mini-Tn5 transposon. The advantages of the system were that one is able to target a specific gene for transposon mutagenesis and that if the entire genome of the organism is known, no cloning procedures are required. Furthermore, since the mini-transposon does not encode its own transposase, it cannot transfer to other sites in the genome and produces a stable genotype. Although it was clear from analysis of the reactions by agarose gel electrophoresis that the reactions had worked, the efficiency of the *In vitro* transposition was poor. Subsequent failures in the electroportations of the target strain of *V. cholerae* to yield mutants led us to consider whether there was sufficient mutated DNA in the reaction product. In order to test this hypothesis we inserted the transposon into the *epsE* gene using conventional gene manipulation techniques and could thereby generate essentially unlimited amounts of the desired DNA either by digestion from a recombinant plasmid with restriction endonucleases and subsequent extraction of the desired DNA fragment from agarose gels or by PCR amplification. Using this DNA the *red/gam* system continued to yield no mutants and we therefore concluded that under the conditions used it was not functional in *V. cholerae*.

There are a number of possibilities why this did not work in *V. cholerae.* It may be that the modification of pkD46 plasmid disabled the *red/gam* genes or that *V. cholerae* produces very effective exonucleases that destroy the linear DNA molecules before they can recombine into the genome. It may also be a strain specific effect and the *red/gam* system may work in some other strains of *V. cholerae*. Clearly more studies must be done before it can be concluded that the *red/gam* system does not function in *V. cholerae*. Further studies should include controlling the modified plasmid, pkD46 *tac/lac* in a system that is known to work for instance in *E. coli* and investigating whether a strain of *V. cholerae* with the unmodified plasmid is inducible with arabinose, this should work if the strain could take up enough arabinose to induce the *red/gam* system. Unfortunately, in the limited time available, these avenues could not be pursued as other goals of the project were more pressing.

Hikojima expression analysis

The *wbeT* gene from an El Tor O1 Ogawa *V. cholerae* strain, VX44945, was amplified and cloned into an inducible expression vector. The resulting plasmid pML-*wbeT* was then electroporated into the O1 classical Inaba strain JS1569 and agglutination tests were performed both on induced and non- induced cultures.

Although full serotype switching was not observed in the induced cultures it could be seen that the strain did clearly agglutinate with Ogawa-specific antiserum. This can be explained by poor expression of the *wbeT* gene from the plasmid in this strain. When the plasmid was inserted

into Phil6973, an El Tor strain, full conversion into the Ogawa serotype was achieved even in the non- induced cultures.

Although useful, the agglutination method used is not a quantitative. The cell concentrations are not standardized and the concentration of the antisera is not known. This can result in differences in the observed agglutination that do not necessarily reflect the levels of antigen expression.

The results show that a culture of an Inaba strain transformed with the pML-*wbeT* plasmid shows both Ogawa and Inaba serotypes. It is possible by several methods to distinguish whether cells in the population have both serotypes present on their surface or whether some cells are fully converted to the Ogawa serotype with others showing no conversion at all. Samples have for example been prepared for further studies in which FACS analysis to determine whether both antigens are present on the same cell. However this study has shown that it is possible to complement *In trans* the *wbeT* gene in an Inaba strain and get mixed expression of the Ogawa and Inaba serotypes. The strain generated in this study is being used for preliminary immunisation studies.

The generated Hikojima strain results from modulation of the level of expression of a fully functional *wbeT* gene. However, it may be possible to by mutagenesis to obtain mutants of the gene that express a gene product with lower enzyme activity that can be expressed at normal levels. The cloned gene obtained in this study will therefore also be used to generate mutants of the *wbeT* genegive products that are less active gene than the wild-type resulting in strains with the Hikojima serotype. Support for this approach comes from the sequence of the *wbeT* genefrom a Hikojima/Inaba strain that had a single point mutation resulting in a full length gene with little or no methylating activity. This point mutation was located at the 472 bp, a TCA to CCA, and resulted in a codon change from serine to proline. Since proline is known to be a helix breaker, it is likely that this mutation situated in a possible a-helix has an impact on the folding of the protein and thereby the enzyme's activity. A reduced activity of the *wbeT* gene's product results in an incomplete methylation of the LPS and therefore expression of both Inaba and Ogawa antigen on the cell surface or a Hikojima phenotype.

JS1569 is a *ctxA* deleted derivative of the classical O1 *V. cholerae* strain 569B and as such is atypical in many respects. This may be reflected in the results of the current study. Previous results from immunisations suggest that immune responses were not as high for this strain as it was for other less attenuated strains it was decided to change the vaccine candidate strain to Phil6973. Further studies will therefore be conducted in the strain Phil6973.

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