**METHODS FOR PRODUCING ENHANCED ANTIGENIC HELICOBACTER SP. AND VACCINES COMPRISING SAME**

**VERFAHREN ZUR PRODUKTION VON VERSTÄRKJT ANTIGEN WIRKENDES HELICOBACTER SP. UND VAKZINE DIE DIESEN ENTHALTEN**

**METHODES DE PRODUCTION D'HELICOBACTER SP. ANTIGENE AMELIORE ET DE VACCINS LE CONTENANT**

<table>
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<th>Designated Contracting States:</th>
<th>• POPE L M ET AL: &quot;Increased protein secretion and adherence to HeLa cells by Shigella spp. following growth in the presence of bile salts&quot; INFECTION AND IMMUNITY, XP002075400</th>
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• DATABASE NCBI TAXONOMY BROWSER [Online] 'Helicobacter pylori' Database accession no. 210

• GRAHAM: 'Helicobacter: A new scientific journal' HELICOBACTER vol. 1, no. 1, 1996, pages 1 - 3

• 94TH AMERICAN SOCIETY OF MICROBIOLOGY MEETING, 1994, DURHAM et al., page 386, Abstract P-96.
This invention relates generally to \textit{in vitro} methods for inducing or enhancing expression of enteric bacterial antigens and/or virulence factors thereby producing antigenically enhanced enteric bacteria, to methods for using antigenically enhanced enteric bacteria and to vaccines comprising antigenically enhanced enteric bacteria.

It is widely recognized that bacteria cultured \textit{in vitro} using conventional media and conditions express characteristics that are different from the characteristics expressed during growth in their natural habitats, which includes \textit{in vivo} growth of normal microflora or pathogens in an animal host. Therefore, such \textit{in vitro} grown pathogenic bacteria might not be good for use as vaccine components. However, if it were possible to define conditions that trigger or enhance expression of virulence factors, relevant physiology, or antigens including outer-surface antigens then important products and therapeutics (e.g., new antigens for vaccines, new targets for antibiotics, and novel bacterial characteristics for diagnostic applications) could be rapidly identified.


Other environmental signals that have been shown to control the expression of coordinately regulated virulence determinants of a wide variety of bacteria in plants and animals include phenolic compounds, monosaccharides, amino acids, temperature, osmolarity, and other ions (Mekalanos, \textit{J. Bacteriol.}, 174:1-7, 1992).

Bacterial pathogens that enter an animal host through the intestine (i.e., oral route) encounter numerous host environment components and conditions that may affect bacterial physiology and expression of virulence factors. These components and conditions include bile, bile acids or salts, stomach pH, microaerophillic conditions (the intestine has high CO$_2$, and low O$_2$), osmolarity and many others yet undefined. Invasive enteric pathogens require \textit{de novo} protein synthesis to accomplish internalization (Headley and Payne, \textit{Proc. Natl. Acad. Sci.}, USA, 87:4179-4183, 1990). Therefore, bacteria may optimally produce these invasive factors only in response to certain environmental signals not ordinarily present \textit{in vitro}. This hypothesis is supported by the recent report that antiserum raised against conventionally grown \textit{C. jejuni} had only a marginal effect on blocking \textit{in vitro} internalization (Konkel, et al., \textit{J. Infect. Dis.}, 168:948-954, 1993). However, immunization of rabbits with extracts of \textit{Campylobacter} grown in the presence of epithelial cell monolayers, a condition enhancing invasiveness, resulted in production of an antiserum that markedly inhibited the internalization of the bacteria.

Researchers have been studying growth of bacteria in the intestinal environment to identify relevant virulence factors. For example, \textit{Campylobacter} strain 81-176 grown in rabbit ileal loops expresses proteins not expressed under conventional laboratory \textit{in vitro} culture conditions (Panigrahi, et al., \textit{Infect. Immun.}, 60:4938-4944, 1992). New or enhanced synthesis of proteins has been seen in \textit{Campylobacter} cultivated with INT 407 cell monolayers as compared to bacteria cultured in the absence of the epithelial cells (Konkel, et al., \textit{J. Infect. Dis.}, 168:948-954, 1993). Furthermore, these changes were temporally associated with increased invasiveness of \textit{C. jejuni}. Other changes such as cellular morphology, loss of flagella, expression of a new outer membrane protein and alteration in cell-surface carbohydrates were induced or enhanced in an avirulent strain of \textit{C. jejuni} when passed intravenously and chorioallantoically through chick embryos (Field, et al., \textit{J. Med. Microbiol.}, 38:293-300, 1993).

Other intestinal components, such as bile acids or salts, are known to be inhibitory for some bacteria, but the bile acids may play another role by affecting virulence expression by the bacterium. \textit{Shigella flexneri} cultured in broth containing sodium chenodeoxycholate demonstrated 3 to 5-fold enhanced infectivity of HeLa cell monolayers. They reported, however, that other bile salts and detergents including cholate, glycocholate, taurodeoxycholate, the CHAPS series, digitonin and Triton X100 and sodium salts thereof, had no effect on the invasiveness of \textit{S. flexneri}. Moreover, their broth containing chenodeoxycholate also had no effect on the invasiveness of \textit{E. coli} or other avirulent strains of \textit{Shigella}.


PCT application publication number WO 93/22423, published November 11, 1993, discloses methods for growing bacteria on lipids, such as phosphatidylserine, or mucus and for the isolation of proteins whose expression is enhanced by growth in the presence of phosphatidylserine. This reference neither discloses nor suggests methods of...
Vaccines against many enteric pathogens, such as *Campylobacter* and *Shigella*, are not yet available but the epidemiology of these disease agents makes such vaccines an important goal. Shigellosis is endemic throughout the world and in developing countries it accounts for about 10 percent of the 5 million childhood deaths annually due to diarrhea. *Campylobacter*, although only recently identified as an enteric pathogen is now recognized as one of the major causes of diarrheal disease in both the developed and underdeveloped countries. An estimated 400 to 500 million *Campylobacter* diarrhea occur yearly, and over 2 million cases occur in the United States.

Shigellosis is a consequence of bacterial invasion of the colonic mucosa. The invasion is associated with the presence of a plasmid found in all invasive isolates (Sansonetti et al., *Infect. Immun.*, 35:852-860, 1982). A fragment of this plasmid contains the invasion plasmid antigen (Ipa) genes, Ipa A, -B, -C, and -D. Ipa A, -C, and -D proteins are essential for the entry process (Baudry et al., *J. Gen. Microbiol.*, 133:3403-3413, 1987).

Ipa proteins are logical vaccine candidates although their protective efficacy has not been clearly established. Ipa B and Ipa C are immunodominant proteins (Hale et al., *Infect. Immun.*, 50:620-629, 1985). Furthermore, the 62 kDa Ipa B protein (the invasin that initiates cell entry and functions in the lysis of the membrane-bound phagocytic vacuole) (High et al., *EMBO J.*, 11:1991-1999, 1992) is highly conserved among *Shigella* species. The prolonged illness observed in malnourished children who have no significant mucosal antibody to *Shigella* Ipa suggests that the presence of mucosal antibody to Ipa may limit the spread and severity of infection.

Though a number of vaccine candidates for *Shigella* have been tested in animals and humans, a successful one has not been found. In spite of the potential significance of Ipa proteins in virulence, most vaccine candidates developed against shigellosis are based on the lipopolysaccharide antigen, which carries the serotype-specific determinants. A parenterally administered polysaccharide-protein conjugate vaccine has also been developed, but is yet to show significant protection in animals (Robbins et al., *Rev. Inf. Dis.*, 13:S362-365, 1991). A similarly administered ribosomal vaccine does induce mucosal immunity, but its protective efficacy remains to be demonstrated (Levenson et al., *Arch. Allergy Appl. Immunol.*, 87:25-31, 1988).

The pathogenesis of *Campylobacter* infections is not as well understood as that of *Shigella* infections. Cell invasion studies in *vitro* (Konkel et al., *J. Infect. Dis.*, 168:948-954, 1993) and histopathologic examinations (Russell et al., *J. Infect. Dis.*, 168:210-215, 1993) suggest that colonic invasion is also important. This conclusion is consistent with the observation that diarrhea caused by *Campylobacter* may be severe and associated with blood in the stool. These activities may be associated with the immunodominant 62 kDa flagellin protein. A recent report indicates that the presence of flagella is essential for *Campylobacter* to cross polarized epithelial cell monolayers (Grant et al., *Infect. Immun.*, 61:1764-1771, 1993).

No specific *Campylobacter* antigens have been established as protective. However, the low molecular weight (28-31 kDa) proteins, or PEB proteins, and the immunodominant flagellar protein are thought to hold promise in this regard (Pavlovskis et al., *Infect. Immun.*, 59:2259-2264, 1992; Blaser and Gotschlich, *J. Bio. Chem.*, 265:14529-14535, 1990). The importance of the flagellar protein is indicated by its association with colonization of the intestine and with the cross-strain protection against infection within Lior subgroups (Pavlovskis et al., *Infect. Immun.*, 59:2259-2264, 1992). However, a flagella protein based *Campylobacter* vaccine may have to include the flagella protein antigen from the 8-10 most clinically relevant Lior serogroups.

Abstract B-147 from the 93rd American Society of Microbiology Meeting, Session 133, 1993, Pope et al., discloses that *Shigella Flexneri* grown in broth containing sodium deoxycholate demonstrates enhanced infectivity of HeLa cell monolayers.

Panigrahi et al., Infection and Immunity, 1992, p4938-4944 describes the human immune response to *Campylobacter jejuni* proteins expressed in vivo.

The NCBI Taxonomy browser identifies that *Campylobacter pylori* and *Helicobacter pylori* are synonymous.

Therefore, objects of the present invention include 1) *in vitro* culture conditions for culturing or treating enteric bacteria which optimally induce or enhance invasive activities and/or certain cellular characteristics including cell surface characteristics, 2) correlated altered invasiveness or cellular characteristics including surface characteristics with changes in antigenic profiles; 3) increased virulence of these organisms in small animal models; and 4) antisera against organisms with enhanced invasiveness or altered characteristics including surface characteristics that are more effective in neutralizing live organisms used for *in vitro* or *in vivo* challenges than antisera prepared against conventionally grown bacteria. This invention addresses these needs and others.

None of the references discussed above teach or suggest the *in vitro* methods of the present invention nor the vaccines of the present invention comprising antigenically enhanced enteric bacteria. Citation or identification of any reference in this section or any other section of this application shall not be construed as indicative that such reference is available as prior art to the invention.
3 SUMMARY OF THE INVENTION

[0022] This invention provides defined culture conditions and components incorporated into growth media of Helicobacter pylori or Helicobacter felis to induce or enhance the presence of virulence factors and other antigens. Preferably, such antigens are immunogenic. More preferably, such immunogenic antigens correlate with indices of virulence.

[0023] The bacteria are grown in the presence of conditions and components simulating certain in vivo conditions to which the organisms are exposed in nature. Methods of the present invention produce antigenically enhanced enteric bacteria with phenotypic changes such as increased total protein per cell, new or increased individual proteins, altered or increased surface carbohydrates, altered surface lipopolysaccharides, increased adhesive ability, increased invasive ability and/or increased intracellular swarming. Moreover, methods of the present invention are adaptable to practical scale-up fermentations for commercial uses.

[0024] Said antigenically enhanced bacteria can be used to produce protective vaccines, such as inactivated whole cell or subunit vaccines, or for diagnostic purposes such as for the production of antibodies and detection of pathogenic bacteria or to produce antibiotics. Further, the antibodies induced by the enhanced enteric bacteria of the present invention may be used as passive vaccines.

[0025] Therefore, an aspect of the present invention is a method for producing Helicobacter pylori or Helicobacter felis according to claim 1.

[0026] A further aspect of the invention is Helicobacter pylori or Helicobacter felis according to claim 4.

[0027] Another aspect of the invention is a vaccine according to claim 4.

[0028] Still a further object of the invention is an in vitro method for detecting a host's production of antibodies to the bacteria in an animal or biological sample therefrom, comprising the steps of contacting a biological sample from a host with bacteria of the present invention having enhanced antigenic properties and screening for antibody:antigen interactions.

[0029] Another object of the present invention relates to a diagnostic kit for detecting a host's production of antibodies to the bacteria.

[0030] The present invention is based, in part, on the surprising discovery that antigenically enhanced bacteria of the invention induce immune responses that are cross-protective against a broader range of strains or serotypes of the same bacterial species than that induced by the same bacteria but grown using conventional culturing conditions. In at least one instance, the immune response induced by the antigenically enhanced enteric bacteria of the invention is cross-protective against a different species of enteric bacteria.

4 BRIEF DESCRIPTION OF THE DRAWINGS

[0031] Figure 1 graphically depicts the effect of bile concentration and the growth phase of the Helicobacter pylori culture on the adhesiveness of Helicobacter pylori NB3-2 cells. H. pylori NB3-2 cells were grown in culture medium containing 0%, 0.025%, 0.05% or 0.1% bile and harvested at 8, 10, 12 and 18 h after inoculation. The invasiveness of these different preparations of H. pylori NB3-2 cells against INT-407 cells are shown. See Example 38 in Section 14 for details.

[0032] Figure 2 graphically depicts the effect of bile concentration and the growth phase of the Helicobacter pylori culture on the adhesiveness of Helicobacter pylori G1-4 cells. H. pylori G1-4 cells were grown in culture medium containing 0%, 0.1% or 0.2% bile and harvested at 6, 8, 10, 12, 14 and 16 h after inoculation. The invasiveness of these different preparations of H. pylori G1-4 cells against INT-407 cells are shown. See Example 38 in Section 15 for details.

5 DETAILED DESCRIPTION OF THE INVENTION

[0033] The methods of the present invention relate to growing Helicobacter pylori or Helicobacter felis bacteria in vitro in the presence of a combination of certain conditions with certain components selected to induce or enhance the expression of antigens and/or virulence factors.

[0034] The terms "components" and "conditions" as used herein and in the claims relate to many factors associated with a bacterium's natural in vivo environment and other factors. Such components and conditions include, but are not limited to, bile, bile acids or salts thereof or their biological precursors such as cholesterol, pH, microaerophilic condition, osmolarity, and harvesting or collecting the bacteria at a desired bacterial growth phase.

[0035] The term "antigens" and its related term "antigenic" as used herein and in the claims includes antigens or antigenic characteristics including, but not limited to, macromolecules contributing to cellular morphology or cell motility; proteins; more particularly surface proteins, lipopolysaccharides and carbohydrates. Preferably said antigens are immunogenic.

[0036] The term "immunogenic" as used herein and in the claims refers to the ability to induce antibody production
in an animal after said animal is exposed to a composition comprising whole bacteria produced by the present invention or a fragment of said whole bacterium.

[0037] The term "antigenically enhanced" or "enhanced antigenic properties" or "enhanced" as used herein and in the claims refers to the antigenic state of enteric bacteria grown according to the methods of the present invention. Such bacteria have higher levels of certain immunogenic antigens and/or new immunogenic antigens as compared to the same bacteria grown using conventional methods.

[0038] The term "conventional" as used herein and in the claims refers to what is known in the prior art.

[0039] The term "microaerophilic conditions" as used herein and in the claims refers to anaerobic conditions or elevated CO₂ levels, such as 5% to 20% CO₂ with 80% to 95% air; or 5% O₂ with 10% CO₂ with 85% N₂.

[0040] The term "virulence" as used herein and in the claims refers to those factors of an enteric bacteria associated with the ability to adhere to and/or to invade and/or to survive in a host and/or cause a pathological condition.

[0041] The term "immuno-cross protective" as used herein and in the claims refers to the ability of the immune response induced by one bacterial strain or serotype, whole cell or otherwise, to prevent or attenuate infection of the same host by a different bacterial strain, serotype, or species of the same genus.

[0042] The term "immuno-cross reactive" as used herein and in the claims refers to the ability of the humoral immune response (i.e., antibodies) induced by one bacterial strain or serotype, whole cell or otherwise, to cross react with (i.e., the antibody binding) a different bacterial strain, serotype, or species of the same genus. Immuno-cross reactivity is indicative of the bacterial immunogen's potential for immuno-cross protection and vice versa.

[0043] The term "host" as used herein and in the claims refers to either in vivo in an animal or in vitro in animal cell cultures. The term "animal" as used herein and in the claims includes but is not limited to all warm-blooded creatures such as mammals and birds (e.g., chicken, turkey, duck, etc.)

[0044] According to the invention, in a vaccine comprising antigenically enhanced bacteria the bacteria may be either live bacteria or may be inactivated and may further comprise an adjuvant, such as, but not limited to, alum, oil-water emulsion, heat labile toxin from enterotoxigenic E. coli (LT) nontoxicigen forms thereof (e.g. mLLT) and/or individual subunits thereof, Bacille Calmette-Guerin (BCG), or Freund's adjuvant and may also further comprise a suitable pharmaceutical carrier, including but not limited to saline, dextrose or other aqueous solution. Other suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, Mack Publishing Company, a standard reference text in this field.

[0045] The term "inactivated bacteria," as used herein and in the claim, refers to bacteria that are incapable of infection and/or colonization and encompasses attenuated as well as killed bacteria. Attenuated bacteria may replicate but cannot cause infection or disease. Inactivation of said bacteria may be accomplished by any methods known by those skilled in the art. For example, the bacteria may be chemically inactivated, such as by formalin fixation, or physically inactivated such as by heat, sonication or irradiation, so that they are rendered incapable of replication and/or infection and/or causing disease.

[0046] An effective amount of the vaccine should be administered, in which "effective amount" is defined as an amount of bacteria that is capable of producing an immune response in a subject. The amount needed will vary depending upon the antigenicity of the bacteria, used, and the species and weight of the subject to be vaccinated, but may be ascertained using standard techniques. In preferred, non-limiting embodiments of the invention, an effective amount of vaccine produces an elevation of antibacterial antibody titer to at least two times the antibody titer prior to vaccination. In a preferred, specific, non-limiting embodiment of the invention, approximately 10⁷ to 10¹¹ bacteria and preferably 10⁹ to 10¹⁰ bacteria are administered to a host. Preferred are vaccines comprising inactivated whole bacteria.

[0047] The term "effective amount" as applied to passive vaccines is an amount of antibody that is capable of preventing or attenuating a bacterial disease or infection. The amount needed will vary depending upon the type of antibody and the antibody titer, and the species and weight of the subject to be vaccinated, but may be ascertained using standard techniques.

[0048] Vaccines of the present invention may be administered locally and/or systemically by any method known in the art, including, but not limited to, intravenous, subcutaneous, intramuscular, intravenous, intraperitoneal, intranasal, oral or other mucosal routes.

[0049] Vaccines may be administered in a suitable, nontoxic pharmaceutical carrier, may be comprised in microcapsules, and/or may be comprised in a sustained release implant.

[0050] Vaccines may desirably be administered at several intervals in order to sustain antibody levels.

[0051] Vaccines of the invention may be used in conjunction with other bacteriocidal or bacteriostatic methods.

[0052] Methods for the detection of antibodies in a host include immunoassays. Such immunoassays are known in the art and include, but are not limited to radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), fluorescent immunoassays, and fluorescence polarization immunoassays (FPIA).

[0053] Another embodiment includes diagnostic kits comprising all of the essential reagents required to perform a desired immunoassay according to the present invention. The diagnostic kit may be presented in a commercially packaged form as a combination of one or more containers holding the necessary reagents. Such a kit comprises a bacteria
of the present invention, in combination with several conventional kit components. Conventional kit components will be readily apparent to those skilled in the art and are disclosed in numerous publications, including Antibodies A Laboratory Manual (E. Harlow, D. Lane, Cold Spring Harbor Laboratory Press, 1989). Conventional kit components may include such items as, for example, microtiter plates, buffers to maintain the pH of the assay mixture (such as, but not limited to Tris, HEPES, etc.), and the like, and other standard reagents.

[0054] Methods of the present invention include growing bacteria in a suitable basal essential culture medium, such as but not limited to commercially available brain heart infusion broth "BHI", Luria broth "LB", sheep blood agar "SBA", Brucella broth, Mueller-Hinton broth, proteose peptone beef extract broth, etc., with various conditions and components including but not limited to 0.025% to 0.2% bile, at a temperature of 37°C until a growth phase at about early log phase, between early log and stationary phases, or at about stationary phase, in air or under microaerophilic conditions, such as but not limited to 5% to 20% CO₂ with 80% to 95% air, or 5% O₂ with 10% CO₂ with 85% N₂; and optionally in the presence of a divalent cation chelator, such as, but not limited to 0 to 100 μM, preferably 25 μM, of BAPTA/AM (2′(ethylenedioxy) diaminine n,n,n',n'-tetraacetic acid/acetoxymethyl ester; Molecular Probes, Eugene, OR); the resulting combination of conditions and components producing antigenically enhanced enteric bacteria.

[0055] The bile salt useful for the present invention is glycocholate (GC).

[0056] Cultures can be prepared as frozen stocks by methods generally known to those skilled in the art and maintained at -80°C for future use. Stocks of Helicobacter pylori can be prepared by growing the organism in brain heart infusion broth ("BHI"). Bacteria can be harvested for freezing by any known method, for instance by swabbing the culture and resuspending in BHI containing 30% glycerol. Cultures for analytical experiments or for production fermentations can be prepared by any generally known methods, such as by growing the organism on BHI with 1.5% agar at 37°C under MC or atmospheric conditions and then transferring a single colony to broth and culturing according to methods of the present invention described herein. Bacteria can be harvested for use by any method generally known to those skilled in the art, such as by centrifugation.

[0057] In further preferred embodiments, antigenically enhanced cells of Helicobacter pylori, preferably of the strain ATCC 49503, NB3-2 or G1-4, are grown in a basal essential culture medium, preferably BHI broth, additionally comprising about 0.05% to about 0.2% bile or about 0.05% glycocholate (GC) at 37°C in a mixture of about 5% to 20% CO₂ with about 80% to 95% air, or about 10% CO₂ with about 85% N₂ and harvested after the growth of the culture has reached about log or about stationary phase. In a more preferred embodiment, the cells are harvested after the culture has reached about log phase.

[0058] Bacteria cultured according to the methods of the present invention have altered morphologies, and/or cell motilities and/or produce certain new proteins, lipopolysaccharides and/or carbohydrates and/or such macromolecules at altered levels compared to cells cultured in basal medium alone. Optimum cultural conditions that enhance cell yield and the indices of pathogenicity can be identified. Utilizing these cultural conditions, virulence-associated antigens that are enhanced or induced can be identified.

[0059] Motility and gross morphological changes can be seen by microscopic examination of either untreated or stained bacteria. It is possible that other morphological changes might result from methods of the present invention as could be seen through electron microscopy or fluorescence microscopy.

[0060] The morphology and mucus-like characteristics of the enteric bacteria cultured according to methods of the present invention suggest that capsule and/or surface layer expression might be induced. To test for capsule production, phenol extracts of surface components, such as proteins, carbohydrates and lipopolysaccharides, can be prepared. The enhanced carbohydrates can be seen by high pressure liquid chromatography (HPLC).

[0061] Protein profiles of outer membranes prepared from enteric bacteria grown under virulence enhancing growth conditions of the present invention can be characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and compared to those from organisms grown in conventional media. SDS-PAGE is conducted to evaluate changes induced in bacterial cellular and extracted proteins in response to antigen enhancing or altering conditions. These data offer qualitative and quantitative information concerning surface changes associated with increased invasiveness or altered antigenicity.

[0062] The immunogenic potential of induced or altered protein antigens can be identified by Western Blotting. Immunogenicity of induced or altered bacterial proteins identified by SDS-PAGE can be evaluated by the generally accepted techniques of Western Blotting as described below. Any source of antibody can be used, such as convalescent immune rabbit or ferret sera (source of antibody from animals infected orally with live organisms grown conventionally or according to methods of the present invention), intestinal mucus (source of IgA antibody), or polyclonal antisera.

[0063] The increased production of several of these bacterial antigens is accompanied with enhancement of properties associated with virulence. These properties include adhesion to and invasion of cultured human intestinal epithelial cells, and Congo red dye binding. The Congo red dye binding assay is a generally accepted method predictive of virulence and is described below and in Andrews et al., (Infect. Immun., 60:3287-3295, 1992); and Yoder (Avian}
DIS., 33:502-505, 1989). Bacterial binding of Congo red indicates ability to bind hemin, this ability to bind hemin is correlated with virulence. Congo red binding also correlates with enhanced bacterial invasion of epithelial cells.

[0064] Methods of the present invention for production of antigenically enhanced bacteria correlate to enhanced virulence in small animal models. With *Helicobacter pylori*, the *Helicobacter felis* gastric colonization model described by Chen et al. (Lancet, 339:1120-1121, 1992) is used to evaluate the vaccine potential of *H. pylori* produced by the methods of the present invention.

[0065] The antigenically enhanced bacteria produced by the methods of the present invention are used to prepare prototype killed whole-cell or subunit vaccines. These vaccines when administered to animals can be shown to induce antibodies and thus establish the vaccines’ protective potential. Elevation or induction of these antigens in a bacterial cell produces cells that make more efficacious vaccines.

[0066] Vaccine candidate preparations produced by the methods of the present invention can be used with various mucosal immunization strategies to induce an intestinal immune response. Successful immunization protocols can then be used to protect animals challenged with the pathogens with or without enhanced antigenicities. Also vaccines can be formulated and tested as combined vaccines,

[0067] The experimental approaches described below enable the detection of changes in surface antigens and other characteristics which are associated with enhanced invasiveness. These changes are both qualitative and quantitative. Most importantly, these experiments establish that the methods of the present invention enhance invasiveness and induce immunogenically relevant antigens. Such antigenically enhanced bacteria induce an immunogenic response which is protective against infections by a broader range of bacterial strains, serotypes and/or species and are therefore useful as efficacious vaccines as compared to conventionally grown bacteria.

[0068] Several well-established models generally known to those skilled in the art exist and are useful to evaluate enhanced antigenic properties, virulence of bacteria and vaccine efficacy as described below.

[0069] Non-limiting examples of the present invention are described below.

6 EXAMPLES: METHODS FOR PRODUCING ENHANCED ANTIGENIC BACTERIA

[0070] Example 1. *Helicobacter pylori* was added to BHI broth plus 4% bovine calf serum. After inoculation the flasks were flushed with 5% O₂, 10% CO₂, 85% N₂ and incubated for 22 h at 37°C with shaking. After this incubation, 2.5 ml of the culture was transferred to a flask containing BHI broth with 4% bovine calf serum or the same medium additionally containing 0.05% sodium glycocholate. These cultures were again flushed with the microaerophilic gas mixture (5% O₂, 10% CO₂, 85% N₂), and incubated 20-24 h at 37°C. The cells were harvested as described above.

[0071] Example 2. *Helicobacter pylori* was added to BHI broth plus 4% bovine calf serum. After inoculation the flasks were flushed with 5% O₂, 10% CO₂, 85% N₂ and incubated for 22 h at 37°C with shaking. After this incubation, 2.5 ml of the culture was transferred to a flask containing BHI broth with 4% bovine calf serum or the same medium additionally containing about 0.1% to about 0.2% bovine bile. These cultures were again flushed with the microaerophilic gas mixture (5% O₂, 10% CO₂, 85% N₂), and incubated 20-24 h at 37°C. The cells were harvested as described above.

7 EXAMPLES: ENHANCED ANTIGENIC BACTERIA

40 EXAMPLE: FACTORS THAT AFFECT THE ADHESIVENESS OF *H. PYLORI* TO ANIMAL CELLS

[0072] Example 3. The adherence of *H. pylori* is enhanced by growth in glycocholate or bile. Cells of *H. pylori* strain NB3-2 or G1-4 were added to BHI broth plus 4% bovine calf serum. After inoculation the flasks were flushed with 10% CO₂, 5% O₂, 85% N₂ and incubated for 22 h at 37°C with shaking. After this incubation, the culture was diluted 1 to 10 to a flask containing 1 liter of the BHI medium with 4% bovine calf serum containing various concentrations of bovine bile (0.025% to 0.2%). These cultures were again flushed with the same gas mixture, and incubated at 37°C. The cells were harvested at various times up to 18 h and their adherence to INT-407 cells assayed using the methods described in Example 28. The results show that culturing with bile enhanced the adhesiveness of *H. pylori* to INT-407 cells (see Figures 12 and 13). For the NB3-2 strain, peak adhesiveness, a 4 to 6 fold increase over that of non-enhanced culture, occurred after about 8 h of growth (Figure 12). For the G1-4 strain, peak adhesiveness, a 2 to 3 fold increase over that of none-enhanced culture, occurred between 12-14 h of growth in 0.2% bile (Figure 13). These “peak” times generally corresponded to the period when the culture of each strain was in log phase growth.

EXAMPLE: VACCINE EFFICACY OF *HELICOBACTER* GROWN ACCORDING TO THE METHODS OF THE PRESENT INVENTION

[0073] Example 4. The protective efficacy of formalin-fixed whole cell *Helicobacter pylori* grown according to the methods of the present invention was determined using the mouse *Helicobacter felis* gastric colonization model de-
scribed by Chen et al. (Lancet, 339:1120-1121, 1992). *Helicobacter pylori* strain G1-4 was grown as a seed culture for about 22 h at 37°C under 10% CO₂, 90% air in BHI media containing 4% bovine calf serum. An aliquot of this culture was used to inoculate a 10-fold volume of the same media containing 0.1% (v/v) bovine bile. After 12-14 h of growth at 37°C, the cells are harvested by centrifugation and resuspended in 1/10 of the original volume of Hank's Balanced Salts Solution (HBSS) at room temperature. Cells were recentrifuged and again suspended in 1/100 of the original volume of HBSS. To the buffered cell suspension, formalin was added to a concentration of 0.075% and the cells inactivated by stirring the suspension at room temperature for 6 h then cooling the solution at 4°C for 18 hours.

**[0074]** Protection potential was routinely measured by administering 3 doses of this inactivated whole cell vaccine orally to 6-8 week old female Balb/c *Helicobacter*-free mice at days 0, 7 and 14 or at days 0, 7 and 21. Doses of $10^9$ bacterial particles per dose were evaluated in combination with the heat labile enteroroxin of *E. coli*. Fourteen days after the third immunizing dose, animals were challenged orally with a single dose ($10^7$ CFU/dose) of live *H. felis*.

**[0075]** Two weeks after challenge the animals were sacrificed and antral stomach segments analyzed for urease activity to determine the presence of *H. felis*. Urease activity was determined by incubating antral tissue samples in 0.5 ml Stuart's Urease Broth (Remel) at room temperature for 4-24 hours. A color change from clear to red occurring within this period was taken as a positive urease result.

**[0076]** As shown in Table 20, administration of enhanced *Helicobacter* whole cell vaccine prepared using *H. pylori* strain G1-4 protected animals against an *H. felis* oral challenge.

**Table 1.**

<table>
<thead>
<tr>
<th>Immunizing Agents</th>
<th>Challenge Organisms (10 CFU)</th>
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<td>PBS + LT</td>
<td><em>H. felis</em></td>
<td>9/9</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> b</td>
<td><em>H. felis</em></td>
<td>2/15</td>
<td>87</td>
</tr>
<tr>
<td>PBS + LT</td>
<td><em>H. felis</em></td>
<td>10/10</td>
<td>0</td>
</tr>
</tbody>
</table>

a All agents given with 10 µg LT (Labile toxin of ETEC) adjuvant as 3 oral doses at 7 day intervals

b Given as $1 \times 10^9$ CFU of Strain G1-4 in a 0.25 ml dose

**[0077]** Results of these experiments show the relevance of enhanced enteric bacterial properties to *in vivo* immunogenicity.

**[0078]** The methods of the present invention produce bacteria capable of inducing an immunogenic response which is protective and therefore are useful as vaccines.

**DEPOSIT OF MICROORGANISM**

**[0079]** The following microorganisms have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, and have the indicated accession numbers:

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Accession No.</th>
<th>Deposit Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helicobacter pylori</em> NB3-2</td>
<td>55714</td>
<td>September 29, 1995</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em> G1-4</td>
<td>55713</td>
<td>September 29, 1995</td>
</tr>
</tbody>
</table>
**MICROORGANISMS**

Optional Sheet in connection with the microorganism referred to on page 51, lines 30-38 of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet.

**Name of depositary institution**

American Type Culture Collection

**Address of depositary institution (including postal code and country)**

12301 Parklawn Drive
Rockville, MD 20852
US

**Date of deposit** September 29, 1995

**Accession Number** 55714

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable). This information is continued on a headed stamped sheet.

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE**

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below must be supported by the International Bureau later. (Specify the general nature of the indications e.g., "Accession Number of Deposit")

**E. □ This sheet was received with the International application when filed (to be checked by the receiving Office)**

(Authorized Office)

□ The date of receipt (from the applicant) by the International Bureau was

(Authorized Office)

Form PCT/WO/134 (January 1981)
Claims

1. A method of producing *Helicobacter pylori* or *Helicobacter felis* bacteria having enhanced antigenic properties which comprises growing a culture of the *Helicobacter* bacteria *in vitro* in brain heart infusion broth culture medium supplemented with bovine calf serum with a combination of conditions comprising:

   a) 0.025% to 0.2% bile or 0.05% glycocholate salt; and 
   b) at a temperature of 37°C; and 
   c) in a microaerophilic condition, wherein the microaerophilic condition comprises i) 5% to 20% CO₂ with 80% to 95% air; or ii) 5% O₂, 10% CO₂ with 85% N₂;

   for a sufficient time so that the culture is in log phase wherein the enhanced antigenic property is a higher level of an immunogenic antigen or a new immunogenic antigen when compared to the antigenic property of bacteria from a culture of the *Helicobacter* species in brain heart infusion broth supplemented with bovine calf serum.

2. The method according to claim 1, wherein the conditions comprise 0.05% glycocholate bile salt.

3. The method according to claim 3, wherein said *Helicobacter* is *Helicobacter pylori* strain, NB3-2 having ATCC Accession No. 55714 or G1-4 having ATCC Accession No. 55713.

4. A *Helicobacter pylori* or *Helicobacter felis* bacterium having enhanced antigenic properties, which is obtainable by culturing *Helicobacter* bacteria *in vitro* in brain heart infusion broth culture medium supplemented with bovine calf serum under a combination of conditions comprising:

   a) 0.025%-2% bovine bile or 0.05% glycocholate salt; and 
   b) at a temperature of 37°C; and 
   c) in a microaerophilic condition, wherein the microaerophilic condition comprises i) 5% to 20% CO₂ with 80% to 95% air; or ii) 5% O₂, 10% CO₂ with 85% N₂;

   for a sufficient time so that the culture is in log phase wherein the enhanced antigenic property is a higher level of an immunogenic antigen or a new immunogenic antigen when compared to the antigenic property of bacteria from a culture of the *Helicobacter* species in brain heart infusion broth supplemented with bovine calf serum.

5. The *Helicobacter* bacterium according to claim 4, wherein said conditions comprise 0.05% sodium glycocholate.

6. The *Helicobacter* bacterium according to claim 4, wherein said *Helicobacter* is *Helicobacter pylori* strain, NB3-2 having ATCC Accession No. 55714 or G1-4 having ATCC Accession No. 55713.
7. A vaccine comprising the Helicobacter bacterium of claim 4, and further comprising pharmaceutically acceptable carrier or diluent.

8. The vaccine according to claim 7, wherein said Helicobacter bacterium is inactivated.

9. The vaccine according to claim 8, wherein said Helicobacter bacterium is inactivated by formalin treatment.

10. The vaccine according to claim 7, wherein said vaccine is suitable for mucosal or parenteral or mucosal and parenteral administration.

11. The vaccine according to claim 7, further comprising an adjuvant.

12. A method for assaying a potential antimicrobial agent comprising, contacting the Helicobacter bacterium of claim 4 in vitro with said agent and assaying the bacteriocidal or bacteriostatic effects of said agent on the Helicobacter bacterium.

13. A method for detecting a host's antibodies to Helicobacter bacteria in an animal or biological sample therefrom, comprising the steps of a) contacting said biological sample with the Helicobacter bacterium of claim 4 in vitro and b) screening for antibody binding of the Helicobacter bacterium or fragment thereof.

14. A diagnostic immunoassay kit for detecting a host's antibodies to Helicobacter bacteria or for detecting Helicobacter bacterium, comprising the Helicobacter bacterium of claim 4.

15. A method for producing anti-Helicobacter antibodies in a non-human animal which comprises using an effective amount of an immunogen comprising the Helicobacter bacterium of claim 4, wherein the anti-Helicobacter antibodies bind said Helicobacter bacterium or a component thereof.

16. An immunogen comprising the Helicobacter bacterium of claim 4, for stimulating an immune response in an animal wherein said immunogen is to be administered to the animal and said immune response prevents, attenuates or cures Helicobacter infections or diseases in the animal.

Patentansprüche

1. Eine Methode zur Produktion der Bakterien Helicobacter pylori oder Helicobacter felis mit verstärkten Antigeneigenschaften, umfassend das Anlegen einer in vitro-Kultur der Helicobacter-Bakterien in Brain Heart Infusion Broth Kulturmehd, das mit bovinem Kälberserum aufgestockt wurde, unter einer Kombination verschiedener Bedingungen, umfassend:

   a) 0,025% bis 0,2% Galle oder 0,05% eines Glykocholats; und
   b) bei einer Temperatur von 37 °C; und
   c) unter mikroaerophilen Bedingungen, wobei mikroaerophile Bedingungen i) 5% bis 20% CO₂ mit 80% bis 95% Luft; oder ii) 5% O₂, 10% CO₂ mit 85% N₂ umfassen;

   über eine ausreichend lange Zeitperiode, so dass sich die Kultur in der log-Phase befindet, worin die verstärkte antige Eigenschaft beim Vergleich der der antigenen Eigenschaft von Bakterien einer mit bovinem Kälberserum aufgestockten Kultur der Spezies Helicobacter in Brain Heart Infusion Broth eine höhere Stufe eines immunogenen Antigens oder eines neuen immunogenen Antigens darstellt.

2. Die Methode gemäß Anspruch 1, worin die Bedingungen 0,05% Gallen-Glykocholat umfassen.

3. Die Methode gemäß Anspruch 3, worin der genannte Helicobacter der Stamm Helicobacter pylori ist und wobei NB3-2 die ATCC-Zugriffs-Nr. 55714 oder G1-4 die ATCC-Zugriffs-Nr. 55713 hat.

4. Ein Helicobacter pylori- oder Helicobacter felis-Bakterium mit verstärkten antigenen Eigenschaften, das man produzieren kann, indem in vitro Kulturen von Helicobacter-Bakterien in Brain Heart Infusion Broth Kulturmehd, das mit bovinem Kälberserum aufgestockt wurde, unter einer Kombination verschiedener Bedingungen angelegt werden, umfassend:
a) 0.025% - 2% Rindergalle oder 0.05% eines Glykocholats; und
b) bei einer Temperatur von 37 °C; und
c) unter mikroaerophilen Bedingungen, wobei mikroaerophile Bedingungen i) 5% bis 20% CO₂ mit 80% bis 95% Luft; oder ii) 5% O₂, 10 % CO₂ mit 85% N₂ umfassen;
über eine ausreichend lange Zeitperiode, so dass sich die Kultur in der log-Phase befindet, worin die verstärkte antigene Eigenschaft beim Vergleich mit der antigenen Eigenschaft von Bakterien einer mit bovinem Kälberserum aufgestockten Kultur der Spezies Helicobacter in Brain Heart Infusion Broth eine höhere Stufe eines immunogenen Antigens oder eines neuen immunogenen Antigens darstellt.

5. Das Helicobacter-Bakterium gemäß Anspruch 4, worin die genannten Bedingungen 0,05% Natriumglykocholat umfassen.

6. Das Helicobacter-Bakterium gemäß Anspruch 4, worin der genannte Helicobacter der Stamm Helicobacter pylori ist und wobei NB3-2 die ATCC-Zugriffs-Nr. 55714 oder G1-4 die ATCC-Zugriffs-Nr. 55713 hat.

7. Ein Impfstoff, umfassend das Helicobacter-Bakterium gemäß Anspruch 4 und weiterhin umfassend einen pharmazeutisch akzeptablen Trägerstoff oder Diluenten.

8. Der Impfstoff gemäß Anspruch 7, worin das genannte Helicobacter-Bakterium inaktiviert wurde.

9. Der Impfstoff gemäß Anspruch 8, worin das besagte Helicobacter-Bakterium durch Formalin-Behandlung inaktiviert wurde.

10. Der Impfstoff gemäß Anspruch 7, wobei sich der genannte Impfstoff zur mikosalen oder parenteralen Verabreichung oder zur mikosalen und parenteralen Verabreichung eignet.

11. Der Impfstoff gemäß Anspruch 7, der außerdem einen Hilfsstoff umfasst.


Revendications

1. Une méthode de production de bactéries Helicobacter pylori ou Helicobacter felis ayant des propriétés antigéniques accrues qui consiste en une culture des bactéries Helicobacter in vitro dans un milieu de culture d'infusion de coeur-cerveau enrichi de sérum de veau sous une combinaison de conditions comprenant :
a) 0.025 % à 0.2 % de bile ou 0.05 % de glycocholate ; et
5 b) à une température de 37 °C ; et
c) en atmosphère microaérophile où l’atmosphère microaréophile comprend i) 5 à 20 % de CO₂ avec 80 à 95 % d’air ; ou ii) 5 % de O₂, 10 % de CO₂ avec 85 % de N₂ ;

pendant une durée suffisante afin que la culture soit en phase de croissance exponentielle dans laquelle les propriétés antigéniques accrues sont à un niveau plus élevé d’un antigène immunogène ou d’un nouvel antigène immunogène comparé aux propriétés antigéniques des bactéries issues d’une culture de l’espèce Helicobacter dans un milieu de culture d’infusion de coeur-cervelle enrichi de sérum de veau.

2. La méthode décrite dans la revendication n° 1 où les conditions comprennent 0,05 % de sel biliaire glycocholate.

3. La méthode décrite dans la revendication n° 3 où ledit Helicobacter est de la souche Helicobacter pylori, NB3-2 ayant le numéro d’accession ATCC 55714 ou G1-4 ayant le numéro d’accession 55713.

4. Une bactérie Helicobacter pylori ou Helicobacter felis ayant des propriétés antigéniques accrues qui peut être obtenue par une culture in vitro de la bactérie Helicobacter dans un milieu de culture d’infusion de coeur-cervelle enrichi de sérum de veau sous une combinaison de conditions comprenant :

a) 0,025 % à 2 % de bile bovine ou 0,05 % de glycocholate ; et
b) à une température de 37 °C ; et
c) en atmosphère microaérophile où l’atmosphère microaréophile comprend i) 5 à 20 % de CO₂ avec 80 à 95 % d’air ; ou ii) 5 % de O₂, 10 % de CO₂ avec 85 % de N₂ ;

pendant une durée suffisante afin que la culture soit en phase de croissance exponentielle dans laquelle les propriétés antigéniques accrues sont à un niveau plus élevé d’un antigène immunogène ou d’un nouvel antigène immunogène comparé aux propriétés antigéniques de bactéries issues d’une culture de Helicobacter dans un milieu de culture d’infusion de coeur-cervelle enrichi de sérum de veau.

5. La bactérie Helicobacter selon la revendication n° 4 où les conditions comprennent 0,05 % de glycocholate de sodium.

6. La bactérie Helicobacter selon la revendication n° 4 où ledit Helicobacter est de la souche Helicobacter pylori, NB3-2 ayant le numéro d’accession ATCC 55714 ou G1-4 ayant le numéro d’accession 55713.

7. Un vaccin comprenant la bactérie Helicobacter de la revendication n° 4 et comprenant également un support ou diluant acceptable du point de vue pharmaceutique.

8. Le vaccin selon la revendication n° 7 dans lequel ladite bactérie Helicobacter est inactivée.

9. Le vaccin selon la revendication n° 8 dans lequel ladite bactérie Helicobacter est inactivée par un traitement au formaldéhyde.

10. Le vaccin selon la revendication n° 7 dans lequel ledit vaccin convient à une administration par voie parentérale ou par les muqueuses ou par les muqueuses et parentérale.

11. Le vaccin selon la revendication n° 7, comprenant de plus un adjuvant.

12. Une méthode pour analyser un agent antimicrobien potentiel comprenant, la mise en contact de la bactérie Helicobacter de la revendication n° 4 in vitro avec ledit agent et l’analyse des effets bactéricides ou bactériostatiques dudit agent sur la bactérie Helicobacter.

13. Une méthode pour déterminer les anticorps d’un hôte anti bactéries Helicobacter chez un animal ou un prélèvement biologique de celui-ci, comprenant les étapes suivantes : a) la mise en contact dudit prélèvement biologique avec la bactérie Helicobacter de la revendication n° 4 in vitro et b) la recherche de la liaison de l’anticorps à la bactérie Helicobacter ou un fragment de celle-ci.

14. Un kit de dosage immunologique diagnostic pour déterminer la production d’anticorps d’un hôte anti bactéries Helicobacter ou pour déterminer les bactéries Helicobacter, comportant la bactérie Helicobacter de la revendication n° 4.
15. Une méthode de production d'anticorps anti-*Helicobacter* chez un animal non humain qui comprend l'utilisation d'une quantité efficace d'un immunogène comportant la bactérie *Helicobacter* de la revendication n° 4, dans lequel les anticorps anti-*Helicobacter* se fixent sur la bactérie *Helicobacter* ou sur un fragment de celle-ci.

16. Un immunogène comprenant la bactérie *Helicobacter* de la revendication n° 4, pour stimuler une réponse immunitaire chez un animal où ledit immunogène doit être administré à l'animal et ladite réponse immunitaire prévient, atténue ou guérit les infections à *Helicobacter* ou les maladies chez l'animal.