Preparations of construct for gene transfer from *Helicobacter pyroli* to transgenic plants

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Degree project in biology, 2007
Examensarbete i biologi 20 p, 2007

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Summary

*Helicobacter pylori* is estimated to infect half of the world's population and causes more or less severe diseases like peptic ulcer and gastric cancer. Treatment failure of the infection is a growing problem world-wide, due to development of antibiotic resistance and many researchers agree that a vaccine against *H. pylori* is one possible way to overcome this problem. Much focus is currently on orally delivered vaccine candidates such as edible safe recombinant protein vaccines to induce a protective immune response. In this study, the gene encoding the highly immunogenic siderophore-mediated iron transport protein TonB from *H. pylori* was cloned in the binary vector pGreen0229. The study included parallel work with whole and truncated TonB. *Agrobacterium tumefaciens* was transformed with the constructs together with the *vir* helper plasmid pSoup. The presence of the selected genes was shown by polymerase chain reaction (PCR) and sequencing. By using the floral dip method, *Arabidopsis thaliana* was infected with the double transformed *Agrobacterium*. The protein was overexpressed in *Escherichia coli*. The presence of the protein was demonstrated by western blot based on pooled human serum containing high concentrations of anti-TonB antibodies. The results of the overexpression procedure showed that the protein was present on the cell surface of the bacteria. Thus, constructs for creating transgenic plants expressing the TonB protein have been produced. Unfortunately, time did not allow the transfer experiments to plants to be completed. These experiments are under way, but the question whether the plants express the protein or not is beyond the scope of this report.
Introduction

In the early 1980’s the common belief was that stress and lifestyle were the two major causes of peptic ulcers. In 1982 Robin Warren and Barry Marshall, two Australian scientists, discovered an unknown bacterium in the stomachs of patients suffering from gastric inflammation and peptic ulcers. They proved that this bacterium, later named *Helicobacter pylori*, causes stomach and duodenal ulcers, and 20 years after the discovery in 2005 they were awarded the Nobel Prize. One convincing experiment was when Marshall infected himself with the bacteria and cured the infection with antibiotics (Waltz & Dennis 2005)

Virulence and disease

*H. pylori* is a gram-negative bacterium that infects the gastric mucosa of almost 50 % of the world population. The infection is asymptomatic in 70-80 % of the infected individuals, but in others it may cause more or less severe conditions like gastric inflammation, peptic and duodenal ulcers and mucosa-associated lymphoid tissue (MALT) lymphoma. The international agency for research and cancer (IARC) declared the pathogen to be a class 1 carcinogen in 1994 based on its association with gastric cancer (Meinke et al. 2006, Kubota et al. 2005). To be able to survive in the stomach, the bacterium has a set of virulence factors. Among the most studied virulence genes and their products are flagellins, vacuolating toxin A (VacA) and cytotoxin-associated protein A (CagA) (Velayudhan et al. 2000). *H. pylori* also produces the enzyme urease to deal with the acidic environment in the stomach. This enzyme is shown to be very important for the colonization of the organism in the mucosa (Zavala-Spinetti et al. 2006).

All bacteria are dependent on iron (Fe$^{2+}$, Fe$^{3+}$) to be able to grow. The free iron concentration in the body is very low due to the iron-binding proteins transferrin and lactoferrin. The bacteria require a system that can remove the iron from these proteins for usage as growth factor. Many pathogens secrete small molecules, siderophores, with high affinity for iron that can form complex with the ion (Worst et al. 1995). The transfer of the complex across the outer membrane is provided by the protein complex consisting of TonB, ExbB and ExbD in *H. pylori* (Velayudhan et al. 2000). Schauer et al. 2007 showed that this complex also is important for nickel uptake into the cell. TonB is a transmembrane protein, consisting of 285 amino acids, that is anchored to the cytoplasmic membrane in the bacterial cell. Its’ C-terminal end interacts with the outer membrane receptors, linking the two membranes together (Karlsson et al. 1991). The molecular weight is 31 kDa.

Treatment and subunit vaccines

Standard treatment of an *H. pylori* infection consists of two antibiotics and one proton pump inhibitor (PPI) as a first-line therapy. If the first-line therapy fails there is a second-line treatment with one PPI, one bismuth component and two antibiotics. However, since more people are treated for the infection, the antibiotic resistance among the strains is increasing and consequently also the failures in eradication of the infection (Manes & Porro 2006). Therefore, many researchers believe that the development of a vaccine would be valuable (Zavala-Spinetti et al. 2006).

Most of the vaccines used today are composed of live attenuated or killed whole bacteria or viruses. A relatively new strategy is to focus on subunit vaccines where one or more important proteins from the pathogen are produced by a non pathogenic organism. The protein can be purified from the harmless organism or be delivered together with the host.
Such harmless vectors can be live or killed bacteria or viruses and transgenic plants. These kinds of vaccines are much safer than the classical ones. The hepatitis B surface antigen was the first recombinant subunit vaccine produced in *Saccharomyces cerevisiae* in 1986 (Ståhl & Liljeqvist 2001), and is used as the Hepatitis B vaccine today (Lou *et al.* 2007).

Potential antigen candidates for a vaccine against *H. pylori* are usually sought among the well known virulence factors like CagA, VacA, urease, adherins and flagella proteins (Vyas & Sihorkar 1999). A recent study showed that the siderophore-mediated iron transporter protein TonB, encoded by *tonB*, is highly immunogenic and thereby ranks as one of the most important vaccine candidates (Storm, M 2006).

**Oral vaccines and transgenic plants**

Most vaccines used today are administered by needles which cause a more efficient systemic immune response as compared to the mucosal sites. Since *H. pylori* affects the mucosa in the stomach, it is important to induce a protective mucosal immune response (Tomonori *et al.* 2007). This can be achieved by delivering the vaccine by the oral route (Holmgren & Czerkinsky 2005). This kind of immunisation is generally inexpensive because no special equipment, like syringes and needles, is needed (Streatfield 2006).

The earliest documented attempt to confer protection of poisonous material by the oral way is from the king of Pontus, about 2000 years ago (Cripps *et al.* 2001). Today, polio and cholera are commonly presented by oral vaccines (Holmgren & Czerkinsky 2005).

A problem to use oral protein-based vaccines is that they are degraded easily due to proteolysis in the stomach. This problem can be solved by delivery of a high amount of antigens, or by protecting the antigens by coating them with products that are degraded slowly in the gastrointestinal tract (Streatfield 2006). One way to deliver large amounts of antigens in an encapsulated form is to use plant-based vaccines which is a relatively new strategy. This kind of vaccines has many benefits, since plants do not have any human or animal diseases. That increases the safeness and reduces the costs because there is no need to screen for bacterial or viral toxins. Plants can also produce large quantity of complicated proteins from higher eukaryotes (Qing *et al.* 2005). No special equipment for delivery is needed which decreases the risk of contamination. Special training of injection techniques and handling of equipment will be less important. An edible vaccine does not need to be stored in the refrigerator which reduces the costs even more, since it is estimated that the cold-storage of today’s vaccines costs $200 to $300 millions a year world-wide (Tomonori *et al.* 2007)

In 1986, the human growth hormone was the first relevant pharmaceutical protein expressed in transgenic tobacco. Since then many recombinant proteins like antibodies, diagnostic proteins, enzymes etc have been expressed in plants like tobacco, potato and maize. In a few cases vaccine candidates, as for example heat-labile toxin B subunit of enterotoxigenic *Escherichia coli*, and the capsid protein of Norwalk virus, have reached the first clinical trial (Streatfield 2006, Ma *et al.* 2003). Thanavala *et al.* 2005, showed that the Hepatitis B virus surface antigen, previously expressed in *Saccharomyces cerevisiae*, also could be expressed in transgenic potatoes and induce a protective immune response in humans. However, no antigen from *H. pylori* has been expressed successfully until Qing *et al.* 2006, managed to express urease subunit B in transgenic rice. Much research is now focussing on using edible transgenic plants like raw fruit, vegetables, leaves and seeds in vaccine development (Streatfield 2006).
The "floral dip method" and *Arabidopsis thaliana* as the model plant

Using *Arabidopsis thaliana* as the model plant has many benefits. It is small, quick-growing and easy to transform genetically. In the late 1980’s, two scientists reported that they had succeeded with transformation of *A. thaliana* with the soil-born *Agrobacterium tumefaciens* by a method called "the floral dip method". The method implies dipping the whole plants except the roots in an *A. tumefaciens* suspension for a few seconds. (Clough 2004).

In the environment, *A. tumefaciens* causes crown galls due to its ability to insert a part of its DNA, the Transfer-DNA (T-DNA) region, into the genome of dicotyledonous plants such as *A. thaliana*. A wounded plant expresses molecules that induce transcription of virulence genes which are located on a plasmid, called the tumor-inducing (Ti) plasmid, in the bacterium. This plasmid also contains the T-DNA region which is defined by the left (LB) and right (RB) borders. Thus, the transfer of the T-DNA from *A. tumefaciens* to dicotyledonous plants dependents of the virulence genes on the Ti-plasmid (Valentine 2003). This opened the idea that Ti-plasmids could be used as vectors to transfer foreign genes into plant cells, by cloning the gene of interest between the border sequences.

However, Ti-plasmids are large and do not contain restriction enzyme sites located only in the T-DNA region. This makes it difficult to clone a gene directly into the T-DNA. One way to solve the problem is to use a binary vector system, where the gene of interest is inserted into a T-region on one plasmid (binary vector) lacking the virulence genes. Another plasmid (*vir* helper) with the virulence genes but without the T-region is constructed. None of the plasmids can function alone, but act *in trans* in the same *Agrobacterium* cell (Gelvin 2003). The plants are then dipped in a culture of the doubly transformed bacteria, and their off-spring can be analysed for the gene and protein expression of interest (Clough 2004) (see figure 4, p. 12).

The pGreen vector system

The binary vector system used in this study is from the pGreen series. These vectors are constructed in an easy-of-use way and exist with different selectable markers and reporter genes within and outside the left and right borders of the T-DNA region. Also, they have different restriction enzyme sites within the T-DNA region. (Hellens *et al*. 2000). The pGreen0229 (used in this study) has a gene encoding for kanamycin resistance which functions as a selectable marker during transformation of *Escherichia coli* and *Agrobacterium tumefaciens*. In the T-DNA region of the plasmid, there is a lacZ gene, with a set of restriction enzymes sites. This is valuable in the cloning and transformation procedure of *E. coli*, since it can be used for blue-white screening. Insertion of a gene fragment within the lacZ region will result in white colonies due to unsuccessful degradation of ß-galactosidase. Plasmids that lack a gene fragment in the lacZ region forms blue colonies from successful degradation of ß-galactosidase. To use blue-white screening, the medium must contain the substance 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (Klug & Cummings 2002). In this study, the *tonB* gene was cloned in a 35S-CaMV cassette which was already cloned in the *EcoRV* site of the lacZ. The cassette has a 35S promoter which is strongly constitutive and will yield replication of the plasmid in *E. coli*, and thereby production of the inserted gene product. There is also a BASTA resistance gene in the T-DNA region. BASTA is an herbicide and untransformed plants will not survive on medium containing BASTA (see fig. 4, p.12).
Aims
The aims of this study were to transform *Arabidopsis thaliana* with the *tonB* gene from *Helicobacter pylori* 26695, by the floral dip method, and to investigate if the plant cells expressed the gene product TonB.
Results

Amplification and control of vectors by transforming competent *Escherichia coli*

The binary vector pGreen0229 has a 35S-CaMV cassette with a 35S promoter (strongly constitutive) cloned into an *Eco*RV restriction enzyme site in the *lacZ* gene of its T-DNA region (see fig. 4, p. 12). The vector also has a kanamycin resistance gene and those bacteria that survive on the selective plates probably have adopted the plasmid. Five single colonies were inoculated and plasmids purified. To verify if the purification had succeeded, a test-cleavage with the restriction enzyme *Eco*RV was done. This reaction cleaves out the 35S-CaMV cassette of ~700 bp from the plasmid, which could be seen in 1% agarose gel (data not shown).

Amplifying whole and truncated *tonB*

Both the whole and truncated part of *tonB* were amplified from *Helicobacter pylori* 26695 using the Invitrogen PCR program. The primers used for these amplifications were constructed so that they contained sites for the restriction enzymes XbaI and BamHI. The transmembrane region of the protein is located in its N-terminus. This region is truncated by one primer pair. The other primer pair amplified the whole gene. This resulted in two different product sizes on the agarose gel. The truncated *tonB* gave a band of ~750 bp while the whole gene resulted in a band of ~850 bp, as expected (data not shown).

Blue / White screening of *Escherichia coli* transformants

The amplicons were cleaved with XbaI and BamHI and ligated into pGreen0229. The plasmids, now with two different inserts, were allowed to transform competent *E. coli* and grown on selective plates containing X-gal. The plasmid containing the truncated *tonB* was named pTrunk and the plasmid containing the whole gene was named pWhole. Both white and blue colonies appeared as expected. 7 of 20 chosen *E. coli* colonies containing pTrunk and 17 of 20 chosen *E. coli* colonies containing pWhole were positive in the PCR analysis (data not shown). 6 of each positive *E. coli* clones were cultivated in LB-medium supplied with 50 μg/ml kanamycin for plasmid purification. PCR screening (using Invitrogen PCR program) for truncated and whole *tonB* from the pTrunk and pWhole were all positive except one, (figure 1a and 1b).

![Figure 1a](image1a.png)  
Figure 1a. PCR of *tonB* from purified pWhole plasmids showing 6 of 6 positive clones. Lane 1; 100 bp molecular size marker, Line 2-7; positive plasmid constructs, Line 8; negative control

![Figure 1b](image1b.png)  
Figure 1b. PCR of truncated *tonB* from purified pTrunk plasmids showing 5 of 6 positive clones. Lane 1-4 + 6; Line 5; negative plasmid, Line 7; negative control, Lane 8; 100 bp molecular size marker
To verify that no mutations had occurred, the inserts in pTrunk and pWhole were sequenced. Six of six samples from pWhole and two of six samples from pTrunk were 100 % identical to *tonB*, comparing to the wild type sequence in BLAST. The remaining four samples showed between 93-97 % identity (data not shown).

**Transforming Agrobacterium tumefaciens**

One of pTrunk and one of pWhole that showed 100 % identity in the sequence analysis was allowed to transform *Agrobacterium tumefaciens* together with the *vir* helper plasmid pSoup. Large amounts of doubly transformed bacteria were grown on the selective plates. Five colonies per construct were analysed by colony PCR, using the TaKaRa PCR program. The first attempt failed, since the positive control was negative. However, distinct bands of about 750 bp were found for two clones carrying the pTrunk plasmid and one of these was chosen to set an over night culture. A new PCR was made for all five clones of the whole gene (boiling time seven minutes instead of five), which yielded expected bands of 850 bp on the agarose gel. One of these clones was selected to set the other over night culture.

**Transforming Arabidopsis thaliana**

Whole (except the roots) *Arabidopsis* plants were dipped in the two different over night cultures. The plants were rested on their side over night before they were transferred to grow in their pots at optimal conditions until seeds could be collected (~ five weeks). The collected seeds (~1 ml of each construct) were washed and plated on ~ 40 selective plates. No seeds started to grow due to a mistake in the washing procedure (see fig. 2).

![Figure 2. Picture of seeds not growing on selective plates. If transformation of Arabidopsis thaliana was succeeded, some seeds would have grown to ~1-2 centimeter high plants.](image)

**Overexpression of TonB**

If the seeds had grown to plants, a positive control would be needed. This was done by overexpressing the protein from the provided *E. coli* BL21 star cells. Since TonB is a transmembrane protein, both the supernatant and the cell pellet were analysed with western blotting. A protein of ~31kDa could be found in the pellet material and not from the purified cytosol material from the lysis procedure (figure 3).

![Image of plate with colonies](image)
Figure 3. Western blot analysis from overexpression of ionB, using human serum and goat anti-human IgG antibodies. Lane 1-5; different concentrations of cell pellet resuspended in equilibration buffer including 8 M urea, Lane 6; supernatant from pellet solved in equilibration buffer excluding 8 M urea, Lane 7; supernatant from pellet solved in equilibration buffer including 8 M urea, Lane 8-9; elution from purified cytosol material, Lane 10; 1:st flow-through of purified cytosol material, Lane 11; 2:nd flow-through of purified cytosol material, Lane 12-13; positive control from whole cell lysate, saved from pilot test, Lane 14, BioRad molecular weight marker.
Discussion

In this study, I attempted to transform *Arabidopsis thaliana* with a gene encoding the highly immunogenic TonB protein of *H. pylori* by the floral dip method, using *Agrobacterium tumefaciens*.

Plasmid constructs for gene transfer to *A. tumefaciens* and *E. coli* were produced, and transformed *A. tumefaciens* was analysed by PCR and sequenced to show that the bacteria contained an intact gene segment. A positive control for possibly transformed plants was performed by over expressing TonB from provided *E. coli* constructs. There were some technical problems with the plasmid construction in the beginning, which took several weeks to resolve. The time available for this project was sufficient to produce the necessary constructs for the transfer to plants. However, the reproduction time of the plants, around ten weeks, restricts the number of experiments possible to do at the plant phase. One transfer experiment was performed but unfortunately no seeds grew. No more seeds were available and therefore the entire transformation from *A. tumefaciens* to *A. thaliana* must be repeated. A similar project with the p24 protein from HIV-1 subtype C expressed in *A. thaliana* (Lindh 2006) succeeded while the first trial of expressing the transmembrane protein MOMP from *Chlamydia trachomatis* (Atterfeldt 2006) failed. The choice to work in parallel with both the whole and truncated part of TonB is based on the failure of the MOMP protein. Both MOMP and TonB are transmembrane proteins which may be difficult to express in plants. The truncated version of my constructs does not include the transmembrane part.

In conclusion, preparation of constructs for creating transgenic plants to express the highly immunogenic TonB protein of *H. pylori* has been completed. Transfer experiments to plants now have to follow and, if successful, subsequent testing of immunogenicity in small animals.
Materials and methods

Bacteria strains and vectors

All bacteria and vectors that were used in this study are listed in table 1 below. The pGreen and pSoup construct is shown in figure 4, p.12.

Table 1. Bacteria and vectors used in this study

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<tr>
<th>Bacteria/Vector</th>
<th>Function</th>
<th>Reference</th>
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<td><em>Helicobacter pylori</em> 26695.</td>
<td>Amplify <em>tonB</em></td>
<td>The Swedish Institute of Infectious Disease Control, Sweden</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> EHA105</td>
<td>Used to transform <em>Arabidopsis thaliana</em></td>
<td>Utah State University, UT, USA</td>
</tr>
<tr>
<td>pGreen0229</td>
<td>Used to clone <em>tonB</em> and transform <em>A. tumefaciens</em></td>
<td>John Innes centre, Norwich Research Park, Norwich, UK</td>
</tr>
<tr>
<td>pSoup</td>
<td>Used to act <em>in trans</em> with pGreen0229</td>
<td>John Innes centre, Norwich Research Park, Norwich, UK</td>
</tr>
<tr>
<td>Chemically competent <em>Escherichia coli</em> DH5α</td>
<td>Was transformed with pGreen0229</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
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<tr>
<td><em>Escherichia coli</em> BL21*-pET28b(+)<em>tonB</em></td>
<td>Used for overexpression of TonB. The protein was HIS-tagged</td>
<td>Swedish Institute for Infectious Disease Control, Sweden</td>
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Plates and media

Luria Bertani agar plates (LB-plates) supplied with kanamycin 50 μg/ml were ordered from Karolinska laboratoriet, Solna, Sweden. (Bacto Tryptone 10 g, yeast extract 5 g, sodium chloride 10 g, bacto agar 15 g and ultrapure H2O up to 1L, adjusted to pH 7.

Luria Bertani medium (LB-medium) supplied with kanamycin 50 μg/ml was ordered from Karolinska laboratoriet, Solna, Sweden. (Bacto Tryptone 10 g, yeast extract 5 g, sodium chloride 10 g and ultrapure H2O up to 1L, adjusted to pH 7.

Murashige and Skoog plates (MS-plates) were prepared from Murashige and Skoog medium powder according to the manufacturer’s recommendations (Duchefa, Haarlem, Netherlands). 10 μg/ml of the herbicide BASTA (Duchefa, Haarlem, Netherlands) and 400 μg/ml Cephatoxime (Duchefa, Haarlem, Netherlands) were added to the medium before autoclaving.
Figure 4. The construction of the binary vector pGreen0229 with the 35S-CaMV cassette, in which truncated and whole tonB were cloned. The 35S-CaMV cassette was already cloned in to an EcoRV site of the lacZ gene of the T-DNA region defined by left and right border (LB and RB). The vir helper plasmid pSoup acts in trans within the same Agrobacterium tumefaciens cell, which allows the whole T-DNA region including the gene of interest to be transferred to the plant genome. For details, see introduction. Picture from www.pGreen.ac.uk

Primers

All primers that were used are listed in table 2 below. HP1341F and HPtrunkF were designed to contain an XbaI site in their 5′ end. HP1341R was designed to contain a BamHI site in its 3′ end. 35SF and 35SR were used for sequencing and annealed before respective after the inserts in pGreen0229. 35SF and 35SR were also used as primers to A. tumefaciens colony PCR, because HP1341F, HP1341R and HPtrunkF were not available.

Table 2. Primers used in this study.

<table>
<thead>
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<th>Name</th>
<th>Sequence (5′ → 3′)</th>
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<tr>
<td>HP1341F</td>
<td>-GAT CTC TAG AAT GAA AAT TTC TCC ATC TCC-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>HP1341R</td>
<td>-CAT CGG ATC TCT AGT CTT TCA AGC TA-</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>HPtrunkF</td>
<td>-GTT TGT CTA GAT TTT AAT GCG CGA AGA AGA-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>35SF</td>
<td>-GAG CAT CGT GGA AAA AGA AGA-</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td>35SR</td>
<td>-CTT ATC GGG AAA CTA CTC ACA CAT T-</td>
<td>Sigma, UK</td>
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PCR programmes and agarose gel electrophoresis analysis

Invitrogen PCR program

The Invitrogen PCR program was used to amplify *tonB*, perform colony PCR on *E. coli* and to check the gene inserts in pGreen0229. Chromosomal DNA was extracted from *H. pylori* 26695 using a DNeasy blood and tissue kit (QIAGEN). The PCR reaction mixture consisted of 1 μl template, 2.5 μl 10 x PCR buffer-Mg, 0.5 μl 10mM dNTP mix, 0.75 μl 50mM MgCl₂, 1.25 μl 10 μM HP1341F respective HPTrunkF as forward primer, 1.25 μl 10 μM HP1341R as reverse primer, 0.22 μl Taq DNA polymerase (5 U/μl) and ultrapure H₂O up to 25 μl (all PCR reagents were from the Taq DNA polymerase kit, Invitrogen). As a negative control, sterile water instead of template was added to one tube and as a positive control, chromosomal DNA from *H. pylori* 26695 was used. The PCR program was set to 180 seconds of incubation at 94°C, 45 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, 90 seconds of extension at 72°C and an extra extension for 10 minutes at 72°C. The samples were then held at 4°C. The number of cycles was 30. PCR products were purified using Qiaquick PCR purification kit (QIAGEN), following the manufacturer’s instructions. In some cases, the DNA concentration was measured using a Nano-drop ND-1000 (Saveen Werner).

The PCR products were analysed on 1% (w/v) agarose gel (Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) dissolved in 1 x TBE buffer (Karolinska laboratoriet, Solna, Sweden). (10 x TBE buffer; 108 g Tris base, 55 g boric acid and 40 ml EDTA 0.5M pH 8.0 up to 1 L double distilled H₂O). 1 μl of 6 x loading dye (Invitrogen) was added per 5 μl sample before loading them onto the gel. As a molecular size marker a 100 bp DNA ladder (Invitrogen) was used. The gel was run 100-120 V, for 30-50 minutes and stained in 1 μg/ml ethidium bromide (Invitrogen) bath for 15 minutes.

TaKaRa PCR program

The TaKaRa PCR program was used for *A. tumefaciens* colony PCR. To extract *A. tumefaciens* DNA, colonies were suspended in 6 ml LB supplied with 50 μg/ml kanamycin and incubated at 28°C, 200 rpm, over night. 0.6 ml of the culture were transferred to empty PCR tubes and centrifuged at 16000 x g, for 3 minutes at room temperature. The supernatants were discarded, the cell pellets were resuspended in 20 μl ultrapure H₂O and boiled for 5 or 7 minutes. After centrifugation at 16000 x g, the supernatant was used as templates. According to the TaKaRa Ex Taq™ (TAKARA BIO INC., Shiga, Japan) general protocol, the PCR reaction mixture was composed of 0.125 μl Ex-Taq (5 U/μl), 2.5 μl 10 x Ex Taq buffer, 2 μl dNTP mix, 10 μl template, 1 μl forward primer 35SF, 1 μl reverse primer 35SR and 8.375 μl ultrapure H₂O. As negative control, sterile water was used and as positive control, plasmid DNA (containing *tonB*) was used. The program was set to 120 seconds of incubation at 98°C, 15 seconds of denaturation at 98°C, 30 seconds of annealing at 55°C, 60 seconds of extension at 72°C and an extra extension for 10 minutes at 72°C. The templates were then held at 4°C. The number of cycles was 25.

The PCR products were analysed on 1% agarose gel consisting of 1 g agarose (Amersham Life Science, Cleveland, OHIO, USA) and 100 ml 1 x TAE buffer (50 x TAE; 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0)). The gel was stained with 1 μl ethidium bromide adding per 10 ml gel (stocksolution EtBr = 10 mg/ml). As a molecular size marker, a 1 kbp DNA ladder (Fermentas) was used. 1 μl of 6 x loading dye (Fermentas) was added per 5 μl sample before loading. The gel was run at 75-120 V for 30-75 minutes.
Transformation of chemically competent E. coli

2 µl vector was added to 100 µl (= one aliquot) chemically competent E. coli DH5α (Invitrogen). The tube was gently mixed before incubated on ice for 30 minutes. The cells were heat-shocked 30 seconds at 42°C and immediately put back on ice for two minutes. 250 µl room temperature SOC medium (Topo XL cloning kit, Invitrogen) was added to the cells. The tube was tightly capped and shaken horizontally for 1 hour at 37°C. The solution was spread on LB-plates supplemented with 50 µg/ml kanamycin and incubated up-side-down over night, at 37°C.

Transformation of Agrobacterium tumefaciens by electroporation

The pSoup vector and two aliquots (100 µl/aliquot) of electrocompetent Agrobacterium tumefaciens were thawed on ice. 5 µl of pSoup and 5 µl of one of each positive pGreen construct were mixed gently with the bacteria and transferred to two different pre-chilled electroporation cuvettes. Electroporation was performed at 2.5 kV, the pulse controller set to 400 W and the capacitance was 25 µF (Gene Pulser II Electroporation System Bio-Rad). 400 µl LB medium was immediately added to the cuvettes after electroporation. The bacteria suspensions were transferred to eppendorf tubes, which were let to incubate at 28°C, 140 minutes, 200 rpm. The suspensions were spread on LB plates with 50 µg/ml kanamycin. Wrapped in foil, the plates were left on the bench at room temperature for four days, up-side-down.

Transforming Arabidopsis thaliana by floral dip

One positive clone of each construct was inoculated in 1 L LB medium with 50 µg/ml kanamycin which were incubated on shake at 28°C, over night. The suspensions were centrifuged at 7000 x g for 15 minutes (Beckman Coulter producent, Avanti J-20XP, Rotor JLA 8.1000), at 4°C and the cell pellets were resuspended in 900 ml 5 % (w/v) sucrose solution (Sigma). Before the plants were dipped in the bacterial suspensions, the surfactant Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) was added to a final concentration of 0.05 % to make the infiltration medium to remain on the plant. 30 plants (growing in a pot) were prepared by cutting off mature silics and overripe flowers. Half of the plants were dipped in the bottle containing Agrobacterium with truncated tonB construct and the rest were dipped in the bottle containing Agrobacterium with whole tonB construct. The whole plant was dipped (up-side-down) in the suspensions for approximately 10 seconds. The plants were allowed to rest on their side in a black plastic bag, containing two small bottles of water (to retain the moisture in the bag), over night. The day after, they were moved to a growing chamber with 16 hours light and 8 hours dark and a temperature of 22°C. After about five weeks in the chamber, the seeds had matured and were collected.

Plasmid preparation

Plasmid transformed E. coli colonies, growing on LB-agar plates with 50 µl/ml kanamycin, were picked and distributed in 50 ml falcon tubes containing five ml LB with kanamycin (50 µl/ml). The tubes were incubated at 37°C, on a shaker, over night. The cells were harvested by centrifugation at 6000 x g for 15 minutes, at room temperature. The plasmids were purified according to manufacturer’s protocol for QIAGEN plasmid mini kit (QIAGEN) or QIAprep® spin miniprep kit (QIAGEN). Plasmids not used immediately were stored at -20°C.
Cleaving procedures

Check of pGreen amplification using EcoRV

The small amount of provided pGreen vector had to be amplified by transformation of E. coli. To check if the plasmid amplification had succeeded, plasmid DNA was cleaved with restriction enzyme EcoRV. The cleavage reaction mixture consisted of 120 units of EcoRV, 0.25 µl BSA buffer, 2.5 µl NE buffer 3, (EcoRV - kit, New England, BioLabs, Ipswich, UK), 10 µl plasmid DNA and 6.25 µl ultrapure H2O. The reactions were incubated 1h at 37°C, and analysed on a 1 % agarose gel with 1 x TBE buffer. The plasmid DNA was stored at -20°C until use.

Producing of sticky ends with BamHI and XbaI

Because of the sensitivity of BamHI, the amplicons were digested by one enzyme at the time, starting with 1 µg of amplified DNA. Three eppendorf tubes contained the cleaving reaction mix of 20 units BamHI, 0.5 µl BSA and 5 µl BamHI buffer, (enzymes and buffer from New England BioLabs). 15 µl purified truncated tonB fragments were added in to the first tube, 16 µl purified tonB fragments were added in the second tube and 27 µl of the pGreen plasmid were added to the third tube. Ultrapure H2O was added to obtain a total volume of 50 µl. The reactions were incubated at 37°C for 1h. The enzyme was then heat-inactivated at 65°C for 20 minutes. The cleaved fragments and the plasmid were purified by QIAquick PCR purification kit (QIAGEN). The DNA was eluted in 60 µl elution buffer.

To use all the BamHI digested fragments and plasmids, 20 µl were distributed in three clean 1.5 ml eppendorf tubes (tube 1-3 = truncated tonB, tube 4-6 = whole tonB and tube 7-9 = plasmids). 20 units XbaI, 0.5 µl BSA, 5 µl NEbuffer 2 and 23.5 µl ultrapure H2O were added to each tube, which were incubated at 37°C for 1h. The enzyme was heat-inactivated 20 min, at 65°C before purification. Tube 1-3, 4-6 and 7-9 were centrifuged on the same spin column to concentrate the DNA. 4 µl of the double digested fragments and plasmids were loaded on a 1% agarose gel with 1 x TBE buffer.

Ligation reaction

The DNA concentration was measured for using in an equation to calculate the correct amount of insert and vector at a molar ratio 5:1 (figure 5). The amount of vector to use was set to 75 ng.

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(\text{ng of vector} \times \text{size of vector (bp)}) / (\text{size of vector (bp)}) \times 5 = \text{ng of insert to use}
\]

ex. (75 ng × 773 bp / 2495) × 5 = 116.2 ng of truncated insert

Figure 5. Equation to calculate amount of inserts to use in ligation reaction

In a clean PCR tube 75 ng of vector DNA, 116.2 ng of truncated tonB fragment, 400 units T4-ligase (Invitrogen), and 1/10 (v/v) of 10 x T4 ligase buffer (Invitrogen) were gently mixed. In a second PCR tube 75 ng of vector, 128 ng of tonB fragment, 400 units T4-ligase and 1/10 (v/v) ligase buffer was mixed. The tubes were incubated at 16°C for 8 h. The enzyme was heat-inactivated at 70°C for 10 min and the ligated samples were stored at 4°C.
Blue / White screening of positive *Escherichia coli* transformants

Colonies of *E. coli* that contained pGreen with inserts were selected with blue / white screening. 100 µl (20 mg/ml) 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (SIGMA) were spread on LB-plates with kanamycin (50 µg/ml) to which the bacterial suspensions were spread on after transformation. The plates were incubated at 37° C over night. 20 white colonies from each transformation were picked and recultured on LB-plates with kanamycin (50 µg/ml).

Sequencing

10 µl of each positive plasmid were sent for sequencing in ABI PRISM™ 3100 Gene Analyser, using primer 35SF and 35SR. The sequences were compared to wild type *tonB* sequence in BLAST.

Seed sterilization

The first sterilization step was brief washing with 70 % ethanol. The supernatant was discarded and 5 % chlorine was added to the seeds, which were then incubated for 15 minutes, at room temperature, vortexing every fourth minute. The chlorine was discarded and autoclaved water was added four times to wash (totally 16 times), vortexing and centrifuging briefly in between washes. This procedure was repeated four times, where the very last water step was replaced with 0.15 % agar solution. All seeds were plated on Murashige and Skoog plates and left at 4° C for 48 hours before they were transferred to the growing chamber.

Overexpression of *TonB*

A pilot test of protein expression with Isopropyl β-D-1-thiogalactopyranoside (=IPTG) was done according to a protocol available from Invitrogen. The induction time with IPTG was set to two hours. A five ml over night culture was transferred to 100 ml fresh LB medium supplied with 50 µg/ml kanamycin, and incubated for two hours at 37° C, until OD_600_ was ~ 0.6. IPTG was added to a final concentration of 1 mM and the culture incubated two hours. The cells were harvested by five minutes centrifugation at 6000 x g. To lyse the bacteria, CellLytic™ B 2X (Sigma) was used according to manufacture’s protocol for large scale extraction. Phenylmethanesulphonylfluoride (PMSF) to a final concentration of 1 mM was added to the lysed suspension. The suspension was centrifuged for 10 minutes, at 16000 x g, at 4° C. The supernatant was saved for purification and the cell pellet was resuspended in equilibration buffer (0.1 M sodium phosphate, pH 8.0, 8 M urea). The saved supernatant was purified with HIS-Select™ Nickel Affinity Gel (protocol available from Sigma) because the protein was HIS-tagged. Fractions from purification of supernatant and cell pellet were analysed with western blotting.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western blotting**

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

Pellets and fractions from protein purification were resuspended in 1 x Sodium Dodecyl Sulfate (SDS) sample buffer (2 x SDS sample buffer is 10 % (w/v) sodium dodecyl sulfate electrophoresis grade, glycerol 2 ml, 0.1 (w/v) bromophenol blue, 2.5 ml 0.5 M Tris-HCl pH 6.8 and deionized water up to 10 ml). 1/10 (v/v) of 1 M dithiothreitol (DDT) was added to the sample buffer before the samples were boiled five minutes. 10-20 µl were loaded on a 10 % Pierce Tris-Hepes gel (Pierce, Rockford, USA) and run at 110 V for 45 minutes in 1 x running buffer (10 x running buffer; Tris base 121 g, Hepes 238 g, SDS, 10 g, ddH₂O up to
1 L). As a molecular weight marker, TriChromRanger™ prestained protein molecular weight marker mix (Pierce, Rockford, USA) or Precision plus protein™ dual color standards (BioRad) was used.

**Western blotting**

On the grey side of a sandwich cassette (mini trans-blot cell set, BioRad), one fiber pad, one Whatman 3 MM filter paper, the gel from SDS-PAGE, one nitrocellulose membrane, and one fiber pad were assembled. All materials were pre-wet in electroblotting buffer consisting of Tris base 3.00 g, Bicine 4.08 g, ethanol 100 ml and ultrapure water up to 1 L, stored at 4°C. A stir ring bar as well as a Bio-ice cooling unit (mini trans-blot cell set, BioRad) were placed in the tank (mini trans-blot cell set, BioRad), which was filled with electroblotting buffer and run for 90 minutes at 40 V. The membrane was then placed in a 50 ml falcon tube containing 0.25 g dried milk powder dissolved in 5 ml TBST buffer (Tris base 2.422 g, NaCl 8.766 g, Tween20 final concentration of 0.05 % and ultrapure water up to 1 L, adjusted with HCl to pH 7.5) and allowed to incubate on an orbital shaker, one hour, at room temperature. The membrane was washed with TBST buffer a few seconds.

Pooled serum samples from 10 patients having antibodies to TonB from *H. pylori* (provided from Lars Engstrand, Swedish Institute for Infectious Disease Control, Solna, Sweden), were diluted 25 x in TBST buffer in which the membrane was incubated on an orbital shaker, over night, at 4°C. The membrane was then washed with TBST for 1 x 10 minutes and 2 x 5 minutes. The membrane was incubated in Goat anti-human IgG conjugated with alkaline-phosphatase (Sigma), diluted 7500 x, for one hour, at room temperature, on shaking. The membrane was washed as before and then rinsed with AP buffer (Tris 12.11 g/L, NaCl 5.84 g/L, MgCl2 1.02 g/L up to 1 L ultrapure water, pH 9.5). 5 ml of AP buffer supplemented with 33 µl nitroblue tetrazolium and 16.5 µl 5-bromo-4-chloro-3-indolyl-phosphate (Promega) was used to develop visible bands on the membrane.
Acknowledgement

Sören Andersson, MD, PhD, Department of virology, immunology and vaccinology, Swedish Institute for Infectious Disease Control, Solna, my head supervisor, for letting me be a part of this project and for your support.

Professor Lars Engstrand, Department of Bacteriology, Swedish Institute for Infectious Disease Control, Solna, my supervisor, for your interest in my work.

Professor Åke Stridh, Department of Natural of Science, Örebro University, for providing the opportunity to work in your laboratory.

Irina Kalbina, PhD, Department of Natural of Science, Örebro University, for your help and great support in the laboratory in Örebro.

Mathilda Lindberg, PhD student, Department of bacteriology, Swedish Institute for Infectious Disease Control, for your help and great support in the laboratory in Solna.

Jimmy Andersson, my boyfriend, for your support and understanding that I have not been at home so much and of course with your financial help.

All of the group members at the Department of bacteriology, Swedish Institute for Infectious Disease Control, Solna and Department of Natural of Science, Örebro University for helping me and giving advice in the daily laboratory work.
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