MEANS OF CONTROLLING INFECTION PERSISTENCE OF HELICOBACTER PYLORI

[0001] FIELD

[0002] The present invention relates to a means of controlling infection persistence of *Helicobacter pylori* (*H. pylori*). In particular, the present invention relates to *H. pylori* which has a modified urease, but is still capable of infection at a reduced persistence.

[0003] BACKGROUND

[0004] *Helicobacter pylori* chronically infects the gastric mucosa of billions of people worldwide, causes peptic ulcer disease in 10% or more of them, and is also implicated as an early critical risk factor for gastric cancer, one of the most frequently lethal malignancies in human populations (Suzuki *et al.*, 2006, *Carcinogenesis*). However, the mechanisms by which *H. pylori* establishes and maintains colonization remains poorly understood. While these mechanisms remain poorly understood it is difficult to consider the development of useful vaccines against *H. pylori*. Moreover, the use of attenuated *H. pylori* as a biological delivery vehicle for peptides will remain elusive.

[0005] One of the first characterised factors essential for colonization by *H. pylori* was urease, an abundant enzyme that decreases the acidity of *H. pylori*'s immediate environment by generating ammonia and carbonate from the urea we secrete as metabolic waste (Marcus & Scott, 2001, *Helicobacter*, 6(2), 93-99; Hu & Mobley, 1990, *Infect. Immun.*, 58(4), 992-998). Although such local control of pH is considered essential, urease-negative *H. pylori* strains

have been shown to be unable to colonise piglets whose acid secretion has been suppressed, suggesting an additional role for urease (Eaton & Krakowka, 1994, *Infect. Immun.*, 62(4), 992-998).

[0006] In order to produce a more effective vaccine strain of *H. pylori* or a strain that could be used as a biological vehicle the inventors have studied the dodecamer surface contribution to urease acid stability and host interactions of *H. pylori*.

[0007] SUMMARY

[0008] The inventors have shown that the region from about amino acid 529 to about amino acid 555 of UreB (GenBank AAD07143.1; SEQ ID NO:1) controls the ability of *Helicobacter pylori* to establish a persistent infection without affecting the urease activity.

[0009] Accordingly, in a first aspect the present invention provides an isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 is altered to produce said modified *Helicobacter pylori* which is unable to establish or maintain a persistent infection.

[0010] In some embodiments, the amino acid sequence between amino acid 529 to amino acid 555 of SEQ ID NO:1 is altered by deletion, mutation or insertion. The alteration may comprise a conservative or non-conservative amino acid change.

[0011] In some embodiments, the amino acids that are altered are selected from the group consisting of 547, 548

and 549 of SEQ ID NO:1. Preferably, the amino acid alteration comprises an insertion of one or more amino acids in-frame into the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1.

[0012] In some embodiments, the insertion comprises between 10 and 100 amino acids. Preferably, between 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54,. 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88,89, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 to 100 amino acids. Most preferably, between about 20 to about 50 amino acids.

[0013] In some embodiments, the genetically modified *Helicobacter pylori* of the present invention will further comprise one or more nucleic acid molecule(s) encoding at least one heterologous antigen or a functional fragment thereof. The nucleic acid molecule may reside extra-chromosomally or will preferably comprise an insertion into the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1. In some embodiments, the insertion is between amino acid 549 and 550.

[0014] In some embodiments, the heterologous antigen or a functional fragment thereof will be from a pathogenic microorganism. Preferably, the pathogenic microorganism will be selected from the group consisting of a virus, a bacterium, a protozoan and a fungus.

[0015] In some embodiments, the heterologous antigen is selected from the group consisting of a human immunodeficiency virus (HIV) antigen, an HTLV antigen, an SIV antigen, an RSV antigen, a PIV antigen, an HSV antigen, a CMV antigen, an Epstein-Barr virus antigen, a Varicella-Zoster virus antigen, a mumps virus antigen, a measles virus antigen, an influenza virus antigen, a poliovirus antigen, a rhinovirus antigen, a hepatitis A virus antigen, a hepatitis B virus antigen, a hepatitis C virus antigen, a Norwalk virus antigen, a togavirus antigen, an alphavirus antigen, a rubella virus antigen, a rabies virus antigen, a Marburg virus antigen, an Ebola virus antigen, a papilloma virus antigen, a polyoma virus antigen, a metapneumovirus antigen, a coronavirus antigen, a *Vibrio cholerae* antigen, a *Plasmodium falciparum* antigen, a *Plasmodium vivax* antigen, a *Plasmodium ovate* antigen, a *Plasmodium malariae* antigen, a *Plasmodium knowlesi* antigen, a *Streptococcus pneumoniae* antigen, *Streptococcus pyogenes* antigen, a *Helicobacter pylori* antigen, a *Streptococcus agalactiae* antigen, a *Neisseria meningitidis* antigen, a *Neisseria gonorrhoeae* antigen, a *Corynebacterium diphtheriae* antigen, a *Clostridium tetani* antigen, a *Bordetella pertussis* antigen, a *Haemophilus* antigen, a *Chlamydia* antigen and an *Escherichia coli* antigen.

[0016] In a second aspect, the present invention provides an immunogenic composition comprising an isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 is altered to produce said modified *Helicobacter pylori* which is unable to establish a persistent infection and a pharmaceutically acceptable carrier.

[0017] In some embodiments, the immunogenic composition further comprises an adjuvant.

[0018] In a third aspect, the present invention provides a method for protecting a mammal against infection with a pathogenic microorganism comprising the step of administering an immunologically effective amount of an immunogenic composition comprising an isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 is altered to produce said modified *Helicobacter pylori* which is unable to establish a persistent infection and a pharmaceutically acceptable carrier.

[0019] In a fourth aspect, the present invention provides a method of controlling the ability of *Helicobacter pylori* to establish a persistent infection comprising the step of altering the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 to produce a modified UreB subunit of urease, wherein said urease retains de-acidification function, but said *Helicobacter pylori* is unable to establish a persistent infection.

[0020] In a fifth aspect, the present invention provides a method of vaccinating a mammal against *Helicobacter pylori* infection comprising the step of administering to said mammal an isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 is altered to produce said modified *Helicobacter pylori* which is unable to establish a persistent infection.

[0021] In a sixth aspect, the present invention provides an immunogenic composition for use in vaccinating a mammal said composition comprising an isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1 is altered to produce said modified *Helicobacter pylori* which is unable to establish a persistent infection and a pharmaceutically acceptable carrier.

[0022] In some aspects, the heterologous antigen will reside in a plasmid vector comprising (a) a nucleotide sequence encoding the heterologous antigen and (b) a control or regulatory sequence operatively linked thereto which is capable of controlling the expression of the nucleotide sequence when the vector is transformed into the modified *Helicobacter pylori* of the present invention.

[0023] Non-limiting examples of a mammal included in the present invention are a primate, a canine, an equine, a bovine, a porcine, an ovine and a rodent.

[0024] The various delivery forms of the compositions are readily prepared for use in the practice of the present invention given the specific types and ratios of specific *Helicobacter pylori*, plasmid vectors and other delivery mechanisms described herein, and those formulation techniques known to those in the formulary arts, such as are described in Remington's Pharmaceutical Sciences, 20th edition, Mack Publishing Company, which text is specifically incorporated herein by reference.

[0025] In a seventh aspect the present invention provides a method of preventing the persistence of

Helicobacter pylori infection in a mammal comprising the step of administering to said mammal an agent capable of affecting the function of the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1.

[0026] BRIEF DESCRIPTION OF FIGURES

[0027] Figure 1. Recombinant regions of urease and selection for enzyme function. a) Molecular structure of urease showing insertion sites on the surface of urease. Urease subunit A (green) and subunit B (blue) associate to form a dodecameric supramolecular molecule (Ha *et al.*, 2001, *Nat Struct Biol*, 8(6), 505-509; Pinkse *et al.*, *J Mass Spectrom.*, 38(3), 315-320). Insertion sites 1, 3, 4, and 8 are indicated in red. Urease activity could not be retained when altered at sites 2, 5, 6, and 7 (pink). b) Selection of bacteria producing functional urease on acidified media supplemented with the urease substrate, urea. The colour change observed on the left side indicated that bacterial colonies were producing functional urease and growing (X47 wild-type). Colour change did not occur on the right side, indicating that inoculated colonies were unable to grow as functional urease was not being produced (X47 ΔureA).

[0028] Figure 2. Recombinant urease activity and acid stability. a) Western Blot analysis of *H. pylori* producing urease recombinant at sites 1, 3, 4 or 8. Lanes 1: Maker (MW: KDa shown); Lane 2: X47 (site 1); Lane 3: X47 (site 3); Lane 4: X47 (site 4); Lane 5: X47 (site 8). Recombinant urease was detected using anti-FLAG antibody directed against UreA (lanes 2 and 3) or UreB (lanes 4 and 5). b) Ability of permeabilised bacteria expressing wild-type or recombinant urease to neutralize acid after

incubation at pH 3 in the presence of urea. To determine acid stability and activity of wild-type and recombinant ureases bacteria were incubated at pH 3 for 0, 2 or 10 min prior to assay of urease activity. After pre-incubation pH was neutralized, urea added as substrate and urease activity at was measured by a change in pH as indicated by a change in the colour of phenol red. Significantly reduced urease activities independent of pre-incubation at pH 3 are annotated " * " (Student's T-test, 2 tailed, equal variance). SEM displayed (n=3).

[0029] Figure 3. Recombinant urease affects bacterial colonisation. a) Persistence over 15 months expressed as percentage of colonized (n=5-15). b) Colonisation level of mice infected with *H. pylori* expressing recombinant urease after 15 months (n=5; median displayed). c) Persistence of *H. pylori* expressing recombinant urease as indicated by anti-*H. pylori* IgG levels (n=12-20; median displayed). Strains were recombinant at either sites 1, 4 or 8 in urease. d) Comparison of anti-*H. pylori* IgG and anti-UreB IgG levels resulting from colonisation of mice for 3 months with X47 expressing wild-type urease, X47 (wt), or urease recombinant at site 8, X47 (site8), (n=10-20; median displayed).

[0030] Figure 4. Recombinant urease is stable in bacteria colonising mice. Western Blot analysis of *H. pylori* extracted from mice after 10 months colonisation, probed with anti-FLAG. Where described, each lane represents protein extracted from a pool of bacteria harvested from an individual mouse. a) lanes 1-3: pools of X47 (wt) from individual mice; lane 4: molecular size marker (MW: KDa shown); lane 5: X47 (site 1); lane 6: X47 (site 4); lane 7: X47 (site 8). b) lanes 1-5: pools of X47 (site 1) from individual mice; lane 7: molecular size

marker; lane 8: X47 (site 1). c) lanes 1-5: pools of X47 (site 4) from individual mice; lane 7: molecular size marker. d) lanes 1-3: pools of X47 (site 8) from individual mice; lane 5: molecular size marker; lane 6: X47 (site 8). To confirm protein sample integrity urease expression was confirmed in wild-type X47 and a pool of X47 (site 1) from mice in which FLAG expression was not detected (annotated *; data not shown). Molecular of standard proteins (KDa) are shown directly adjacent to the marker.

[0031] Figure 5. A schematic showing insertion sites at the urease locus of DNA coding epitopes and linkers. Insertions were made in DNA corresponding to insertion after amino positions 102 (site 1) and 238 (site 3) of UreA (GenBank AAD07144.1; SEQ ID NO:3), and amino acid positions 1 (site 4) and 549 (site 8) of UreB (GenBank AAD07143.1 SEQ ID NO:1). Insertions at sites 3 and 4 correspond to the C and N terminals of UreA and UreB respectively. DNA coded HA(T): hemagglutinin T cell eptitope; HA(B) hemagglutinin B cell epitope; SR linker: semi-random linker; linker: GPSL linker; FLAG: FLAG epitope; STOP: STOP codon.

[0032] Figure 6. Alignment showing conservation of ureases at region of site 8. Alignment of *H. pylori* UreB (sp|P69996; SEQ ID NO:23) at the region of site 8, for which the crystal structure has been determined (PDBe Entry: 1e9y), and ureases from different species. UniProtKB/Swiss-Prot numbers are displayed.

[0033] BRIEF DESCRIPTION OF THE SEQUENCES

[0034] The following nucleic acid and amino acid sequences are referenced throughout the description of the

present invention:

[0035] SEQ ID NO: 1 - Amino acid sequence of the UreB subunit of the urease from *Helicobacter pylori* (GenBank AAD07143.1);

[0036] SEQ ID NO: 2 - Nucleotide sequence encoding the UreB subunit of the urease from *Helicobacter pylori*;

[0037] SEQ ID NO: 3 - Amino acid sequence of the UreA subunit of the urease from *Helicobacter pylori* (GenBank AAD07144.1);

[0038] SEQ ID NO: 4 - Nucleotide sequence of the UreA subunit of the urease from *Helicobacter pylori*;

[0039] SEQ ID NO:5 - Primer UF5-234

[0040] SEQ ID NO:6 - Primer UR2-234

[0041] SEQ ID NO:7 - Primer UR2-56

[0042] SEQ ID NO:8 - Primer UF5-56

[0043] SEQ ID NO:9 - Primer rpsL-F

[0044] SEQ ID NO:10 - Primer ermR

[0045] SEQ ID NO:11 - Primer si1aF3

[0046] SEQ ID NO:12 - Primer si3F3

[0047] SEQ ID NO:13 - Primer si4F3

[0048] SEQ ID NO:14 - Primer si8F3

[0049] SEQ ID NO:15 - Primer HAFLAG

[0050] SEQ ID NO:16 - Primer HAFLAG (rc)

[0051] SEQ ID NO:17 - Primer UF1

[0052] SEQ ID NO:18 - Primer UR6

[0053] SEQ ID NO:19 - Primer si1aR1

[0054] SEQ ID NO:20 - Primer si3R1

[0055] SEQ ID NO:21 - Primer si4R1

[0056] SEQ ID NO:22 - Primer si8R1

[0057] SEQ ID NO:23 - Amino acids 535 to 575 of UreB (sp|P69996)

[0058] SEQ ID NO:24 - Amino acids 535 to 575 of UreB (sp|P42823)

[0059] SEQ ID NO:25 - Amino acids 535 to 575 of UreB (sp|Q93PJ4)

[0060] SEQ ID NO:26 - Amino acids 535 to 575 of UreB (tr|Q17YJ8)

[0061] SEQ ID NO:27 - Amino acids 535 to 575 of UreB (tr|Q4CHE3)

[0062] SEQ ID NO:28 - Amino acids 535 to 575 of UreB (sp|Q9KG59)

[0063] SEQ ID NO:29 - Amino acids 535 to 575 of UreB (sp|Q07397)

[0064] SEQ ID NO:30 - Amino acids 535 to 575 of UreB (tr|Q1XLS1)

[0065] SEQ ID NO:31 - Amino acids 535 to 575 of UreB (sp|Q5KYM1)

[0066] SEQ ID NO:32 - Amino acids 535 to 575 of UreB (sp|Q5FB23)

[0067] SEQ ID NO:33 - Amino acids 535 to 575 of UreB (tr|QOVKY1)

[0068] SEQ ID NO:34 - Amino acids 535 to 575 of UreB (sp|Q733J6)

[0069] SEQ ID NO:35 - Amino acids 535 to 575 of UreB (sp|Q2JQ88)

[0070] SEQ ID NO:36 - Amino acids 535 to 575 of UreB (tr|Q4IXD2)

[0071] SEQ ID NO:37 - Amino acids 535 to 575 of UreB (tr|052305)

[0072] SEQ ID NO:38 - Amino acids 535 to 575 of UreB (sp|Q21P94)

[0073] SEQ ID NO:39 - Amino acids 535 to 575 of UreB (sp|P73061)

[0074] SEQ ID NO:40 - Amino acids 535 to 575 of UreB (sp|Q2SYF7)

[0075] SEQ ID NO:41 - Amino acids 535 to 575 of UreB (tr|Q1FX09)

[0076] SEQ ID NO:42 - Amino acids 535 to 575 of UreB (tr|Q05PQ2)

[0077] SEQ ID NO:43 - Amino acids 535 to 575 of UreB (tr|Q11EW4)

[0078] SEQ ID NO:44 - Amino acids 535 to 575 of UreB (tr|QOAC98)

[0079] SEQ ID NO:45 - Amino acids 535 to 575 of UreB (tr|AOFWY4)

[0080] SEQ ID NO:46 - Amino acids 535 to 575 of UreB (sp|Q8DMV6)

[0081] SEQ ID NO:47 - Amino acids 535 to 575 of UreB (sp|Q2SDQ1)

[0082] SEQ ID NO:48 - Amino acids 535 to 575 of UreB (sp|Q5LSQ2)

[0083] SEQ ID NO:49 - Amino acids 535 to 575 of UreB (tr|Q2BLC2)

[0084] SEQ ID NO:50 - Amino acids 535 to 575 of UreB (tr|Q161S8)

[0085] SEQ ID NO:51 - Amino acids 535 to 575 of UreB (sp|Q46IY3)

[0086] SEQ ID NO:52 - Amino acids 535 to 575 of UreB (tr|O30337)

[0087] SEQ ID NO:53 - Amino acids 535 to 575 of UreB (sp|Q8XXT1)

[0088] SEQ ID NO:54 - Amino acids 535 to 575 of UreB (sp|P77837)

[0089] SEQ ID NO:55 - Amino acids 535 to 575 of UreB (sp|Q3KIT2)

[0090] SEQ ID NO:56 - Amino acids 535 to 575 of UreB (tr|A0L6F2)

[0091] SEQ ID NO:57 - Amino acids 535 to 575 of UreB (sp|Q31B49)

[0092] SEQ ID NO:58 - Amino acids 535 to 575 of UreB (tr|Q9Z369)

[0093] SEQ ID NO:59 - Amino acids 535 to 575 of UreB (tr|Q1YUB5)

[0094] SEQ ID NO:60 - Amino acids 535 to 575 of UreB (tr|Q2ZGT0)

[0095] SEQ ID NO:61 - Amino acids 535 to 575 of UreB (tr|A0JRH4)

[0096] SEQ ID NO:62 - Amino acids 535 to 575 of UreB (sp|Q7V1B6)

[0097] SEQ ID NO:63 - Amino acids 535 to 575 of UreB (tr|Q0LFJ0)

[0098] SEQ ID NO:64 - Amino acids 535 to 575 of UreB (tr|Q1FJ39)

[0099] SEQ ID NO:65 - Amino acids 535 to 575 of UreB (sp|Q47G55)

[0100] SEQ ID NO:66 - Amino acids 535 to 575 of UreB (sp|Q8YQZ0)

[0101] SEQ ID NO:67 - Amino acids 535 to 575 of UreB (sp|Q9RYJ4)

[0102] SEQ ID NO:68 - Amino acids 535 to 575 of UreB (sp|Q3J770)

[0103] SEQ ID NO:69 - Amino acids 535 to 575 of UreB (tr|Q0FQX6)

[0104] SEQ ID NO:70 - Amino acids 535 to 575 of UreB (sp|P42885)

[0105] SEQ ID NO:71 - Amino acids 535 to 575 of UreB (sp|Q7V3V2)

[0106] DEFINITION OF TERMS

[0107] As used herein, certain terms may have the following defined meanings. As used in the specification and claims, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof. Similarly, use of "a compound" for treatment or preparation of medicaments as described herein contemplates using one or

more compounds of this invention for such treatment or preparation unless the context clearly dictates otherwise.

[0108] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

[0109] All strains *Helicobacter pylori* are included in the scope of the present application as long as they have a functional urease gene. Particularly preferred strains of *Helicobacter pylori* include strains deposited at the American Type Culture Collection (ATCC) as ATCC Accession Nos: 43504; 43504D-5; 43526; 43579; 43629; 49396; 49503; 51110; 49503; 51111; 51407; 51652; 51653; 51932; and 53727. Also see, for example, US Pat. No: 5,459,041 incorporated herein by reference. Other preferred strains of *Helicobacter pylori* include strains deposited at the National Measurement Institute under Accession Nos. V09/009101; V09/009102; V09/009103; V10/014059; V10/014060 and V09/009104.

[0110] As used herein the term "isolated" is meant to describe a *Helicobacter pylori* cell, a polynucleotide or a polypeptide that is in an environment different from that in which the *Helicobacter pylori* cell, polynucleotide or polypeptide naturally occurs. An isolated genetically modified *Helicobacter pylori* cell may be present in a mixed population of *Helicobacter pylori* cells.

[0111] A "genetically modified" *Helicobacter pylori* refers to a *Helicobacter pylori* bacterium that differs in its pheno- and/or genotype from that of the corresponding wild type *Helicobacter pylori* in that it comprises an alteration to the urease gene present in *Helicobacter pylori*. On the one hand, the genetically modified *Helicobacter pylori* maintains urease activity, but on the other hand the factors required for establishing a persistent infection are 'knocked-out' or 'altered'. Methods for the genetic modification of the *Helicobacter pylori* are well-known in the art; cf. for example Sambrook, J. and Russell, D. W. (2001), "Molecular Cloning--A Laboratory Manual", Cold Spring Harbor Laboratory Press, New York, 3rd Edition.

[0112] Genes encoding urease of *Helicobacter pylori* have been described and sequenced (Labigne *et al.*, (1991), *J. Bacteriol.*, 173: 1920-1931). Of the seven genes involved in urease expression and secretion, only two genes encode the two structural subunits urease A and B of the urease enzyme; ureA (SEQ ID NO:3) and ureB (SEQ ID NO:1). These two polypeptides form a polypeptide complex having urease activity.

[0113] Urease activity can be determined a number of ways. For example, it is known that urease converts urea into ammonium carbonate, which then decomposes into

ammonia and carbon dioxide. Consequently, in the past, one test for detecting the presence of *Helicobacter pylori* included the steps of contacting a sample of gastric material with a composition containing urea and an indicator, namely a pH indicator that changes colour when there is a rise in pH. If urease is present within the gastric material it breaks down the urea, which results in the formation of ammonia after further decomposition and causes the pH indicator to change colour. *Helicobacter pylori* urease activity can also be detected by orally administering urea to a subject with subsequent monitoring of the exhaled dioxide and ammonia. Various test for urease activity are described in U.S. Pat. No. 4,748,113 and US Pat. Applic. No. 20030082664, which are incorporated herein by reference.

[0114] The term "functional fragment" when used herein with reference to urease, refers to any fragment of the *Helicobacter pylori* urease (i.e. a molecule which is reduced in size or truncated compared with the naturally occurring form) that still has the ability to convert urea into ammonium carbonate. Determination of the ability of the urease or functional fragment thereof to convert urea into ammonium carbonate may in this connection take place both qualitatively and quantitatively (i.e. as a quantifiable measurement).

[0115] The term "nucleic acid" used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

[0116] The terms "peptide," "polypeptide," and "protein" are used interchangeably herein, and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones.

[0117] "Percent identity (homology)" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, *Proc. Natl. Acad. Sci. USA*, 87:2264-2268, 1990, modified as in Karlin and Altschul (*Proc. Natl. Acad. Sci. USA* 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (*J. Mol. Biol.* 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score=50, wordlength=3, to obtain amino acid sequences homologous to a reference polypeptide (eg., SEQ ID NO: 2). To obtain gapped alignments for comparison purposes, Gapped BLAST is utilised as described in Altschul *et al.* (*Nucleic Acids Res.* 25:3389-3402, 1997). When utilising BLAST and Gapped BLAST programs, the default parameters of the respective programs (eg., XBLAST and NBLAST) are used. These maybe found on the World Wide Web at the URL "ncbi.nim.nih.gov."

[0118] As used herein, the term "exogenous nucleic acid" refers to a nucleic acid that is not normally or naturally found in and/or produced by *Helicobacter pylori* in nature. As used herein, the term "endogenous nucleic acid" refers to a nucleic acid that is normally found in and/or produced by *Helicobacter pylori* in nature. An

"endogenous nucleic acid" is also referred to as a "native nucleic acid" or a nucleic acid that is "native" to *Helicobacter pylori*.

[0119] The term "heterologous nucleic acid," as used herein, refers to a nucleic acid wherein at least one of the following is true: (a) the nucleic acid is foreign ("exogenous") to (i.e., not naturally found in) *Helicobacter pylori*; (b) the nucleic acid comprises a nucleotide sequence that is naturally found in (e.g., is "endogenous to") *Helicobacter pylori* (e.g., the nucleic acid comprises a nucleotide sequence that is endogenous to *Helicobacter pylori*) but is either produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell, or differs in sequence from the endogenous nucleotide sequence such that the same encoded protein (having the same or substantially the same amino acid sequence) as found endogenously is produced in an unnatural (e.g., greater than expected or greater than naturally found) amount in the cell; (c) the nucleic acid comprises two or more nucleotide sequences or segments that are not found in the same relationship to each other in nature, e.g., the nucleic acid is recombinant.

[0120] "Recombinant," as used herein, means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems. Generally, DNA sequences encoding the structural coding sequence can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of synthetic oligonucleotides, to provide a synthetic nucleic acid

which is capable of being expressed from a recombinant transcriptional unit contained in a cell or in a cell-free transcription and translation system. Such sequences can be provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA comprising the relevant sequences can also be used in the formation of a recombinant gene or transcriptional unit. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions, and may indeed act to modulate production of a desired product by various mechanisms.

[0121] Thus, e.g., the term "recombinant" polynucleotide or "recombinant" nucleic acid refers to one which is not naturally occurring, e.g., is made by the artificial combination of two otherwise separated segments of sequence through human intervention. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

[0122] Similarly, the term "recombinant" polypeptide refers to a polypeptide which is not naturally occurring, e.g., is made by the artificial combination of two otherwise separated segments of amino sequence through human intervention. Thus, e.g., a polypeptide that comprises a heterologous amino acid sequence is recombinant.

[0123] By "vector" is meant a recombinant nucleic acid, generally recombinant DNA, which has been generated for the purpose of the expression and/or propagation of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

[0124] The terms "DNA regulatory sequences," "control elements," and "regulatory elements," used interchangeably herein, refer to transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, protein degradation signals, and the like, that provide for and/or regulate expression of a coding sequence and/or production of an encoded polypeptide in a *Helicobacter pylori* cell.

[0125] The term "transformation" is used interchangeably herein with "genetic modification"" and refers to a permanent or transient genetic change induced in a *Helicobacter pylori* cell following introduction of new nucleic acid. Genetic change ("modification") can be accomplished either by incorporation of the new DNA into the urease gene of the *Helicobacter pylori* cell, or by transient or stable maintenance of the new DNA as an episomal element such as a plasmid or expression vector, which may contain one or more selectable markers to aid in their maintenance in the recombinant *Helicobacter pylori* cell. Suitable methods of genetic modification include

transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, and the like. A general discussion of these methods can be found in Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995.

[0126] "Transforming nucleic acid sequence" as used herein means a plasmid vector, or other expression cassette containing a nucleic acid sequence encoding a an antigen. In some embodiments of the present invention the nucleic acid sequence can encode for one or more antigens. "Transforming nucleic acid sequence" can also be used to mean a "transgene" in accordance with certain embodiments of the present invention. In another embodiment of the present invention the transforming nucleic acid sequence includes nucleic acid sequence encoding for a promoter and/or other regulatory elements.

[0127] "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. As used herein, the terms "heterologous promoter" and "heterologous control regions" refer to promoters and other control regions that are not normally associated with a particular nucleic acid in nature. For example, a "transcriptional control region heterologous to a coding region" is a transcriptional control region that is not normally associated with the coding region in nature.

[0128] The term "conservative amino acid substitution" refers to the interchangeability in proteins of amino acid

residues having similar side chains. For example, a group of amino acids having aliphatic side chains consists of glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains consists of serine and threonine; a group of amino acids having amide-containing side chains consists of asparagine and glutamine; a group of amino acids having aromatic side chains consists of phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains consists of lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains consists of cysteine and methionine. Exemplary conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

[0129] The genetically modified *H. pylori* of the present invention is incapable of establishing or maintaining a persistent infection. A "persistent infection" is an infection in which *H. pylori* is not cleared or eliminated from the infected mammal, even after the induction of an immune response. Persistent infections can be chronic infections or latent infections. Latent infection is characterized by the lack of demonstrable infectious *H. pylori*. Chronic infection is characterized by the continued presence of the *H. pylori* following the primary infection. While acute infections are relatively brief (lasting a few days to a few weeks) and resolved from the body by the immune system, persistent infections can last for example, for months, years, or even a lifetime. These infections may also recur frequently over a long period of time, involving stages of silent and productive infection without cell killing or even producing excessive damage to the host cells. Persistent infections often involve stages of both silent and

productive infection without rapidly killing or even producing excessive damage of the host cells. It is usually considered that the acute phase for *H. pylori* infection lasts for about 10-14 days and a chronic infection is established after about three months leading to a lifelong colonisation or persistence. Thus, in the present invention the genetically modified *H. pylori* of the present invention is capable of establishing an initial infection, but this infection is not maintained for more than about 12, 11, 10, 9, 8, 7, 6, 5, 4, 3 or 2 months as compared to an infection with a wild type *Helicobacter pylori* which can remain lifelong.

[0130] A "vaccine composition", "vaccine", "immunogenic composition", and similar terms refer to a composition comprising a strain of live genetically modified *Helicobacter pylori* that expresses at least one antigen, such that when administered to a mammal, the bacteria will elicit an immune response in the mammal against the antigen(s) expressed in the *Helicobacter pylori* and, thereby, provide at least partial protective immunity against organism from which the antigen was isolated. Such protective immunity may be evidenced by any of a variety of observable or detectable conditions, including but not limited to, diminution of one or more disease symptoms, shorter duration of illness, diminution of tissue damage, regeneration of healthy tissue, clearance of pathogenic microorganisms from the mammal, and increased sense of well being by the mammal. Although highly desired, it is understood by persons skilled in the art that no vaccine is expected to induce complete protection from a disease in every individual that is administered the vaccine or that protective immunity is expected to last throughout the lifetime of an individual without periodic "booster" administrations of a vaccine composition. It is also

understood that a live vaccine comprising a genetically modified *Helicobacter pylori* described herein may be, at the discretion of a healthcare professional, administered to an individual who has not presented symptoms of disease, but is considered to be at risk of infection or is known to already have been exposed to a disease, e.g., by proximity or contact with infected mammals or contaminated air, liquids, or surfaces.

[0131] A "therapeutically effective amount" of a genetically modified *Helicobacter pylori* of the present invention or an antigen expressed by said *H. pylori* as described herein is understood to comprise an amount effective to elicit the desired response but insufficient to cause a toxic reaction. A desired response, for example, may constitute the formation of a sufficient and/or acceptable detectable antibody titer level in a blood sample. The dosage and duration of treatment of the preparation to be administered to a mammal will be determined by the health professional attending the mammalian subject in need of treatment, and will consider the age, sex and weight of the subject, and the specific *Helicobacter pylori* and nucleic acid molecule being expressed.

[0132] The terms "oral", "enteral", "enterally", "orally", "non-parenteral", "non-parenterally", and the like, refer to administration of a genetically modified *Helicobacter pylori* of the present invention to a mammal by a route or mode along the alimentary canal. Examples of "oral" routes of administration of a vaccine composition include, without limitation, swallowing liquid or solid forms of a vaccine composition from the mouth, administration of a vaccine composition through a nasojejunal or gastrostomy tube, intraduodenal

administration of a vaccine composition, and rectal administration, e.g., using suppositories that release a live bacterial vaccine strain described herein to the lower intestinal tract of the alimentary canal.

[0133] The term "inducing immune tolerance to an antigen," comprises mucosal delivery of an antigen by an isolated, genetically modified *Helicobacter pylori* secreting an antigen for the preparation of a medicament, medical food or nutraceutical for mucosal delivery to treat an immune response related disease in a mammal, wherein said antigen is preferably continuously present in said mammal.

[0134] A "heterologous" antigen is one not native to *Helicobacter pylori*, i.e., not expressed by *Helicobacter pylori* in nature or prior to introduction into *Helicobacter pylori*.

[0135] "Detectable immune response" as used herein is either an antibody (humoral) or cytotoxic (cellular) response formed in a mammal in response to an antigen that can be measured using routine laboratory methods including, but not limited to enzyme-linked immunosorbant assays (ELISA), radio-immune assays (RIA), Enzyme-linked ImmunoSPOT (ELISPOT), immunofluorescent assays (IFA), complement fixation assays (CF), Western Blot (WB) or an equivalent thereto.

[0136] DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0137] Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified methods and may, of course, vary. It is also to be understood that the

terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting which will be limited only by the appended claims.

[0138] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety. However, publications mentioned herein are cited for the purpose of describing and disclosing the protocols, reagents and vectors which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0139] Furthermore, the practice of the present invention employs, unless otherwise indicated, conventional techniques of pharmacology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. See, eg., Coligan *et al.* "Current protocols in Protein Science" (1999) Volume I and II (John Wiley & Sons Inc.); Sambrook *et al.*, (Molecular Cloning: A Laboratory Manual, 2nd & 3rd Editions, Cold Spring Harbor Laboratory press (1989) (2001); and Bailey, J.E. and Ollis, D.F., Biochemical Engineering Fundamentals, McGraw-Hill Book Company, NY, 1986; "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Animal Cell Culture" (R. I. Freshney, ed., 1987); the series "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D. M. Weir & C. C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J. M. Miller & M. P. Calos, eds., 1987); "Current

Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987, and periodicals) "Polymerase Chain Reaction" (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J. E. Coligan et al., eds., 1991).

[0140] In the broadest aspect, the present invention provides an isolated, genetically modified *Helicobacter pylori*. In some embodiments, the *Helicobacter pylori* is initially isolated from clinical samples taken from a mammalian subject which is chronically infected with *H. pylori*. The urease *ure*B gene (SEQ ID NO:1) is then modified by deletion, mutation or insertion such that the urease remains functional ie capable of converting urea into ammonium carbonate, which then decomposes into ammonia and carbon dioxide.

[0141] The region of the urease that is modified is from about amino acid 529 to about amino acid 555 of UreB (GenBank AAD07143.1; SEQ ID NO:1). In some embodiments, the region is between 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554 and 555. In some embodiments, the amino acids that are altered are selected from the group consisting of 547, 548 and 549 of SEQ ID NO:1.

[0142] The alterations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter *et al.*, (1986), *Nucl. Acids Res.*, 13:4331; Zoller *et al.*, (1987), *Nucl. Acids Res.*, 10:6487), cassette mutagenesis (Wells *et al.*, (1985), *Gene*, 34:315), restriction selection mutagenesis (Wells *et al.*, (1986), *Philos. Trans. R. Soc.*

London SerA, 317:415) or other known techniques can be performed on cloned DNA to produce an urease variant DNA.

[0143] In some embodiments, genomic DNA obtained from an isolate of *Helicobacter pylori* is inserted into a suitable shuttle vector, e.g., a shuttle plasmid with selectable markers, e.g., antibiotic markers, to assess their transformability. Broadly, a suitable shuttle vector will include one, two, three or more of the following features, a cloning site, a *Helicobacter pylori* origin of replication, an *E. coli* origin of replication, and an antibiotic resistance gene and/or selectable marker. Art-known vectors suitable for this purpose, or readily adaptable for this purpose include, for example, the recombinant shuttle plasmid pHR106 described by Roberts et al. (*Appl Env Mircobiol.*, 54: 268-270 (1988)); the PJIR 750 and PJIR 751 plasmids described by Bannam *et al.* (*Plasmid*, 29:233-235 (1993)); the promoterless PPSV promoter selection vector of Matsushita *et al.* (Plasmid, 31, 317-319 (1994)),; the shuttle plasmids pJIR1456 and pJIR1457, described by Lyras *et al.* (*Plasmid*, 39, 160-164 (1988)); and the pAK201 shuttle vector described by Kim *et al.* (*Appl Environ Microbiol.*, 55, 360-365(1989)), the contents of which are incorporated herein by reference in their entireties. Removal of the *Helicobacter pylori* origin of replication converts the shuttle vector into a suicide vector.

[0144] Alternatively, homologous recombination can be used to introduce a mutation or exogenous sequence into the urease gene.

[0145] Once the alteration has been made then nucleic acid transfer protocols are used including transformation/transfection, electroporation, liposome

mediated nucleic acid transfer, N-[1-(2,3-Dioloyloxy)propyl]-N,N,N-trimethylammonium methylsulfate meditated transformation, and others. One skilled in the art will be readily able to select the appropriate tools and methods for genetic modifications of the *Helicobacter pylori* according to the knowledge in the art and design choice.

[0146] In some embodiments, the present invention is directed at mucosal delivery of an immune dominant antigen secreted by the isolated, genetically modified *Helicobacter pylori* of the present invention. The purpose of the delivery is the suppression of local and systemic T-cell responses. This approach of antigen-delivery has the capacity for potentiating oral tolerance even in the setting of established hypersensitivity. Thus it is applicable for the prevention of disease caused by pathogens (ie vaccination) but also the treatment of autoimmune and/or allergic diseases.

[0147] Accordingly, in some embodiments, the amino acid alteration comprises an insertion of one or more amino acids in-frame into the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO:1, which encode one or more antigens or a functional fragments thereof. Preferably the insertion is between amino acid 549 and 550.

[0148] In some embodiments, the insertion will comprise between 10 and 100 amino acids. Preferably, between about 20 to about 50 amino acids.

[0149] In some embodiments, the heterologous antigen or a functional fragment thereof will be from a pathogenic microorganism. Preferably, the pathogenic microorganism

will be selected from the group consisting of a virus, a bacterium, a protozoan and a fungus.

[0150] In some embodiments, the heterologous antigen is selected from the group consisting of a human immunodeficiency virus (HIV) antigen, an HTLV antigen, an SIV antigen, an RSV antigen, a PIV antigen, an HSV antigen, a CMV antigen, an Epstein-Barr virus antigen, a Varicella-Zoster virus antigen, a mumps virus antigen, a measles virus antigen, an influenza virus antigen, a poliovirus antigen, a rhinovirus antigen, a hepatitis A virus antigen, a hepatitis B virus antigen, a hepatitis C virus antigen, a Norwalk virus antigen, a togavirus antigen, an alphavirus antigen, a rubella virus antigen, a rabies virus antigen, a Marburg virus antigen, an Ebola virus antigen, a papilloma virus antigen, a polyoma virus antigen, a metapneumovirus antigen, a coronavirus antigen, a *Vibrio cholerae* antigen, a Plasmodium falciparum antigen, a *Plasmodium vivax* antigen, a *Plasmodium ovate* antigen, a *Plasmodium malariae* antigen, a *Plasmodium knowlesi* antigen, a *Streptococcus pneumoniae* antigen, *Streptococcus pyogenes* antigen, a *Helicobacter pylori* antigen, a *Streptococcus agalactiae* antigen, a *Neisseria meningitidis* antigen, a *Neisseria gonorrhoeae* antigen, a *Corynebacterium diphtheriae* antigen, a *Clostridium tetani* antigen, a *Bordetella pertussis* antigen, a *Haemophilus* antigen, a *Chlamydia* antigen and an *Escherichia coli* antigen.

[0151] Preferably, the antigen is displayed at the surface of the *Helicobacter pylori* or is secreted.

[0152] In one aspect of the invention there is provided a method for inducing immune tolerance to an antigen, comprising mucosal delivery of said antigen by an

isolated, genetically modified *Helicobacter pylori* secreting an antigen for the preparation of a medicament, medical food or nutraceutical for mucosal delivery to treat an immune response related disease in a mammal, wherein said antigen is preferably continuously present in said mammal.

[0153] Preferably, the immune tolerance is induced in a mammal selected from the group consisting of a dog, a cat, a mouse, a rat, a pig, a cow, a sheep, a horse and a human. Preferably, the mammal is human. Preferably, the immune tolerance is mucosal tolerance.

[0154] Mucosa as used here can be any mucosa such as oral mucosa, rectal mucosa, urethral mucosa, vaginal mucosa, ocular mucosa, buccal mucosa, pulmonary mucosa and nasal mucosa. Mucosal delivery as used throughout the application encompasses the delivery to the mucosa. Oral mucosal delivery includes buccal, sublingual and gingival routes of delivery. Accordingly, the present invention relates to method in which said mucosal delivery is chosen from the group consisting of rectal delivery, buccal delivery, pulmonary delivery, ocular delivery, nasal delivery, vaginal delivery and oral delivery. Preferably, said mucosal delivery is oral delivery and said tolerance is oral tolerance.

[0155] Mucosal tolerance as used here throughout the application is the inhibition of specific immune responsiveness to an antigen in a mammal (including humans), after that said mammal has been exposed to said antigen via the mucosal route. Preferably, said mucosal tolerance is systemic tolerance. The subsequent exposure of the antigen can be every exposure known to the person skilled in the art, such as exposure by parenteral

injection, by mucosal delivery, or by endogenous production such as in the case of auto-antigens. Oral tolerance is the inhibition of specific immune responsiveness to an antigen in a mammal (including humans), after that mammal has been exposed to said antigen via the oral route.

[0156] The present invention also relates to a method or use as described herein, wherein said induction of immune tolerance is at least 1.5, preferably 2, more preferably 3 times or more relative to before said induction. Alternatively, said antigen is tolerated at least 1.5, 2, 3 times or more relative to before said induction. The induction of immune tolerance can be measured by methods known in the art. Preferably, said induction of immune tolerance can be measured by modulation of a cytokine level in said animal. As such, the modulation can be an increase of a cytokine level, for instance said increase of a cytokine level is at least 1.5, 2, 3 times or more relative to before said induction, e.g. IL-10 or TGF-beta. Alternatively, said modulation is a decrease of the level of a particular cytokine level, for instance said decrease of the cytokine level is at least 1.5, 2, 3 times or more relative to before said induction, e.g. IL-12, IL-17 and IFN-gamma. The cytokines which are modulated may be chosen from any relevant cytokines, preferably said cytokines are chosen from the group consisting of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-23, TNF-alpha, IFN-gamma, IFN-alpha, MCP-1, TGF-beta, RANK-L and Flt3L.

[0157] The invention will now be further described by reference only to the following non-limiting examples. It should be understood, however, that the examples following are illustrative, and should not be taken in any way as a

restriction on the generality of the invention described herein.

[0158] EXAMPLE 1 MUTATION BY INSERTION

[0159] To test the possible involvement of the urease surface in host-pathogen interactions, the UreA/UreB structure (Ha *et al.*, 2001, *supra*) was analysed *in silico* to identify surface regions that might tolerate the insertion of two epitope tags (Figure 1a).

[0160] Mutant *H. pylori* strains were then produced by chromosomal replacement of wild-type *ure*A *or ure*B gene DNAs with mutant DNAs with in-frame insertions encoding tag sequences flanked by semi-random six amino acid linkers at eight sites in the urease (Figure 4) using standard PCR techniques.

[0161] Briefly, all PCR constructions used 26695 genomic DNA as template for initial amplifications. Primers used in this study are shown in Table 1 and the combinations used are shown in Table 2. The principles of splicing by overlap PCR have been previously reported (Shevchuk *et al.*, 2004, *Nucleic Acid Res.*, 32(2), e19. To construct rpsL.ermB cassette (Dailidiene *et al.*, 2006, *Appl. Environ. Microbiol.*, 72(9), 5908-5914) flanked by regions for homologous recombination 3 stages of PCR were

SEQUENCES OF OLIGONUCLEOTIDES USED IN THIS STUDY

TABLE 1

Primer	Sequence (5' – 3')	SEQ ID NO:
UF5-234	gtgtttaatccatagttataaagcatcccattggcctcaataggggtat	SEQ ID NO:5
UR2-234	ttcaatagctataaattatttaataagtaacggtggcggtaaaaccct	SEQ ID NO:6
UR2-56	ttcaatagctataaattatttaataagtaagtgagcttggcgcaactcttta	SEQ ID NO:7
UF5-56	gtgtttaatccatagttataaagcatcgtgagcggtagtgtcgttgaat	SEQ ID NO:8
rpsL-F	gatgctttataactatggattaaacac	SEQ ID NO:9
ermR	ttacttattaaataatttatagctattgaa	SEQ ID NO:10
si1aF3	ggcccttctttaggaaaaatttcaaatctttcaaagctvnnvnnvnnvnnvnnvnnggcctcaataggggtatgcacggtt	SEQ ID NO:11
si3F3	ggcccttctttaggaaaaatttcaaatctttcaaagctvnnvnnvnnvnnvnnvnnctccttaattgtttttacatagttgt	SEQ ID NO:12
si4F3	ggcccttctttaggaaaaatttcaaatctttcaaagctcatttcttactccttaattgtttttaca	SEQ ID NO:13
si8F3	ggcccttctttaggaaaaatttcaaatctttcaaagctvnnvnnvnnvnnvnnvnngccatccacgaacacatggtaagtt	SEQ ID NO:14
HAFLAG	tttatcatcgtcatctttataatctaagctaggcccaggatagctcccttcttttttcggttaaccataagctaggcccttctttaggaaaaatttcaaat	SEQ ID NO:15
HAFLAG (rc)	atttgaaatttttcctaaagaagggcctagcttatggttaacccgaaaaagaagggagctatcctgggcctagcttagattataaagatgacgatgataaa	SEQ ID NO:16
UF1	cgactttggttaacccgcaaatcccat	SEQ ID NO:17
UR6	gggcgtggtggattatgtgtatta	SEQ ID NO:18
si1aR1	gattataaagatgacgatgataaannbnnbnnbnnbnnbnnbaatggtaaattagttcctggtga	SEQ ID NO:19
si3R1	tttatcatcgtcatctttataatcvnnvnnvnnvnnvnnctccttaattgtttttacatagttgt	SEQ ID NO:20
si4R1	gattataaagatgacgatgataaannbnnbnnbnnbnnbnnbaaaaagattagcagaaaagaatatgtt	SEQ ID NO:21
si8R1	gattataaagatgacgatgataaannbnnbnnbnnbnnbnnbaaagaagtaacttctaaaccagcca	SEQ ID NO:22

TABLE 2

PRIMER COMBINATIONS USED TO PRODUCE MODIFIED DNA

Description	Stage	Product	Primer 1	Primer 2	Template
rpsL.ermB site 1					
	1	A	UF1	UR2-234	genomic
	1	B	rpsL-F	ermR	genomic*
	1	C	UF5-234	UR6	genomic
	2	Fusion	UF1	ermR	A+B
	2	Fusion	rpsL-F	UR6	B+C
	3	Fusion	UF1	UR6	AB+BC
rpsL.ermB site 8					
	1	A	UF1	UR2-56	genomic
	1	B	rpsL-F	ermR	genomic*
	1	C	UF5-56UR6	UR6	genomic
	2	Fusion	UF1	ermR	A+B
	2	Fusion	rpsL-F	UR6	B+C
	3	Fusion	UF1	UR6	AB+BC
Site 1					
	1	A1	UF1	Si1aR1	genomic
	2	A2	UF1	HAFLAG (rc)	A1
	1	B1	Si1aF3	UR6	genomic
	2	B2	HAFLAG	UR6	B1
	3	Fusion	UF1	UR6	A2+B2
Site 3					
	1	A1	UF1	Si3R1	genomic
	2	A2	UF1	HAFLAG (rc)	A1
	1	B1	Si3F3	UR6	genomic
	2	B2	HAFLAG	UR6	B1
	3	Fusion	UF1	UR6	A2+B2
Site 4					
	1	A1	UF1	Si4R1	genomic
	2	A2	UF1	HAFLAG (rc)	A1
	1	B1	Si4F3	UR6	genomic
	2	B2	HAFLAG	UR6	B1
	3	Fusion	UF1	UR6	A2+B2
Site 8					
	1	A1	UF1	Si8R1	genomic
	2	A2	UF1	HAFLAG (rc)	A1
	1	B1	Si8F3	UR6	genomic
	2	B2	HAFLAG	UR6	B1
	3	Fusion	UF1	UR6	A2+B2

* Genomic DNA from X47 harbouring rpsL.ermB at the *mda*B locus

performed. Stage 1 involved the amplification of the rspL.ermB cassette, and flanking regions for homologous recombination at the urease locus. Stage 2 involved the addition of either flanking region to the rpsL.ermB cassette using 2 way SOE PCR, Stage 3 involved using these products as template to add both flanking regions to the rpsL.ermB cassette using 2 way SOE PCR (Table 2). To produce DNA encoding HA (aa 150-159 and aa 110-120 of Influenza virus A/PR/8/34 hemagglutinin protein; Casares *et al.*, 1997, *Viral Immunol.*, 10(3), 129-136) and FLAG (DYKDDDDK) epitopes separated by a four amino acid linker, flanked by semi-random six amino acid linkers and regions for homologous recombination, 3 stages of PCR were performed using AccuPrime™ Pfx Supermix (Invitrogen). Stage 1 involved the addition of flanking linkers, stage 2 involved the addition of HA and FLAG epitopes, stage 3 involved the addition of flanking regions for homologous recombination using 2-way SOE PCR (Table 2). Thermocycling conditions were as follows: 94°C for 15 s, 56°C for 20 s, 68°C for 3.5 min (10 cycles). After the addition of primers an additional 35 cycles of 94°C for 15 s, 62°C for 20 s, 68°C for 3.5 min were performed, followed by a final extension of 3 min. Extension times were varied according to PCR product lengths.

[0162] Streptomycin resistant *H. pylori* strain X47 was used for all experiments (Kleanthous *et al.*, 1995, *Gut*, 37, A94. Bacteria were grown on Brain Heart Infusion (BHI) based agar plates supplemented with 5% horse blood and when appropriate, with erythromycin (10 µg/mL) or streptomycin (10 µg/mL) in an atmosphere containing 5% CO_2.

[0163] Overnight cultures of *H. pylori* grown on BHI based agar plates were subcultured onto plates supplemented with DENT (Oxoid) in lawns of approximately 2

cm in diameter. PCR products were *Dpn*I treated to remove residual genomic DNA and purified QIAQuick PCR Purification Kit (Quiagen) prior for use in transformation. Transformation was performed by the addition of approximately 1 µg of purified PCR product after growth of bacterial lawns for 6-8 hrs. After overnight incubation putative transformants were streaked on selective media.

[0164] *H. pylori* producing functional urease were selected on BHI based agar plates supplemented with 7% (v/v) horse serum, phenol red (100 mg/L), and urea (600 mg/L). Media was acidified to pH 3 using 1M HCl to select for *H. pylori* producing functional urease, as illustrated in Figure 2.

[0165] Four of eight candidate sites yielded mutant urease enzymes that allowed *H. pylori* to grow and metabolize urea (Figure 1b). Insertions at the remaining four sites did not result in isolation of bacteria expressing functional ureases under these conditions.

[0166] *H. pylori* were harvested from mouse stomachs, grown for 4 d, harvested and resuspended in SDS-PAGE loading buffer. Standard SDS-PAGE and Western Blot methodologies were performed (Sambrook & Russell, 2001, *Molecular Cloning, supra*). Electrophoresis was performed using SDS-PAGE on a discontinuous 10% gel. For Western Blotting proteins were transferred to PVDF Immuno-Blot PVDF (0.2 µM) membrane (Biorad). Membranes were blocked overnight at 2% Blocking Reagent (Roche) in Maleic acid buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5, 20°C) supplemented with 0.2 % (v/v) Tween 20. To detect FLAG membranes were probed with a 1:1000 dilution of monoclonal anti-FLAG (Sigma Aldrich) in 1% Blocking Reagent

supplemented with 0.1% (v/v) Tween 20 for 2 hours at room temperature. For detection membranes were incubated with rabbit anti-mouse IgG conjugated to horse radish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) under identical conditions for 1 h at room temperature. Detection was performed using Chemiluminescent Peroxidase Substrate-3 (Sigma-Aldrich) and the FujiFilm LAS-3000 Imager. Urease was similarly probed using a 1:200 dilution anti-urease alpha subunit (bc-14; Santa Cruz Biotechnology) and detected using a 1:2500 dilution of rabbit anti-goat HRP conjugate antibody (Jackson ImmunoResearch Laboratories, Inc.).

[0167] Western blot analysis confirmed that *H. pylori* producing recombinant urease at sites 1, 3, 4 or 8 contained insertions of epitope tags (Figure 2a). The pH of the mouse stomach lumen, which *H. pylori* must traverse to establish gastric mucosal infection is between 3 and 4 (McConnell *et al.*, (2008), *J Pharm Pharmacol.*, 60 (1), 63-70). To determine if these insertions in urease's surface exposed loops altered its activity or stability we assayed enzymatic activity in bacteria expressing wild-type or mutant urease after exposure to acid (pH 3). One of the mutant ureases (insertion at site 1) was more sensitive than wild type, and the three other mutant ureases were similar to wild type in their sensitivity to this acid treatment (Figure 2b; Student's T-test; $p<0.05$).

[0168] EXAMPLE 2 EXPERIMENTAL INFECTION OF MICE

[0169] C57BL/6, *Helicobacter* free, mice were purchased from the Animal Resource Centre (Perth, Western Australia). Studies were performed with approval from the UWA Animal Ethic Committee (approval no. 07/100/598). Eight week old mice were orogastrically inoculated with

approximately 1.0 x 10^9 *H. pylori* harvested from an overnight agar plate based culture into BHI broth (Oxoid). Mice were inoculated with pools of 3 independent, genetically characterised clones expressing wild type or recombinant urease. To determine the level of colonisation, stomachs were harvested from sacrificed animals, opened, and residual food removed. Opened stomachs were suspended in 500 µL PBS and homogenised using 5 mm stainless steel beads for 25 seconds at setting of 30 (Qiagen Tissue Lyser). Samples were then homogenised for a further 2 min at setting of 10 to facilitate bacteria release from the tissue. Serial dilutions of homogenates were plated on BHI based agar plates supplemented amphotericin B (8 µg/mL), trimethoprim (5 µg/mL) and vancomycin (6 µg/mL), Nalidixic acid (10 µg/mL), polymyxin B (10 µg/mL) and bacitracin (200 µg/mL) (Dailidiene *et al.*, (2006), *Appl Environ Microbiol.*, 72 (9), 5908-5914). Bacterial growth was determined 5-7 days post plating.

[0170] Sera were collected from mice at different time points and assessed for the presence of urease specific IgG. Nunc 96 well maxisorb plates were coated with 10 µg/mL of Urease B protein (expressed from plasmid pILL927 and purified as described in reference Ferrero *et al.*, (1994), *Infect. Immuno.*, 62(11), 4981-4989) in 100 µL carbonate buffer and incubated overnight at 4°C. Plates were washed 5 x with PBS supplemented with 0.05% (v/v) Tween 20 (PBST) and then blocked with 200 µL of PBS supplemented with 2% BSA (w/v) for 2 hours at 37°C. Following 2 x washes with PBST a 1:20 dilution mouse sera in 100 µL of PBST supplemented with 2% (w/v) BSA was added to duplicate wells and the plates incubated for 1 hour at room temperature. Subsequently, plates were washed with 5 x with PBST and then a 1:1000 dilution of anti-mouse IgG

alkaline phosphatase (Sigma Aldrich) in 100 µL PBST supplemented with 2% (w/v) was added to each well and the plates incubated at room temperature for 1 hour. After 5 x washes with PBST, 200 µL of nitrophenyl phosphate substrate in diethanolamine buffer added to each well and the plates incubated for 40 min at room temperature in the dark before absorbance was measured at 405nm.

[0171] *H. pylori* were harvested after growth for 24 h on BHI base agar plates and were rinsed in cold saline (0.9% v/v). The bacterial suspension was diluted to an OD_{600} of 4 and 15 µL was added to 15 µL of saline supplemented with Tween 20 (0.2% v/v). To each sample 90 µL of KCl (200 mM; pH 3) was added and samples were incubated for 10 min while shaking at 300 rpm at room temperature. Subsequently the solution was neutralized by the addition of 120 µL of PBS (pH 6.8). 150 µL of each sample was added to 25 µL of phenol red sodium salt (80 mg/mL) and warmed to 37°C. The reaction was initiated by the addition of 75 µL of 0.5 M urea and the change in pH was measured by reading absorbance at 560 nm every 70 s.

[0172] The ability of each mutant urease-producing *H. pylori* strain to colonise C57BL/6 mice was tested. The strain with site 3-mutant urease did not colonize mice (data not shown) and were not further studied, whereas each of the other three mutant strains colonized mice as efficiently as the wild-type in short-term (< 3 months) infections. However, persistence of *H. pylori* with site 8-mutant urease was greatly reduced over longer periods of time (Figure 3a). In confirmation, bacterial titers in mouse stomachs and anti-*H. pylori* IgG in serum were each much reduced relative to wild type and the other two mutants in the case of ten month infection by this strain (Figure 3b, c). Important in our infection protocol, each

inoculation used a pool of three independent transformant clones, which rules out concerns of possible attenuation by secondary mutations distinct from the insertions within urease. Further tests showed that nearly all bacteria recovered from mice 10 months after inoculation still expressed the expected mutant urease, thereby supporting the expectation that success in colonization was not due to loss of inserted DNAs. As the sole exception, just one mouse initially infected with the site 1 mutant strain seemed to produce revertant urease at ten months (Figure 4). It was also striking that reduced urease activity measured *in vitro* did not correlate well with reduced colonisation ability, in that the site 1 mutant strain, whose urease was less acid stable than the others colonized mice as well as wild-type, and the site 8 mutant, whose urease activity was similar in acid resistance to that of wild type was nevertheless less persistent in mouse colonization. Rather, we propose an alternative role for the urease surface around site 8 (Figure 1a), important during chronic infection.

[0173] In summary, a structure-based insertion mutagenesis of the urease complex identified two discrete regions on the enzyme surface that are needed for colonisation or persistence of *H. pylori*, site 3 and site 8 (Figure 1a). The inability of recombinant *H. pylori* to colonise mice due to an insertion at site 3 gives further support for an alternative role of urease, different from acid neutralisation and warrants further investigation. In addition, our results suggest that the site 8 region is not essential for the de-acidification function of urease, but is rather involved in an alternative function required for persistence in the host. Site 8 is located in the turn of a beta-meander at the surface of the urease complex. No insertions or deletions are found at this site in

homologous urease sequences in diverse organisms (Figure 6) and we therefore propose that the precise structure of this beta-meander is important for urease's alternative role during long-term colonisation. Sites 3 and -8 have in common that both are located in the proximity of the rotation axis that connects three alpha/beta trimers. Since enzymatic activity relies on the integrity of the alpha/beta trimer, the insertion of tags at sites 3 and -8 could modify the trimer/trimer interaction surface without compromising the enzymatic activity.

[0174] Adaptive immune responses change dramatically during the establishment and maintenance of chronic *H. pylori* infection, in particular at the site of infection. Urease site 8 overlaps with an established *H. pylori* $CD4^+$ T-cell epitope in response to which splenic lymphocytes produce cytokine IL-4 (Shi *et al.*, (2007), *Vaccine*, 25(14), 2583-2590), a promoter of Th_2 responses and driver of antibody production, whereas sites 1, 3 and 4 do not coincide with any known B or T cell epitopes. We note that *H. pylori* expressing urease recombinant at site 8 infected animals at 3 months of infection, when their bacterial load is similar to that of bacteria expressing wild type urease (Figure 3a), exhibit a weaker humoral response against the urease B subunit (UreB), but a normal response against total *H. pylori* antigen (Figure 3d). Since urease constitutes up to 10% of bacterial protein (Marcus & Scott, (2001), *Helicobacter*, 6(2), 93-99; Hu & Mobley, (1990), *Infect Immun.*, 58(4), 992-998) the removal of an abundant Th_2 driver may prevent adequate immune modulation by *H. pylori* important in persistence and thereby facilitate bacterial clearance. Alternatively, given that binding of *H. pylori* to CD74 (MHC class II) on the gastric epithelium increases IL-8 secretion and up regulation of

inflammatory cytokines (Beswick *et al.*, (2006), *Infect Immun.*, 74(2), 1148-1155)) we can imagine direct interaction of the urease site 8 region with CD74-type host cell receptors affecting host cell signalling and thereby promoting chronic infection. This hypothesis is supported by the observation that CD74 receptors function as trimers (reviewed in Beswick *et al.* (2009), *World J Gastroenterol.* 15:2855) and insertion of tags at sites 3 and -8 likely affect the trimerisation of alpha/beta trimers.

[0175] In conclusion, surface properties of the urease complex, distinct from urease activity *per se*, were found to be important for *H. pylori* colonisation and persistence. In the light of the many failed efforts to develop anti-*H. pylori* vaccines, a better molecular understanding of factors important in persistence should contribute to development of new, much needed therapeutic approaches.

CLAIMS

1. An isolated, genetically modified *Helicobacter pylori* comprising a functional urease, wherein the contiguous amino acid sequence between amino acid 529 and amino acid 555 of SEQ ID NO: 1 is altered to produce said modified *Helicobacter pylori* which is unable to establish or maintain a persistent infection.

2. The isolated, genetically modified *Helicobacter pylori* according to claim 1, wherein the amino acid sequence between amino acid 529 to amino acid 555 of SEQ ID NO:1 is altered by insertion of one or more than one additional contiguous amino acid(s).

3. An isolated, genetically modified *Helicobacter pylori* comprising a functional urease comprising a modified UreB subunit, wherein said modified UreB subunit comprises the amino acid sequence set forth in SEQ ID NO: 1 wherein one or more than one additional contiguous amino acid residue(s) have been inserted in a region between amino acid 529 and amino acid 555 of SEQ ID NO: 1, and wherein said modified *Helicobacter pylori* has reduced ability to establish or maintain a persistent infection relative to a *Helicobacter pylori* expressing an unmodified urease.

4. The isolated, genetically modified *Helicobacter pylori* according to claim 3, wherein said modified UreB subunit comprises an amino acid sequence set forth in SEQ ID NO: 1 wherein at least 10 additional contiguous amino acids residues have been inserted in a region between amino acid 529 and amino acid 555 of SEQ ID NO: 1.

5. The isolated genetically modified *Helicobacter pylori* according to claim 3 or 4, wherein between 10 to 100

additional contiguous amino acid residues have been inserted in a region between amino acid 529 and amino acid 555 of SEQ ID NO: 1.

6. The isolated genetically modified *Helicobacter pylori* according to any one of claims 3 to 5, wherein between 20 to 50 additional contiguous amino acid residues have been inserted in a region between amino acid 529 and amino acid 555 of SEQ ID NO: 1.

7. The isolated genetically modified *Helicobacter pylori* according to any one of claims 3 to 6, wherein 49 additional contiguous amino acid residues have been inserted in a region between amino acid 529 and amino acid 555 of SEQ ID NO: 1.

8. The isolated, genetically modified *Helicobacter pylori* according to any one of claims 3 to 7, wherein the insertion is between amino acid residues 549 and 550 of SEQ ID NO: 1.

9. The isolated genetically modified *Helicobacter pylori* according to any one of claims 2 to 8, wherein the amino acid insertion is a heterologous antigen or a functional fragment thereof.

10. The isolated genetically modified *Helicobacter pylori* according to claim 9, wherein the heterologous antigen or functional fragment thereof is from a pathogenic microorganism.

11. The isolated genetically modified *Helicobacter pylori* according to claim 10, wherein the pathogenic microorganism is selected from the group consisting of a virus, a bacterium, a protozoan and a fungus.

12. The isolated genetically modified *Helicobacter pylori* according to claim 10 or claim 11, wherein the heterologous antigen is selected from the group consisting of a human immunodeficiency virus (HIV) antigen, an HTLV antigen, an SIV antigen, an RSV antigen, a PIV antigen, an HSV antigen, a CMV antigen, an Epstein-Barr virus antigen, a Varicella-Zoster virus antigen, a mumps virus antigen, a measles virus antigen, an influenza virus antigen, a poliovirus antigen, a rhinovirus antigen, a hepatitis A virus antigen, a hepatitis B virus antigen, a hepatitis C virus antigen, a Norwalk virus antigen, a togavirus antigen, an alphavirus antigen, a rubella virus antigen, rabies virus antigen, a Marburg virus antigen, an Ebola virus antigen, a papilloma virus antigen, a polyoma virus antigen, a metapneumovirus antigen, a coronavirus antigen a *Vibrio cholerae* antigen, a *Plasmodium falciparum* antigen, a *Plasmodium vivax* antigen, a *Plasmodium ovate* antigen, a *Plasmodium malariae* antigen, a *Plasmodium knowlesi* antigen, a *Streptococcus pneumoniae* antigen, *Streptococcus pyogenes* antigen, a *Helicobacter pylori* antigen, a *Streptococcus agalactiae* antigen, a *Neisseria meningitidis* antigen, a *Neisseria gonorrhoeae* antigen, a *Corynebacterium diphtheriae* antigen, a *Clostridium tetani* antigen, a *Bordetella pertussis* antigen, a *Haemophilus* antigen, a *Chlamydia* antigen and an *Escherichia coli* antigen.

13. An immunogenic composition comprising the isolated genetically modified *Helicobacter pylori* according to any one of claims 1 to 12 and a pharmaceutically acceptable carrier.

14. The immunogenic composition according to claim 13, further comprising an adjuvant.

15. A method of controlling the ability of *Helicobacter pylori* to establish a persistent infection comprising the step of inserting one or more than one additional contiguous amino acid(s) between amino acid residues 529 and amino acid 555 of SEQ ID NO : 1 to produce a modified UreB subunit of urease in said *Helicobacter pylori*, wherein said urease retains de-acidification function, but said *Helicobacter pylori* has a reduced ability to establish or maintain a persistent infection relative to a *Helicobacter pylori* expressing an unmodified urease.

16. A method of producing a genetically modified Helicobacter pylori having reduced ability to establish or maintain persistent infection, said method comprising:

inserting a nucleic acid encoding one or more than one contiguous amino acid residue(s) in-frame into a nucleic acid encoding a UreB subunit of *H. pylori* urease to thereby produce a recombinant UreB gene encoding a modified UreB subunit comprising one or more than one contiguous amino acid residue(s) in a region of SEQ ID NO: 1 between amino acid 529 and amino acid 555, and

introducing the recombinant UreB gene into *H. pylori* to produce a modified *H. pylori* expressing a functional urease protein comprising the a modified UreB subunit, wherein said functional urease has de-acidification function, and wherein said modified *H. pylori* has a reduced persistence relative to a *H. pylori* expressing an unmodified urease.

17. The method of claim 16, wherein the nucleic acid is inserted in-frame between nucleotide residues 1588 and 1662 of SEQ ID NO: 2.

18. The method according to claim 16 or claim 17, comprising inserting nucleic acid encoding between 10 and 100 contiguous amino acid residues in-frame into nucleic acid encoding a UreB subunit of *H. pylori* urease to thereby produce a recombinant UreB gene encoding a modified UreB subunit comprising the between 10 and 100 contiguous amino acid residues in a region of SEQ ID NO: 1 between amino acid 529 and amino acid 555.

19. The method according to any one of claims 16 to 18, wherein the nucleic acid encoding one or more than one contiguous amino acid residue(s) is inserted in-frame into nucleic acid encoding a UreB subunit of *H. pylori* urease to thereby produce a recombinant UreB gene encoding a modified UreB subunit comprising the one or more than one contiguous amino acid residue(s) in a region of SEQ ID NO: 1 between amino acid 549 and amino acid 550.

20. The method according to claim 23, wherein the nucleic acid is inserted in-frame between nucleotide residues 1647 and 1648 of SEQ ID NO: 2.

21. A method of vaccinating a mammal against *Helicobacter pylori* infection comprising administering to said mammal the isolated, genetically modified *Helicobacter pylori* according to any one of claims 1 to 12 or the immunogenic composition according claim 13 or 14.

22. Use of the isolated, genetically modified *Helicobacter pylori* according to any one of claims 1 to 12 in the preparation of a medicament for vaccinating a mammal against *Helicobacter pylori* infection.

23. The isolated, genetically modified Helicobacter pylori according to any one of claims 1 to 12 or the

immunogenic composition according to claim 13 or 14 or the method according to any one of claims 15 to 21 or the use according to claim 22 substantially as herein before described with reference to the Examples and/or Drawings.