Title: ACID-TOLERANT SALMONELLA TYPHI, DOUBLE ARO MUTANTS THEREOF AND USE AS AN ORAL VACCINE FOR TYPHOID FEVER

Abstract

Acid-tolerant Salmonella typhi and double aro mutants of Salmonella typhi are disclosed which are useful for preparing a single dose oral vaccine against typhoid fever and as a carrier of genes which express protective antigens cloned from other pathogens. Also disclosed is a method of enrichment of acid-tolerant Salmonella typhi.
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ACID-TOLERANT SALMONELLA TYPHI, DOUBLE ARO MUTANTS THEREOF AND USE AS AN ORAL VACCINE FOR TYPHOID FEVER

The development of this invention was supported by the University of Maryland, Baltimore, Maryland.

FIELD OF THE INVENTION

The present invention relates to acid-tolerant Salmonella typhi and to double aro mutants of Salmonella typhi which are useful for preparing a single dose oral vaccine against typhoid fever and as a carrier of genes which express protective antigens cloned from other pathogens. The present invention also relates to a method of enrichment of acid-tolerant Salmonella typhi.

BACKGROUND OF THE INVENTION

I. Salmonella typhi

Salmonella typhi, the causative agent of typhoid fever, is a highly host-adapted microorganism which is only fully pathogenic for humans. Typhoid fever is a disease of the reticuloendothelial system. The clinical manifestations of acute typhoid fever usually begin with abdominal discomfort, malaise, fever and headache that increase in a step-wise fashion. Bronchitic cough, nausea, abdominal pains, splenomegaly, rose spots (rare) and leukopenia are also associated with clinical typhoid fever although the frequencies of these signs and symptoms vary. Without early antibiotic intervention, S. typhi infection of humans can lead to intestinal hemorrhage or to the development of a perforated small intestine, which results in life threatening peritonitis. The antibiotics of choice for the treatment of typhoid
fever are amoxicillin, trimethoprim/sulphamethoxazole, chloramphenicol, or ciprofloxacin.

II. The Pathogenesis of Salmonella typhi

Knowledge of the steps involved in the pathogenesis of typhoid fever comes from the following sources (Levine, M., Microbiol. Rev., 47:510-550 (1983)):


2. a chimpanzee model of S. typhi infection used in the 1950s and early 1960s at the Walter Reed Army Institute of Research (Gaines et al, J. Infect. Dis., 118:293-306 (1968));


III. Dose Response Studies in Volunteers

In the late 1950s and 1960s, careful dose-response studies in volunteers were carried out with the following three wild-type S. typhi strains:
(1) Quailes (isolated from a chronic carrier in Maryland who was responsible for a family outbreak);

(2) Zermatt (the strain responsible for a large epidemic in that Swiss ski resort town); and

(3) Ty2 (isolated in 1915).

These three wild-type strains, all of which had been maintained in the laboratory for years, were fed to volunteers in a dose of $10^7$ viable organisms in 45 ml of milk. No bicarbonate or other buffer was given to neutralize the gastric acid in the stomachs of the volunteers (Hornick et al, N. Engl. J. Med., 283:686-691 (1970)). With each strain there was a high rate of infection and of clinical illness. Quailes strain caused typhoid fever in 53% of the volunteers, Zermatt strain caused typhoid fever in 55% of the volunteers and Ty2 caused typhoid fever in 33% of the volunteers. In these studies, typhoid fever was defined as clinical illness manifested by a temperature of 103°F for at least 36 hours. Many other volunteers in these studies had milder illness accompanied by positive cultures. This was considered typhoid infection but not typhoid fever.

Extensive dose-response experiments in volunteers have been carried out with the Quailes strain (Hornick et al, N. Engl. J. Med., 283:686-691 (1970)). In these studies, the S. typhi organisms were also fed in 45 ml of milk without bicarbonate or other buffer. Using the same stringent definition of typhoid fever as the studies discussed above, the Quailes strain caused typhoid fever in 28% of 116 volunteers who ingested $10^7$ viable organisms, 50% of 32 volunteers who ingested $10^7$ viable organisms, 89% of 9 volunteers who ingested $10^8$ viable organisms, and 95% of 40 volunteers who ingested $10^9$ viable organisms. In subsequent
challenge studies carried out in the early 1970s to assess the efficacy of vaccine candidates (Levine et al, J. Infect. Dis., 133:424-429 (1976); and Gilman, J. Infect. Dis., 136:717-723 (1977)) the recorded attack rate of typhoid fever was approximately 45-55% in non-immunized volunteers challenged with $10^5$ viable Quailes strain organisms. Thus, even when given without buffer, a dose of $10^5$ or $10^7$ viable organisms of a wild-type S. typhi strain causes full-blown typhoid fever in a sizable proportion of individuals.

As discussed in more detail below, it has been found in the present invention that the Quailes strain is sensitive to the effects of low pH. Hence, it is quite probable that the pH sensitive characteristic of the Quailes strain might have reduced its capacity to cause disease because of reduced survival through the gastric environment of the stomach and reduced survival in the acidified phagolysosome.

IV. The Host-S. typhi Relationship During Typhoid Fever

As discussed above S. typhi, unlike Salmonella typhimurium which has a much broader host range (fully pathogenic for mice, guinea, pigs, chickens, calves, pigs, and rabbits), is virulent only for humans (Carter et al, Infect. Immun., 10:816-822 (1984)). Following ingestion of virulent S. typhi by the human host, the bacilli reach the small intestine where they invade through the epithelial surface (Takeuchi, Curr. Top. Pathol., 54:1-27 (1971)). The typhoid bacilli then enter the lymph circulation, spill into the blood circulation via the thoracic duct and cause a silent primary bacteremia (Gaines et al, J. Infect. Dis., 118:293-306 (1968)). The uptake of the typhoid bacilli by fixed macrophages of the
reticuloendothelial system during the primary bacteremia leads to dissemination within the liver, spleen, bone marrow and lymph nodes (Gaines et al, J. Infect. Dis., 118:293-306 (1968)). During the incubation period (typically 8-12 days), the typhoid bacilli are localized in phagosomes within the macrophages where they remain viable and slowly proliferate. At the end of the incubation, the bacteria emerge from the macrophages of the reticuloendothelial system and give rise to a secondary bacteremia that is accompanied by the onset of clinical symptoms (Hornick et al, N. Engl. J. Med., 283:686-691 (1970)).

V. Live Oral Typhoid Fever Vaccines

The development of live oral typhoid fever vaccines have been highly desired in the art. Typhoid fever currently results in 500,000 deaths each year and causes a great deal more morbidity (Institute of Medicine: New Vaccine Development. Establishing Priorities, Vol II, Appendix D-14, pp. 1-10 (1986)). The World Health Organization has targeted the improvement of vaccines against typhoid fever as a priority (World Health Organization Publication WHO/CDD/RES86.8 rev.1 (1987)). A single dose live oral vaccine, would potentially provide a suitable prophylactic tool for control of typhoid fever in endemic areas.

It has been proposed, therefore, that a single dose, highly immunogenic live oral typhoid fever vaccine would also be highly suited as a carrier of genes expressing protective antigens cloned from other pathogens (Formal et al, *Infect. Immun.*, 34:746-750 (1981); Forrest et al, *J. Infect. Dis.*, 159:145-146 (1989); Clements et al, *Infect. Immun.*, 46:564-569 (1984); and Curtiss et al, *Vaccine*, 6:155-160 (1988)). Such hybrid live oral vaccine strains expressing foreign protective antigens would be expected to elicit immune responses against the cloned foreign antigens thereby providing protection against the pathogens from which the foreign antigens originated. Hence, a live oral typhoid fever vaccine carrying foreign antigens has the potential of very broad applications.

As discussed in more detail below, prior to the present invention, a single dose live oral typhoid fever vaccine that is both safe and can elicit effective humoral immune responses as well as effective cell-mediated immune responses has not been available. As a consequence, there has been much focus on the development of such a single dose live oral typhoid fever vaccine.

### A. Inactivated Typhoid Fever Vaccines

Whole cell inactivated *S. typhi* bacteria have been used for decades as parenteral vaccines (Levine et al, *Rev. Infect. Dis.* 11(supplement 3):S552-S567 (1989)). Parenteral inactivated typhoid fever vaccines have been found to be significantly protective in controlled field studies. While parenteral typhoid fever vaccines confer a moderate level of protection upon vaccinated children and adults in endemic areas (Levine et al, *Rev. Infect.*
Dis., 11(supplement 3): S552-S567 (1989)), they cause marked systemic and local adverse reactions at such a high frequency that they are not practical public health tools (Ashcroft et al, Am. J. Hyg., 79:196-206 (1964); Yugoslav Typhoid Commission Bull. Wld. Hlth. Org., 30:623-630 (1964); and Hejlec et al, Bull. Wld. Hlth. Org., 34:321-339 (1966)). In several controlled field trials sponsored by the World Health Organization in the 1960s, the inactivated parenteral typhoid fever vaccines were found to cause fever in approximately 25% of the volunteers (of whom about 15% had to be absent from school or work). Local adverse reactions were observed in approximately 50% of the volunteers (Ashcroft et al, Am. J. Hyg., 79:196-206 (1964); Yugoslav Typhoid Commission Bull. Wld. Hlth. Org., 30:623-630 (1964); and Hejlec et al, Bull. Wld. Hlth. Org., 34:321-339 (1966)). In experimental challenge studies in volunteers it was found that the protective effect of inactivated parenteral typhoid fever vaccines could be overwhelmed by increasing the challenge inoculum (Hornick et al, N. Engl. J. Med., 283:686-691 (1970)).

B. Live Oral Typhoid Fever Vaccines

Live oral typhoid fever vaccines provide higher levels of protection against challenge with wild-type S. typhi than those achieved by parenteral inactivated vaccines if both humoral as well as effective cell-mediated immune responses are elicited during vaccination. In order for S. typhi to stimulate effective cell-mediated immunity, the typhoid bacilli must grow and survive for extended periods within macrophages, whereupon S. typhi antigens are processed and expressed on the surface of the macrophage in association with major histocompatibility antigens
HLA-A, B, and C (MHC I) or HLA-DP, DQ, and DR (MHC II). The modified structure of the processed antigen in association with MHC I stimulates CD8+ T-lymphocytes. Further, the modified structure of the processed antigen in association with MHC II stimulates CD4+ T-lymphocytes. Such stimulated T-lymphocytes become activated and it is these activated T-lymphocytes that effect the cell-mediated immune response.

Live oral vaccines are at present the only means known by which effective numbers of activated T-lymphocytes, and hence effective cell-mediated immunity, can be elicited. Live oral typhoid fever vaccines, if fully attenuated, will have the added advantage of being much less reactogenic than the parenteral typhoid fever vaccines tested by the World Health Organization in the 1960s.

1. Early Live Oral Typhoid Fever Vaccines

Rechallenge of volunteers previously infected with wild-type S. typhi has shown that S. typhi infection only provided 22% protection. Notwithstanding the poor immunogenicity of wild-type S. typhi, there has been much progress in the use of attenuated strains of S. typhi as live oral vaccines to prevent typhoid fever. Attenuated S. typhi induce better protective immunity than wild-type S. typhi. However, each of the candidate vaccines so far tested in humans has exhibited one or more serious deficiencies that make it suboptimal.

The candidate live oral typhoid fever vaccine strains which have heretofore been tested in clinical studies in man include the:
(i) streptomycin-dependent \textit{S. typhi}, strain 27V (Levine et al, \textit{J. Infect. Dis.}, 133:424-429 (1976));


(iii) recombinant \textit{Vi} mutant of Ty21a with restored expression of the \textit{Vi} antigen, strain Ty21a-\textit{Vi} \textsuperscript{+} (Cryz et al, \textit{Infect. Immun.}, 57:3863-3868 (1990));

(iv) recombinant \textit{galE} \textit{Vi} \textsuperscript{−} mutant of Ty2, strain EX462 (Hone et al, \textit{Infect. Immun.}, 56:1326-1333 (1988)); and

(v) auxotrophic mutants of CDC10-80, strains 541Ty and 543Ty (Levine et al, \textit{J. Clin. Invest.}, 79:888-902 (1987)).

As discussed in more detail below, the above listed strains are disadvantageous in that they possess one or more of the following: insufficient attenuation, poor immunogenicity, the requirement to be administered in multiple doses to achieve serological responses and protection, poor yield after lyophilization, loss of potency following lyophilization, and a lack of precise knowledge of the genetic alterations responsible for attenuation.
a. Streptomycin-Dependent Strain

The streptomycin-dependent *S. typhi* vaccine strain 27V is well-tolerated when given in doses of greater than $10^{10}$ organisms with bicarbonate buffer. However, administration of multiple doses is required to stimulate protective immunity (Levine et al, *J. Infect. Dis.*, 133:424-429 (1976)). More importantly, while this vaccine strain is capable of eliciting protection when administered as multiple doses of freshly-harvested organisms, following lyophilization (freeze drying), which is necessary to prepare a practical formulation, the vaccine loses its potency (Levine et al, *J. Infect. Dis.*, 133:424-429 (1976)).

b. *galE* Vi- Mutant of Ty2

buffer-containing liquid suspension of Ty21a organisms provides the highest level of protection; lyophilized vaccine in enteric-coated capsules provides an intermediate level of protection; and gelatin-coated capsules provide poor protection. A comparison of two, three and four doses of Ty21a vaccine in enteric-coated capsules given within one week showed that the best protection occurred with four doses (Ferreccio et al, J. Infect. Dis., 159:766-769 (1989)).

Thus, the drawbacks of Ty21a include the necessity to administer multiple doses to achieve a moderate level of protection, a high loss of viability following lyophilization and a lack of precise knowledge about the mutations responsible for attenuation.

c. Recombinant Vi⁺ Mutant of Ty21a

Strain Ty21a has been modified by recombinant DNA techniques so as to restore the Vi polysaccharide antigen, giving rise to strain Ty21a-Vi⁺ (Cryz et al, Infect. Immun., 57:3863-3868 (1990)). While strain Ty21a-Vi⁺ remained well-tolerated, this strain is not highly immunogenic after ingestion of a single dose or three doses given every other day.

d. Recombinant galE Vi⁻ Mutant of Ty2

Strain EX462, a mutant of pathogenic strain Ty2 that, like Ty21a, harbors a deletion in the galE gene and is Vi⁻, has been developed (Hone et al, Infect. Immun., 56:1326-1333 (1988)). However, when strain EX462 was fed to 4 adult volunteers in a dose of $5 \times 10^8$ organisms, 2 of the 4 volunteers developed full-blown typhoid fever (Hone et al, Infect. Immun.,
56:1326-1333 (1988)). These results demonstrate that the mutation in the \textit{gale} gene and the lack of Vi polysaccharide, by themselves, are not responsible for the extraordinary safety of Ty21a. Thus, the attenuation of Ty21a is not solely dependent on the \textit{gale} gene and lack of Vi, i.e., other mutations caused by the non-specific treatment with NG contribute to the safety of Ty21a.

The decision to construct a \textit{gale} mutant of \textit{S. typhi} was made after a \textit{gale} mutant of \textit{S. typhimurium}, prepared by recombinant DNA techniques, proved to adequately attenuate \textit{S. typhimurium} for mice, even when doses as high as $5 \times 10^5$ were given orally (Hone et al, \textit{J. Infect. Dis.}, 156:167-174 (1987)). Taken together, these results demonstrate that mouse studies with mutants of \textit{S. typhimurium} are not reasonably predictive of how analogous mutants of \textit{S. typhi} will behave in humans. In addition, this demonstrates the fundamental differences between \textit{S. typhi} pathogenesis and \textit{S. typhimurium} pathogenesis.

e. \textbf{Auxotrophic mutants of CDC10-80}

The potential that \textit{in vivo} transfer of DNA might lead to a reversion of a mutant candidate live oral typhoid fever vaccine has led to the belief that inclusion of two mutations well-separated on the chromosome is advantageous (Stocker, \textit{In: Development of Vaccines and Drugs Against Diarrhea}, Holmgren et al, eds., Studentlitteratur, Lund, Sweden (1986); Edwards et al, \textit{J. Bacteriol.}, 170:3991-3995 (1989); and Levine et al, \textit{J. Clin. Invest.}, 79:888-902 (1987)). Reversion to wild-type would require transfer of large segments of DNA (Sanderson et al, \textit{In: Escherichia coli and Salmonella typhimurium}...
2:877-918 (1984)). Such an event is thought to be highly unlikely.


Strain 543Ty is a variant of 541Ty which lacks the Vi polysaccharide antigen that usually covers *S. typhi*.

Strains 541Ty and 543Ty are well-tolerated when ingested by North American adults in doses as high as $10^{10}$ viable organisms with bicarbonate buffer, but fail to stimulate serological responses in 90% of the volunteers (Levine et al, *J. Clin. Invest.*, 79:888-902 (1987)). These two vaccine strains are considered to be hyperattenuated due to the minimal rate of seroconversion of O-antibody (10%), since seroconversion correlates with protection in Ty21a immunized individuals. In the event that attenuated *S. typhi* is exploited as a carrier to generate serum and mucosal antibody specific for cloned antigens, it is important that the carrier itself elicits serum and mucosal antibody responses.

Another drawback of these two vaccine strains is the method by which they were constructed (U.S. Patent 4,735,801; and Edwards et al, *J. Bacteriol.*, 170:3991-3995 (1989)). "Classical" genetic methods, i.e., non-recombinant, were used to construct strains 541Ty and 543Ty. More specifically, phage P22 was used to transduce *aroA*DEL407, *hisG46* and *delpurA155* mutations from *S. typhimurium* mutant strains to *S. typhi*. The drawbacks to this methodology are that the *aroA* and *purA* deletions are not well defined and could extend beyond the limits of the mutated allele,
thus altering flanking sequences. Because of the potential changes to flanking sequences, one cannot be sure if immunologically important alleles have not been lost. In addition, phage P22 usually transfers about 40,000 base pairs, i.e., 1% of the *Salmonella* chromosome, with each transduction event. Depending on the recombination event that ensues, small amounts, or even all, of this introduced DNA will become part of the chromosome of the *S. typhi* recipient. This could lead to many silent changes to the *S. typhi* chromosome that adversely affect vaccine potential.

The silent transposon insertion zih-908: Tn10 has also been used to introduce delpurA155 into *S. typhi* (U.S. Patent 4,735,801; and Edwards et al, *J. Bacteriol.*, 170:3991-3995 (1989)). Selection for tetracycline-sensitive derivatives using fusaric acid-resistance, removed the Tn10 insertion. Unfortunately, spontaneous excision of Tn10 in the majority of cases can lead to inversion and/or deletion of adjacent DNA (Ross et al, *Cell*, 16:721-731 (1979); and Kleckner, In: *Mobile DNA*, Berg et al, eds., American Society for Microbiology, Washington, D.C., pp. 227-268 (1989)). Thus, strains made in this fashion might carry an undefined inversion and/or deletion at the point where Tn10 excision occurred.

It is clear, therefore, that the methods used to construct 541Ty and 543Ty were far from optimal since multiple undefined changes might have been introduced in addition to the desired changes.

These two strains did, however, establish that some specific auxotrophic mutants of *S. typhi* are attenuated in humans and provided evidence that double mutants are stable in human volunteers. This has led to the preference, by regulatory authorities, for
double mutants over single mutants as live oral typhoid fever vaccines for humans.

2. Recent Live Oral Typhoid Fever Vaccines
   a. Block in Chorismate Synthesis is Pleiotrophic

Chorismic acid is an important biochemical precursor of the aromatic amino acids tryptophan, tyrosine and phenylalanine, ubiquinone and menaquinone, and the vitamins p-aminobenzoic acid (hereinafter "PABA") and 2,3-dihydroxybenzoic acid (hereinafter "DHB") (Pittard, In: Escherichia coli and Salmonella typhimurium, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984)).

Ubiquinone and menaquinone are required for H₂S production and oxidative phosphorylation (Poole et al, In: Escherichia coli and Salmonella typhimurium, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984)).


DHB is utilized for the synthesis of enterochelin (enterobactin), an iron chelator produced by

b. Mammalian Cells Do Not Contain Sufficient PABA and DHB to Allow for the Growth of Aro Mutants


c. Strains Bearing Aro Mutations Are Safe And Immunogenic


Studies in calves that were orally administered approximately 10^10 organisms of a S. typhimurium aroA mutant, showed that the mean temperature of the vaccinated calves increased from 39.1°C to 39.9°C for approximately one to three days after vaccination

Strains of S. typhi that carry the aroA mutation also have been shown to lose their virulence in mice (Dougan et al, Mol. Gen. Genet., 207:403-405 (1987)). Even though aroA mutants of Salmonella are highly attenuated for mice, immunization with these mutant strains affords high levels of protection (Hoiseth et al, Nature, 291:238-239 (1981); Smith et al, Am. J. Vet. Res., 45:1858-1861 (1984); and O'Callaghan et al, Infect. Immun., 56:419-423 (1988)).

d. Multiple Aro Mutations Produce the Same Effect

Because the products of the aro genes, which are well separated on the Salmonella chromosome, are the only means by which strains of Salmonella can assimilate chorismic acid, loss of any one of these genes produces the same effect (Davis et al, Arch. Exp. Pathol. Pharmacol., 220:S.1-S.15 (1954); Davis et al, J. Bacteriol., 64:729-748 (1953); Weiss et al, J. Am. Chem. Soc., 78:2894-2898 (1955); Pittard, In: Escherichia coli and Salmonella typhimurium, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984); and Dougan et al, Mol. Gen. Genet., 207:403-405 (1987)). The exceptions are the aroF, aroG and aroH genes, which encode isoenzymic forms of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, which catalyze the first step of chorismate
synthesis, i.e., conversion of phosphoenol pyruvate and erythrose 4-phosphate to 3-deoxy-D-arabino-heptulosonate 7-phosphate; and the aroL gene and an unidentified gene that encode Shikimate kinase II and Shikimate kinase I, respectively, which catalyze the fifth step of chorismate synthesis, i.e., conversion of shikimate to shikimate 3-phosphate (Pittard, In: Escherichia coli and Salmonella typhimurium, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984)).

e. Development of Double Aro Mutants of \textit{S. typhi}

Double \textit{aro} mutations are described in U.S. Patent 4,735,801. The future development of double \textit{aro} mutants of \textit{S. typhi}, particularly \textit{aroA aroD} double mutants, is the direction of choice (Stocker, \textit{Vaccine}, 6:141-145 (1988)) since the chance of \textit{in vivo} reversion is negligible.

A method has been developed for the construction of strains of \textit{Salmonella} harboring two independent \textit{aro} mutations in different \textit{aro} genes, in particular, construction of \textit{aroA} and \textit{aroC} double mutants of \textit{Salmonella} (Dougan et al, \textit{Mol. Gen. Genet.}, 207:403-405 (1987); and Dougan et al, \textit{J. Infect. Dis.}, 158:1329-1335 (1988)). The method involved the introduction of a the first deletion using P22-mediated transduction of \textit{aroA::Tn10} into the target \textit{Salmonella} strain and selection for the loss of \textit{Tn10} in the \textit{aro} mutant by imprecise excision of the transposon resulting in tetracycline-sensitivity and aromatic auxotrophy. Then, a modified \textit{aroC} gene, having inserted therein a kanamycin-resistance encoding gene, was used as the means for introduction of the second deletion. Hence, kanamycin-resistance
was used as positive selection for the introduction of
the second mutant allele. Mercury-resistance could be
used in place of kanamycin-resistance for the
development of human vaccine, since
kanamycin-resistance is an unacceptable property for
a live oral typhoid fever vaccine (Dougan et al, J.
Infect. Dis., 158:1329-1335 (1988)).

European Patent Publication 322237 describes a
similar method for the construction of double aro
mutants. The method thereof relies on the use of
imprecise excision for the removal of
antibiotic-resistance markers during the construction
of the mutants. Specific examples of double aro
mutants described therein are S. typhi aroA aroE and
S. typhi aroA aroC double mutants.

3. Summary of Live Oral Typhoid
Fever Vaccine Development

To summarize, the common theme seen in the
development of live oral typhoid fever vaccines in
past is the desire to inactivate genes in order to
safely attenuate S. typhi without overly affecting
immunogenicity. A variety of techniques have been
used to create mutants that display the desired
deficiency. These prototype vaccines have resulted in
the belief that the attenuating mutations introduced
into a candidate live oral typhoid fever vaccine must
be defined in molecular terms, be non-reverting, and
be located in at least two well-separated genes for
added safety.

However, no specific attention has been given to
the wild-type parent S. typhi strain from which the
attenuated mutants have been derived. Thus,
heretofore no attempt has been made to enhance any
phenotypic feature of wild-type S. typhi that might be
beneficial in vaccine strains subsequently derived from such enhanced wild-type S. typhi strains. The present invention seeks, inter alia, to address these issues.

C. The Gastric Acid Barrier as a Defense Against Bacterial Enteropathogens

The attack rate of clinical illness that follows ingestion of bacterial enteropathogens and the severity of clinical illness is a function of the inoculum size, i.e., the number of viable organisms ingested (Hornick et al, N. Engl. J. Med., 283:686-691 (1970); and Levine et al, In: Acute Enteric Infections in Children: New Prospects for Treatment and Prevention, Holme et al, eds., Amsterdam, Elsevier/North Holland, pp. 443-459 (1981)). The critical factor is the number of pathogenic bacteria that survive transit through the gastric acid barrier to reach the small intestine. That is, when fasting volunteers ingested 10⁶ V. cholerae O1 El Tor Inaba with bicarbonate buffer or with a meal that included a glass of milk, the attack rate for diarrhea was > 90%. 100% of these volunteers had positive cultures. This demonstrated intestinal colonization. In contrast, when fasting volunteers ingested 10⁶ V. cholerae O1 El Tor Inaba with plain water, none of the volunteers became colonized or had diarrhea. This demonstrates the effectiveness of the gastric acid barrier as a natural non-specific defense against bacterial infection.
D. The Modulating Effect of the Gastric Acid Barrier on the Immunogenicity and Protective Efficacy of Live Oral Bacterial Vaccines

The gastric acid barrier that represents a potent non-specific defense against bacterial enteropathogens also plays an important role in determining the immunogenicity and protective efficacy of live oral enteric vaccines. The first demonstration of the critical role of the gastric acid barrier and the attention that must be paid to it was obtained with attenuated Shigella vaccines. The evidence can be summarized as follows:

1. buffering the contents of the stomach in fasting volunteers with 2.0 grams of bicarbonate buffer several minutes before ingesting live oral Shigella vaccine significantly increased the rate of recovery of vaccine organisms from coprocultures of volunteers and results in significantly greater serological responses (DuPont et al, J. Infect. Dis., 125:5-11 (1972));

2. buffering the gastric contents of volunteers with either 2.0 grams of bicarbonate buffer or with 240 ml of milk containing 0.8 grams of bicarbonate buffer significantly enhanced the survival of attenuated Shigella vaccine organisms through the stomach and into the intestine, as determined by recovery of vaccine organisms on coprocultures (Levine et al, Am. J. Epidemiol., 96:40-49 (1972); and

3. attenuated Shigella live oral vaccines are highly efficacious when children ingested the vaccine with bicarbonate buffer to ensure survival through the gastric acid

E. The Effect of Formulation on the Immunogenicity and Protective Efficacy of Attenuated *S. typhi* Oral Vaccines

Galenic formation plays a critical role of the vaccine in determining immunogenicity and efficacy. Different formulations relate to strategies by which vaccine organisms, following ingestion, are transported through the stomach to reach the small intestine in a viable state.

typhoid fever in a field trial in Santiago, Chile (Levine et al, Lancet, 1:1049-1052 (1987)).

In order to explore the effect of additional doses of attenuated Ty21a vaccine to school children within an eight day period, children in Chile were randomized to receive two, three or four doses of Ty21a vaccine in enteric-coated capsules given within a period of eight days (Ferreccio et al, J. Infect. Dis., 159:766-769 (1989)). The incidence rate was lowest in the group that received four doses of vaccine. Since the doses were spaced only two days apart, the increased protection conferred by the four doses cannot be due to a booster effect but, rather, must be due to delivering more viable attenuated bacteria to the small intestine from whence, upon release of the capsules, the bacteria can translocate to the lamina propria and mesenteric lymph nodes as a prerequisite to further spread within the reticuloendothelial system. The requirement for multiple doses of Ty21a is reflected in the discovery in the present invention of the sensitivity of Ty21a to low pH. Thus, to overcome this sensitivity, large numbers of organisms must be given in multiple doses to achieve an effective immune response.

Lastly, the third year of surveillance is now coming to an end in another field trial of Ty21a in Santiago, Chile in which the efficacy of a buffer-containing liquid (lyophilate rehydrated in the field) has been compared to an enteric-coated capsule formulation. Chilean school children vaccinated with three doses of the buffer-containing liquid formulation had a significantly lower incidence rate of typhoid fever than children who ingested three doses of vaccine in enteric-coated capsules. This emphasizes, once again, the fact that the gastric acid.
barrier plays a critical role in determining the effective immunizing dose of strain Ty21a.

F. Summary of Vaccine Organisms During Passage through the Stomach

In summary, the above observations highlight the importance of assuring that, after oral immunization, live vaccine organisms survive transit through the hostile gastric environment to reach the small intestine in a viable state. Failure to optimize this facet of oral immunization has practical consequences in that larger doses of vaccine or more doses of vaccine are required to obtain a desired level of protective efficacy. While this important aspect of oral immunization with attenuated bacteria has received attention in recent years, the modifications to protect the vaccine organisms from the ravages of gastric acid and proteolytic enzymes heretofore, have been entirely based on modifying the formulations of vaccine and have included the use of different buffers (Levine et al, *Lancet*, 1:1049-1052 (1987); DuPont et al, *J. Infect. Dis.*, 125:5-11 (1972); Levine, *Am. J. Epidemiol.*, 96:40-49 (1972); and Mel et al, *Bull. Wid. Hlth. Org.*, 45:457-464 (1971)) or the enteric-coating of capsules containing the vaccine (Levine et al, *Lancet*, 1:1049-1052 (1987)).

G. Intracellular Survival of Salmonella in Macrophages

Survival and growth intracellularly following ingestion by macrophages, including fixed phagocytes of the reticuloendothelial system, is also considered a desirable feature of vaccine strains.

The macrophage is a professional phagocytic cell that takes up antigens, including microorganisms, for
their removal from the host (Metchnikoff, In: Lectures in Comparative Pathology of Inflammation, Kegan, Paul, Trench, Truber and Co., London (1893); and Moulder, Microbiol. Rev., 49:298-337 (1985)). The phagocytic process involves the binding of antigen to receptors on the macrophage surface, either to the C3 receptor or to the Fc receptor, which causes the membrane to invaginate and engulf the antigen into what is called the phagosome. The phagosome is internalized into the cytoplasm of the macrophage where, under normal circumstances, it becomes fused to the lysosomes. After fusion, the lysozymes release their antigen-inactivating contents into the phagosome (Klebanoff, In: Inflation: Basic Principals and Clinical Correlates, Gallin et al, eds., Raven, New York (1988); and Elsbach et al, In: Inflation: Basic Principals and Clinical Correlates, Gallin et al, eds., Raven, New York (1988)).

"Rough" strains of S. typhimurium (Holme et al, J. Gen. Microbiol., 52:45-54 (1968)), i.e., those which lack complete lipopolysaccharide and which do not stimulate protective cell-mediated immunity, are rapidly killed by macrophages. Rapid killing of such "rough" strains by macrophages is thought to result in hyperattenuation of these strains (Germanier, Infect. Immun., 2:309-315 (1970)).

S. typhi are representative of a group of bacteria that survive for extended periods within macrophages and elicit cell-mediated immunity (Olitzki et al, Path. Microbiol. (Basel), 27:175-201 (1964); and Levine et al, J. Clin. Invest., 79:888-902 (1987)). Other bacteria of this type include non-typhoidal Salmonella spp, Mycobacteria spp, Listeria monocytogenes, Francisella spp, Legionella

Many mechanisms have evolved to provide pathogenic microorganism with a means to survive within the macrophage (Moulder, \textit{Microbiol. Rev.}, 49:298-337 (1985)). \textit{Rickettsia} (a genus obligate intracellular bacteria) escape the phagosome and grow in the cytoplasm of the macrophage (Moulder, \textit{Microbiol. Rev.}, 49:298-337 (1985)). As a result, \textit{Rickettsia} avoids the killing mechanisms of the lysosomal contents. Other intracellular bacteria, such as \textit{M. tuberculosis}, avoid macrophage killing by preventing lysosome-phagosome fusion (Moulder, \textit{Microbiol. Rev.}, 49:298-337 (1985)). \textit{S. typhi} does not employ either of these two methods, but instead is able to survive the killing effect of lysosome-derived cationic peptides, called defensins, \(H_2O_2\) and oxygen radicals (also released by the lysosome into the phagosome), and acidification of the phagosome by a yet to be elucidated mechanism (Olitzki et al, \textit{Path. Microbiol.} (Basel), 27:175-201 (1964)).

H. \textbf{The \textit{S. typhimurium} Mouse Typhoid Model}

One class of mutants of \textit{S. typhimurium} that display reduced survival in macrophages was found to be more sensitive to polymorphonuclear cell-derived defensins (Fields et al, \textit{Science}, 243:1059-1062 (1989)). The mutations mapped in the \textit{phoP} gene. The \textit{phoP} gene, which along with the \textit{phoQ} gene, has been shown to be a regulator of several genes, including transcriptional activation of the \textit{pagA}, \textit{pagB} and \textit{pagC} genes, and repression of another unidentified group of genes, called the \textit{prg} genes (Miller et al, \textit{J. Bacteriol.}, 172:2485-2490 (1990)). The \textit{phoP/phoQ}
activated genes are thought to provide _S. typhimurium_ with resistance to macrophage-derived defensins (Groisman et al, _Proc. Natl. Acad. Sci. USA_, 86:7077-7081 (1989); Miller et al, _Proc. Natl. Acad. Sci. USA_, 86:5054-5058 (1989)). The fact that _phoP_ and _phoQ_ mutants were also found to be attenuated reflects the importance of the resistance to macrophage killing in the pathogenesis of _S. typhimurium_ (Miller et al, _Proc. Natl. Acad. Sci. USA_, 86:5054-5058 (1989)).

The killing activity of phagocyte-derived defensins is seen only in neutral to slightly alkaline conditions. Hence, the killing role of the defensins is probably most important immediately after phagocytosis, i.e., before the phagosome becomes acidified (Lehrer et al, _Infect. Immun._, 42:10-14 (1983)). This might indicate that the _phoP_, _phoQ_ regulon activates the _pag_ genes early in the phagocytosis process but, then, represses these genes once _S. typhimurium_ becomes internalized within the macrophage. This postulation is supported by the more recent observation showing that when a chemically-induced _phoP_ constitutive mutation (phoP<sup>c</sup>) (Kier et al, _J. Bacteriol._, 130:420-428 (1977)), is introduced into a mouse-virulent strain of _S. typhimurium_ by P22-mediated transduction (Miller et al, _J. Bacteriol._, 172:2485-2490 (1990)), the resulting strain becomes as attenuated as _phoP_/ _phoQ_ mutants, but remains resistant to defensins (Miller et al, _J. Bacteriol._, 172:2485-2490 (1990)). The _phoP<sup>c</sup>_ strain was also found to be highly immunogenic when given intraperitoneally, even at doses as low as 15 viable organisms. However, the _phoP<sup>c</sup>_ mutation remains genetically undefined, other than it maps at the _phoP_/ _phoQ_ locus. Also, the _phoP<sup>c</sup>_ mutation was found to
revert at a significant frequency (Miller et al, J. Bacteriol., 172:2485-2490 (1990)). Thus, it is unclear why such a mutant strain is attenuated yet immunogenic.

Resistance to oxidative stress can be induced by exposure of *S. typhimurium* to low levels of H₂O₂. This response is under the regulation of oxyR (Christman et al, Cell, 41:753-762 (1985)). An adaptive response like this one might provide *S. typhimurium* with an additional mechanism to escape the killing caused by the oxidative burst within the macrophage. Several proteins that are induced as a consequence of the H₂O₂ adaptive response have been identified.

Another adaptive response that is well described in *S. typhimurium* is the heat shock response (Neidhardt et al, In: *Escherichia coli and Salmonella typhimurium*, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984)). When *S. typhimurium* are subjected to heat shock stress, e.g., growth at 37°C to 42°C, the regulation of many of the cell's protein and carbohydrate synthesis pathways becomes altered. In particular, a class of proteins called the heat shock proteins are induced during such a response.

*Mycobacterium leprae* and *Mycobacterium tuberculosis* also have been shown to express heat shock response proteins, particularly a 65kD protein which is homologous to the *E. coli* heat shock protein, GroEL, and is the immunodominant antigen of these two pathogens. The expression of the 65kD GroEL-like protein of *M. leprae* and *M. tuberculosis* in the macrophage is thought to induce a specific cell-mediated immune response and lead to macrophage lysis (Shinnick et al, Infect. Immun., 56:446-451 (1988)). Such a process might provide *M. spp* with a
means to spread to neighboring cells (Kaufmann, Immunol Today, 9:168-173 (1988)).

Listeriolyticin, a major virulence factor of Listeria monocytogenes, is under the control of the heat shock protein response and is expressed within the macrophage (Sokolovic et al, Infect. Immun., 57:295-298 (1989)). Since S. typhimurium produces the same heat shock response proteins, and in addition, the groEL proteins play a role in survival within the macrophage (Buchmeier, Abstr. Ann. Mtg. Amer. Soc. Microbiol. (1990)), it is possible that other heat shock proteins are involved in virulence.

A further adaptative response, the so-called acid-tolerance response (hereinafter "ATR"), has also been described for S. typhimurium (Foster et al, J. Bacteriol., 172:771-778 (1990)). S. typhimurium grown at pH 5.8 in minimal medium were found to adapt to the killing effects of acidic medium (pH 3.3). A mutant of S. typhimurium has been described that constitutively expresses acid tolerance, suggesting that there is a genetic basis to this response. It was postulated that the ATR might provide S. typhimurium with a means of surviving the acidified phagolylosome and acidic environments outside the host. To date, no virulence or immunogenicity data of strains defective in the ATR are available. Therefore, it is not clear how important this response is in pathogenesis of S. typhimurium in mice or vaccine development.

I. S. typhimurium Differs in Many Ways from S. typhi

While the mouse typhoid model has provided useful information on the pathogenesis of S. typhimurium in mice, the differences between this mouse-virulent
organism and human-adapted *S. typhi* are vast. Thus, one must treat comparisons cautiously. To be more specific, the clinical manifestations in humans elicited by these organisms are incomparable. *S. typhimurium* causes gastroenteritis and the growth of the bacilli in humans is restricted mainly to the intestinal lumen and mucosal layer. In particular, *S. typhimurium* invades the mucosa and is phagocytosed by polymorphonuclear cells that infiltrate the site of invasion, not by the macrophages, as is the case for *S. typhi* (Hook, *In: Principles and Practice of Infectious Disease*, Churchill Livingstone Inc. (1989)). Only rarely is there a generalized systemic *S. typhimurium* infection of individuals, except in AIDS patients who are more susceptible. This is in contrast to that seen with *S. typhi* (Hornick et al, *New Engl. J. Med.*, 283:686-691 (1970); and Hornick et al, *New Engl. J. Med.*, 283:739-746 (1970)). In addition, sublethal infection by *S. typhimurium* provides high levels of protection (Collins et al, *Infect. Immun.*, 6:451-458), whereas infection by *S. typhi* provides very little protection against subsequent challenge (Hornick et al, *New Engl. J. Med.*, 283:686-691 (1970); and Hornick et al, *New Engl. J. Med.*, 283:739-746 (1970)).

In addition to the clinical and immunological differences, *S. typhimurium* harbors large plasmids (typically about 90,000 base pairs, which represents 2% extra genetic material), called the virulence plasmid because of its virulence enhancing properties. Strains of *S. typhimurium* that have lost the virulence plasmid are attenuated in mice (Gulig et al, *Infect. Immun.*, 55:2891-2901 (1987)). There are at least two regions that express this virulence enhancing property (Norel et al, *Molec. Microbiol.*, 3:733-743
(1989)). However, to date the plasmid has not been found to be associated with the ability of S. typhimurium to survive within the macrophage (Gulig et al, Infect. Immun., 56:3262-3271 (1987)). The absence of the virulence plasmid and the failure to find homologous sequences in S. typhi underlines a major difference between these two species.

Finally, the gale mutation does not safely attenuate S. typhi, whereas gale mutants of S. typhimurium are highly attenuated in mice (Hone et al, Infect. Immun., 56:13-20 (1988); and Hone et al, J. Infect. Dis., 156:167-174 (1987)). This highlights the limitation in drawing comparisons between the pathogenesis of these two species of Salmonella.

Thus, while S. typhi and S. typhimurium display some similarities in their host-parasite relationship in the human and mouse respectively, there are sufficient differences that do not always enable predictable comparisons to be made.

**SUMMARY OF THE INVENTION**

Accordingly, an object of the present invention is to provide acid-tolerant S. typhi.

A further object of the present invention is to provide double aro mutants of S. typhi.

Another object of the present invention is to provide an oral vaccine against typhoid fever.

Still another object of the present invention is to provide a single dose oral vaccine against typhoid fever.

Yet another object of the present invention is to provide a single dose oral vaccine against typhoid fever which is useful as a carrier of genes expressing foreign antigens cloned from other pathogens and that raises protective immune responses in humans against
the pathogen from which the foreign antigens were derived.

An additional object of the present invention provide a method for immunizing a subject against typhoid fever.

A further object of the present invention is to provide a method for simultaneously immunizing a subject against typhoid fever and another pathogen.

Yet another object of the present invention is to provide a method for enrichment of acid-tolerant S. typhi useful as a live oral typhoid fever vaccine.

An additional object of the present invention provide a method for enrichment of acid-tolerant attenuated S. typhi expressing cloned foreign antigens useful as a carrier vaccine.

These and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met in one embodiment by substantially pure acid-tolerant Salmonella typhi.

In another embodiment, the above-described objects of the present invention have been met by an aroC, aroD double mutant of Salmonella typhi.

In an additional embodiment, the above-described objects of the present invention have been met by a vaccine against typhoid fever comprising:

(A) a pharmaceutically effective amount of an acid-tolerant typhoid fever vaccine strain of Salmonella typhi; and

(B) a pharmaceutically acceptable carrier or diluent.
In yet another embodiment, the above-described objects of the present invention have been met by a vaccine against typhoid fever comprising:

(A) a pharmaceutically effective amount of an aroC, aroD double mutant of Salmonella typhi; and

(B) a pharmaceutically acceptable carrier or diluent.

In still another embodiment, the above-described objects of the present invention have been met by a method of immunizing a subject against typhoid fever comprising orally administering a pharmaceutically effective amount of an acid-tolerant typhoid fever vaccine strain of Salmonella typhi.

In a further embodiment, the above-described objects of the present invention have been met by a method of immunizing a subject against typhoid fever comprising orally administering a pharmaceutically effective amount of an aroC, aroD double mutant of Salmonella typhi.

In a still further embodiment, the above-described objects of the present invention have been met by a method for enrichment of acid-tolerant Salmonella typhi comprising the steps of:

(A) culturing Salmonella typhi at an adaptive pH of about 4.5 to 5.5, at about 20 to 42°C and without aeration,

(B) screening for viable acid-tolerant Salmonella typhi by:

(i) culturing an aliquot of the resulting Salmonella typhi of step (A) at a selective pH of about 2.0 to 3.5 at about 30 to 40°C; and

(ii) determining viability of the cultured Salmonella typhi of step (i), wherein
if the cultured Salmonella typhi of step (i) is viable;

(C) harvesting the resulting Salmonella typhi of step (A), so as to isolate and enrich for acid-tolerant Salmonella typhi.

**BRIEF DESCRIPTION OF THE DRAWINGS**

In Figures 1-4, B = BamHI; Bg = BglII; E = EcoRI; RV = EcoRV; C = ClaI; F = BamHI, BglII fusion; Fi = BamHI, SauIIIa fusion; H = HindIII; P = PstI; and S = SalI.

Figure 1 shows a BglII-linearized scaled diagrammatic representation of plasmid pCVD1001 (8.2 kb) and its restriction map. Plasmid pCVD1001 was derived from pAROD-TYPHI, a 17.6 kb cosmid constructed in the 6.4 kb cosmid vector pHC79. The position and relative size of the 0.756 kb coding region of the arOD gene is also shown. Further, the position of the transposon Tn1725 (9.0 kb) insertion in pCVD1001 that resulted in plasmids pCVD1006 (17.2 kb) and pCVD1007 (17.2 kb) are shown. The Tn1725 insertions in pCVD1006 and pCVD1007 are indicated by the arrows along with the plasmid designation that corresponds to each insertion. Transposon Tn1725 has two EcoRI digestion sites, each located 15 bp from the transposon ends. Therefore, a Tn1725 insertion effectively creates a EcoRI digestion site.

Figure 2 shows a BamHI- and BglII-linearized scaled diagrammatic representation of plasmids pCVD1010 (5.35 kb) and pCVD1013 (8.45 kb) and their restriction maps. Plasmid pCVD1010 carries the 0.75 kb HindIII (converted to SalI) to EcoRI (located in the Tn1725 insertion) fragment of pCVD1006 and the 0.9 kb EcoRI (located in the Tn1725 insertion) to
BamHI fragment of pCVD1007. These fragments were joined by ligation at the EcoRI site. The resulting fused fragment form a 1.65 kb hybrid fragment which is flanked by SalI and BamHI sites, and is inserted into SalI- and BglII-linearized pGP704 (3.7 kb). The BamHI and BglII sites are lost as a result and the fusion point is designated as "F". By fusing the fragments from pCVD1006 and pCVD1007, 0.35 kb of the aroD gene is deleted in pCVD1010 and the derivatives thereof. The altered aroD gene, called delaroD1013, is designated as \( \Delta D \). Plasmid pCVD1013 is identical to pCVD1010, but the 3.8 kb PstI fragment of pKTN701 (6.8 kb), which carries the cat gene, has replaced the 0.7 kb PstI fragment of pCVD1010, which carries the bla gene of pGP704.

Figure 3 shows an EcoRI-linearized scaled diagrammatic representation of pCVD1003 (6.4 kb) and its restriction map. A 3.7 kb BglII fragment of the 11.9 kb cosmid, pBRD138, was inserted into the BamHI site of pUC9 (2.7 kb). The fusion joint of the BglII ends of pBRD138 and the BamHI site of pUC9 is designated as "F". The location of the 1.077 kb aroC gene of S. typhi, which spans from 124 bp upstream of the PstI site in the S. typhi DNA to 299 bp downstream of the NruI site, also located in the S. typhi DNA, is shown. Plasmid pCVD1015 (not shown) has the SalI fragment of pCVD1003 deleted. Plasmid pCVD1015, which has a unique NruI site, was further derivised by deleting the 0.654 kb PstI to NruI fragment located in the aroC gene to create pCVD1016 (not shown). The 0.654 kb PstI to NruI deletion in plasmid pCVD1016 results in an altered aroC gene, designated delaroC1019.

Figure 4 shows an EcoRI-linearized scaled diagrammatic representation of plasmids pCVD1011
(5.3 kb) and pCV1019 (6.7 kb) and their restriction maps. Plasmid pCVD1011 was derived by inserting the 1.6 kb SalI to EcoRI fragment of pCVD1016 (which carries delaroC1019) into SalI- and EcoRI-linearized pGP704. The deletion modified arcC gene, designated delaroC1019, located on pCVD1019, is shown as \( \Delta C \). Plasmid pCVD1019 is identical to pCVD1011, but with the 4.6 kb BamHI to PstI fragment, which carries the cat gene of pKTN701 has replaced the 2.1 kb PstI fragment of pCVD1011, which carries the bla gene of pGP704.

Figure 5 shows the series of events that led to the introduction of delaroC1019 into the chromosome of S. typhi. First, plasmid pCVD1019 is introduced into S. typhi by electroporation, whereupon it undergoes a homologous recombination event. Then, plasmid pCVD1019 becomes integrated into the chromosome of S. typhi. This results in formation of a stable cointegrate (H236.1). This cointegrate is resistant to chloramphenicol. Next, a second homologous recombination event results in the curing of the cointegrate. There are two possible outcomes of this second recombination event. The first (1.) is due to recombination on the same side of delaroC1019 as the first recombination event. This results in an arcC' genotype (H239.1). The second (2.) is the result of recombination on the opposite side of delaroC1019 as the first recombination event. This results in a delaroC1019 genotype (H238.1). Plasmid pCVD1019 cannot replicate in S. typhi and thus is lost after replication.

Figure 6 shows the nucleotide sequence of the arcC gene of S. typhi. The coding region for the arcC gene starts with an ATG codon (position 1) and ends with a TGG codon (position 1077). The deletion
mutation delaroC1019 is underlined and is flanked by a *Pst*I site (CTGCA<sup>126</sup>G; digests at position 124) and an *Nru*I site (TCG<sup>778</sup>GCA; digests at position 778).

Figure 7 shows the nucleotide sequence of the *aroD* gene of *S. typhi*. The coding region of the *aroD* gene starts with an ATG codon (position 1) and ends with a GCC codon (position 775). # denotes that the preceding underlined sequence gives the approximate position of the Tn<sub>1725</sub> insertion in pCVD1007. * denotes that the preceding underlined sequence gives the approximate position of the Tn<sub>1725</sub> insertion in pCVD1006. The position of the *EcoRV* digestion site (GATATC) is shown for reference to Figure 1.

**DETAILED DESCRIPTION OF THE INVENTION**

As discussed above, in one embodiment, the above-described objects of the present invention have been met by substantially pure acid-tolerant *Salmonella typhi*.

In another embodiment, the above-described objects of the present invention have been met by an *aroC, aroD* double mutant of *Salmonella typhi*.

In an additional embodiment, the above-described objects of the present invention have been met by a vaccine against typhoid fever comprising:

(A) a pharmaceutically effective amount of an acid-tolerant typhoid fever vaccine strain of *Salmonella typhi*; and

(B) a pharmaceutically acceptable carrier or diluent.

In yet another embodiment, the above-described objects of the present invention have been met by a vaccine against typhoid fever comprising:
(A) a pharmaceutically effective amount of an
aroC, aroD double mutant of Salmonella
typhi; and

(B) a pharmaceutically acceptable carrier or
diluent.

In still another embodiment, the above-described
objects of the present invention have been met by a
method of immunizing a subject against typhoid fever
comprising orally administering a pharmaceutically
effective amount of an acid-tolerant typhoid fever
vaccine strain of Salmonella typhi.

In a further embodiment, the above-described
objects of the present invention have been met by a
method of immunizing a subject against typhoid fever
comprising orally administering a pharmaceutically
effective amount of an aroC, aroD double mutant of
Salmonella typhi.

In a still further embodiment, the
above-described objects of the present invention have
been met by a method for enrichment of acid-tolerant
Salmonella typhi comprising the steps of:

(A) culturing Salmonella typhi at an adaptive pH
of about 4.5 to 5.5, at about 20 to 42°C and
without aeration,

(B) screening for viable acid-tolerant
Salmonella typhi by:

(i) culturing an aliquot of the resulting
Salmonella typhi of step (A) at a
selective pH of about 2.0 to 3.5 at
about 30 to 40°C; and

(ii) determining viability of the cultured
Salmonella typhi of step (i), wherein
if the cultured Salmonella typhi of
step (i) is viable,
(C) harvesting the resulting Salmonella typhi of step (A), so as to isolate and enrich for acid-tolerant Salmonella typhi.

Heretofore, there has not been an attenuated S. typhi live oral typhoid fever vaccine strain that has been both clinically well-tolerated and able to stimulate a strong immune response in the majority of vaccinees.

In the present invention, a method for the enrichment of acid-tolerant S. typhi has been designed so as to obtain "substantially pure" acid-tolerant S. typhi. As used herein the expression "substantially pure" acid-tolerant S. typhi means that greater than about 75% of the organisms survive after 90 min at pH 3.0.

Enrichment of acid-tolerance of S. typhi is believed in the present invention to increase survival of S. typhi and its attenuated derivatives during their passage through the gastric environment of the stomach and within the acidified phagolysosome of infected macrophages, thereby enabling more viable organisms to reach the site of invasion and enhancing the ability of the vaccine strain to stimulate cell-mediated immunity.

The acid-tolerant S. typhi of the present invention can be used to produce a live oral typhoid fever vaccine. To achieve this end, any mutation or any combination of known mutations that cause safe attenuation, but retain effective immunogenicity can be introduced into acid-tolerant wild-type S. typhi.

The particular wild-type S. typhi strain from which the acid-tolerant vaccine is derived and which can be employed in the immunization method of the present invention is not critical thereto. For example, the wild-type S. typhi may be any well-known

Mutations that cause safe attenuation, but retain effective immunogenicity, can be introduced using non-specific mutagenesis, such as N-methyl-N'-nitro-N-nitrosoguanidine; classic genetic techniques such as Tn10 mutagenesis, P22-mediated transduction and conjugational transfer; or site-directed mutagenesis using recombinant DNA techniques. Recombinant DNA techniques are preferable since strains constructed by recombinant DNA techniques are far more defined. Examples of such mutations that cause safe attenuation, but retain effective immunogenicity include:


(3) specific virulence-defective mutations such as phoP/phoQ (Groisman et al, Proc. Natl. Acad. Sci. USA, 86:7077-7081 (1989); and Miller et al, Proc. Natl. Acad. Sci. USA, 86:5054-5058 (1989)) or phoP (Miller et al,
mutations;

(4) mutations that effect the heat shock
response that result in temperature
sensitivity, such as htpR (Neidhardt et al,
(1981), or increased macrophage sensitivity,
such as groEL (Buchmeier et al, Ann. Mtg.
Amer. Soc. Microbiol. (1990));

(5) mutations that increase sensitivity to the
antimicrobial activity of macrophages and
other professional phagocytes, such as par
or prgs mutations (Miller et al., J.

Preferable mutations are those that result in safe
attenuation but do not affect immunogenicity. Double
aroC, aroD mutants are most preferred. The double
mutants may be insertion mutants, deletion mutants or
a combination thereof, although deletion mutants are
preferred.

The aroC gene is 1.077 kb in size (see Figure 6).
Thus, the size of the deletion in the aroC mutant may
range from 0.001 to 100 kb, preferably, from 0.001 to
1.077 kb. While intracistronic deletions in aroC can
range from 1 base pair to 1.077 kb in size, deletions
can also be made that extend beyond the aroC gene,
i.e., extracistronic deletions of up to 100 kb.
However, the latter is not preferable.

The aroD gene is 0.755 kb in size (see Figure 7).
Thus, the size of the deletion in the aroD mutant may
range from 0.001 to 100 kb, preferably, from 0.001 to
0.755 kb. While intracistronic deletions in aroD can
range from 1 base pair to 755 base pairs in size,
deletions can also be made that extend beyond the aroD
gene, i.e., extracistronic deletions of up to 100 kb. However, the latter is not preferable.

Deletions can be made in the S. typhi aroC gene using convenient restriction sites, such as AsuI (position 121), BbvI (positions 311, 955), BssHII (positions 753, 755, 757), SmaI (position 487), and XmaI (position 485) or by site-directed mutagenesis with oligonucleotides (Sambrook et al., eds., In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Publications (1989)).

Deletions can be made in the S. typhi aroD gene using convenient restriction sites, such as AluI (positions 118, 449),ClaI (positions 337, 785), EcoRV (position 161), FokI (position 53) and DdeI (position 180) or by site-directed mutagenesis using oligonucleotides (Sambrook et al., eds., In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Publications (1989)).

Inactivation of the aroC gene and aroD gene can also be carried out by an insertion of foreign DNA using any of the above-mentioned restriction sites or by site-directed mutagenesis with oligonucleotides (Sambrook et al., eds., In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Publications (1989)). The typical size of an insertion that can inactivate aroC and aroD genes of S. typhi is from 1 base pair to 100 kb, although insertions smaller than 100 kb are preferable. The insertion can be made anywhere inside the aroC or aroD gene coding region or between the coding region and the promoter.

Other methods for aroD or aroC inactivation include transfer into S. typhi deletions or insertions made in S. typhimurium aroC or aroD genes or E. coli aroC or aroD genes, transposon-generated deletions, and imprecise excision of DNA insertions. The latter
two methods are more likely to make deletions that extend beyond the aroD or aroC gene and therefore are not preferable.

Alternatively, acid-tolerance can be introduced into a live oral typhoid fever vaccine strain by transfer of DNA sequences encoding acid-tolerance. Although the genetic basis of acid-tolerance has not yet been determined, those skilled in the art can readily identify the gene or genes involved using conventional techniques, such as transposon mutagenesis, transcriptional fusions in addition to gene cloning and sequencing methodologies. The cloned genes can then be introduced into a vaccine strain of S. typhi, or a wild-type strain of S. typhi which is employed to produce a vaccine strain, by recombinational crosses or as part of an expression cassette on plasmid vectors.

The particular S. typhi vaccine strain from which the acid-tolerant S. typhi vaccine strain is derived and which can be employed in the immunization method of the present invention is not critical thereto. For example, the S. typhi vaccine strain may be any well-known vaccine strain, such as the Ty21a, Ty21a-Vi⁺, 541Ty, 543Ty, 27V strains, in addition to the CVD906 strain developed in the present invention.

Another alternative is to identify the gene regulator of acid-tolerance using transposon mutagenesis and other genetic methodologies, such as gene transfers between S. typhimurium and S. typhi, and to alter the regulator in such a way as to cause constitutive expression of acid-tolerance in the resulting S. typhi strain. If the regulator controls gene expression by a negative control, then deletion inactivation would result in constitutive expression of acid-tolerance. If the regulator controls gene
expression by a positive control, then those skilled in the art can design modifications that cause an irreversible gene activation by the repressor that would result in constitutive expression of acid-tolerance.

The *S. typhi* of the present invention can also be used as carriers of genes expressing protective antigens cloned from other pathogens. As used herein the expression of "protective antigens" means antigens or epitopes thereof which give rise to protective immunity against infection by the pathogen from which they are derived.

The other pathogens from which genes encoding protective antigens would be cloned are not critical to the present invention. Examples of such other pathogens include protozoan, viral and bacterial pathogens.

Examples of protective antigens of protozoan pathogens include the circumsporozoite antigens of *Plasmodium spp.* (Sadoff et al, *Science*, 240:336-337 (1988)), such as the circumsporozoite antigen of *P. berghei* or the circumsporozoite antigen of *P. falciparum*; and gp63 of *Leishmania spp.* (Russell et al, *J. Immunol.*, 140:1274-1278 (1988)).


Examples of protective antigens of bacterial

Antigens introduced into the carrier must be well-expressed. This can be achieved by constructing an expression cassette which includes a transcriptional promoter, a ribosomal binding site, and a start codon fused to the gene of interest. Examples of well-known promoters useful in the present invention include the lambda P, ptac, ptrp or plac promoters (Hoopes et al, In: Escherichia coli and Salmonella typhimurium, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984); Reznikoff et al, In: Maximizing Gene Expression, Reznikoff, ed., Butterworths (1986); and Miller et al, eds., In: The Operon, Cold Spring Harbor Publications (1978)).

An alternative to this approach is to fuse the gene or portion thereof, that encodes the foreign protective antigen, into well-expressed genes, such as lamb (Charbit et al, Gene, 70:181-189 (1988)), the non-toxic B-subunit of the heat labile toxin of ETEC (Guzman-Verduzco et al, J. Bacteriol., 169:5201-5208 (1987); and Schodel et al, Infect. Immun., 57:1347-1350 (1989)) or the major flagella subunit, flagellin (Newton et al, Science, 244:70-72 (1989)).

The pharmaceutically effective amount of the *S. typhi* of the present invention to be administered will vary depending on the age, weight and sex of the subject. Generally, the dosage employed will be about $5.0 \times 10^3$ to $5.0 \times 10^9$ viable organisms, preferably about $1.0 \times 10^5$ to $5.0 \times 10^9$ viable organisms.

The particular pharmaceutically acceptable carrier or diluent employed is not critical to the present invention. Examples of diluents include buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine et al, *J. Clin. Invest.*, 79:888-902 (1987); and Black et al *J. Infect. Dis.*, 155:1260-1265 (1987)), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine et al, *Lancet, II*:467-470 (1988)). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g. sucrose, or polyvinylpyrrolidone.

As discussed above, in the present invention, a sensitive but inexpensive method has been developed that enables the isolation and enrichment of acid-tolerant *S. typhi* strains. The method of the present invention depends on the ability of *S. typhi* strains to initially adapt to acidic conditions, i.e., growth at about pH 4.5 to 5.5, preferably about pH 4.9 to 5.1, more preferably about pH 5.0, without aeration, i.e., in an oxygen tension of less than about 1.0% (v/v) to anaerobic conditions, at about 20
to 42°C, preferably about 35 to 39°C, generally to an
$\text{OD}_{600}$ of about 0.2 to 0.5, preferably about 0.3.

The method described is one which gives the
greatest enrichment of acid-tolerance. Hence, the pH
range is important since the peak of acid-tolerance
enrichment occurs at pH 5.0 ± 0.1. The oxygen tension
is important since only low acid-tolerance enrichment
occurs when $S. \text{typhi}$ is grown in the presence of
greater than 1.0% (v/v) to atmospheric pressure
oxygen. The temperature that best facilitates
acid-tolerance enrichment is 37°C ± 2°C. However,
other temperatures, while not optimal, do allow some
enrichment of acid-tolerant $S. \text{typhi}$. The $\text{OD}_{600}$ ranges
give highest acid-tolerance enrichment. However,
enrichment does occur at other $\text{OD}_{600}$ values.

Thereafter, in order to screen for viable
acid-tolerant $S. \text{typhi}$, the bacteria are grown at a
selective pH, about the acidic levels of the stomach
at the time of immunization, i.e., at about pH 2.0
to 3.5, preferably about pH 2.9 to 3.1, at about 30 to
40°C, preferably about 35 to 39°C, and viability is
determined, e.g., as described in In: Manual of
Methods for General Bacteriology, American Society for
Microbiology, Gerhardt et al, eds., Washington, D.C.

The $S. \text{typhi}$ can be cultured in any well-known
nutrient medium, such as L-broth (Difco, Detroit, MI),
Brain-Heart Infusion (Difco, Detroit, MI), Nutrient
broth (Difco, Detroit, MI), M9 medium (Miller, In:
Experiments in Molecular Biology, Cold Spring Harbor
Press (1979)) supplemented with 0.2% (w/v) casamino
acids (Difco, Detroit, MI), Peptone broth (Difco,
Detroit, MI) and Tryptone broth (Difco, Detroit, MI).

The $S. \text{typhi}$ of the present invention are stored
at -70°C suspended in 50% (v/v) glycerol, 0.5% (w/v)
Tryptone, 0.25% (w/v) Yeast extract, 0.01% (w/v) PABA, and 0.1% (w/v) casamino acids (pH 5.0), or lyophilized in 5.0% (w/v) sucrose in Aro-Broth (pH 5.0).

The following examples are provided for illustrative purposes only and are in no way intended to limit the scope of the present invention.

**EXAMPLE 1**

**Acid Tolerance**

I. Acid-Tolerance Response in Minimal E Medium

It is known that a higher yield of acid-tolerant S. typhimurium, strain LT2, can be obtained when these bacilli are grown with aeration in minimal E medium comprising 0.2% (w/v) MgSO₄·7H₂O, 0.2% (w/v) citric acid·1H₂O, 1.0% (w/v) K₂HPO₄ anhydrous, and 0.35% (w/v) NaH₂PO₄·4H₂O (pH 7.6) as compared to when grown in minimal E medium (pH 5.8) (Foster et al, *J. Bacteriol.*, 172:771-778 (1990)). Hence, the level of acid-tolerant S. typhi strain ISP1820 that are produced using these same conditions was assayed.

Viability counts were determined as described in *In: Manual of Methods for General Bacteriology*, American Society for Microbiology, Gerhardt et al. eds., Washington, D.C. (1981). More specifically, 10-fold serial dilutions of the cell suspension (0.1 ml of cells in 0.9 ml of 0.9% (w/v) NaCl (saline)) were prepared. Then, 100 µl samples were spread evenly onto L-agar plates comprising L-broth (pH 3.0) comprising 1.0% (w/v) Tryptone (Difco, Detroit, MI), 0.5% (w/v) Yeast extract (Difco, Detroit, MI), and 0.5% (w/v) NaCl (Sigma, St. Louis, MO); and containing 1.5% (w/v) agar (Difco, Detroit, MI). Next, the plates were incubated overnight at 37°C with aeration and colonies were counted. Viability counts were performed on duplicates of each
sample. The viability count is the number of colonies on a given plate multiplied by 10 (to convert the results to counts per ml of sample) and multiplied by the dilution factor (to convert the results to the original sample numbers). Viability counts were taken at 0 min and 90 min after the cells were suspended in the pH 3.0 medium. The results were expressed as the number of viable organisms at 90 min divided by the number of viable organisms at 0 min and multiplied by 100 so as to express the result as a % survival. The results are shown in Table 1 below.

**TABLE 1**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MEDIUM</th>
<th>pH</th>
<th>% SURVIVAL AFTER 90 min AT pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2:</td>
<td>E</td>
<td>7.6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8</td>
<td>68.0</td>
</tr>
<tr>
<td>ISP1820:</td>
<td>E</td>
<td>7.6</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

As shown in Table 1 above, only low levels of acid-tolerant *S. typhi* ISP1820 were obtained using E medium. This is in contrast to the high levels
observed for \textit{S. typhimurium} LT2. The results in Table 1 above demonstrate that there is an intrinsic physiological difference in the ATR between \textit{S. typhimurium} LT2 and \textit{S. typhi} ISP1820. Notwithstanding this difference, it is clear that acid-tolerant \textit{S. typhi} can be enriched 5000-fold using this approach. Because of the failure of minimal E medium to produce a high level of acid-tolerant \textit{S. typhi} similar to that seen when \textit{S. typhimurium} is grown in minimal E medium, there was a need to design a method that maximized the enrichment of acid-tolerant \textit{S. typhi}.

II. Growth Medium for Enhanced Acid-Tolerance of \textit{S. typhi}

In an effort to find growth conditions that produced a higher percentage of acid-tolerant \textit{S. typhi} than that seen after growth in minimal E medium, other nutrient rich media were tested.

Aerated growth in CFB medium comprising 0.15\% (w/v) Yeast extract (Difco, Detroit, MI), 1.0\% (w/v) casamino acids (Difco, Detroit, MI), 0.005\% (w/v) MgSO$_4$ (Sigma, St. Louis, MO) and 0.005\% (w/v) MnCl$_2$ (Sigma, St. Louis, MO), at 25°C has been shown to enhance expression of \textit{V. cholerae} virulence factors (Hall et al, \textit{Microbial. Path.}, \textbf{4}:257-265 (1988)). Thus, the capacity of CFB medium to produce cultures highly enriched with acid-tolerant \textit{S. typhi} was tested as follows.

10 ml of CFB medium at various pH (see Table 2), were inoculated with 10$^6$ colony forming units (c.f.u.) of LT2 and ISP1820 and incubated for 16 hr at 25°C with aeration. The cultures were then subcultured by diluting 100-fold into fresh CFB medium (of the same pH) and incubated at 37°C with aeration until the OD$_{600}$
reached 0.4. This typically required about 2 hr during which the pH of the medium remained unchanged. After the additional incubation, the cells were pelleted, then resuspended in 5.0 ml of CFB medium (pH 3.0). A 0.1 ml sample of this cell suspension in CFB medium (pH 3.0) was immediately transferred to 0.9 ml of phosphate-buffered saline (hereinafter "PBS") (which neutralized the pH) so as to measure the starting viability. After 3 hr, 1.0 ml of the culture was transferred to a 1.5 ml microfuge tube and pelleted by centrifugation at 4000 x g. Next, the cells were resuspended in 1.0 ml of PBS, and viability counts were determined as described above. The results are shown in Table 2 below.

**TABLE 2**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MEDIUM</th>
<th>pH</th>
<th>% SURVIVAL AFTER 3 hr AT pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2:</td>
<td>CFB</td>
<td>7.0</td>
<td>0.02%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>38.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>18.54%</td>
</tr>
<tr>
<td>ISP1820:</td>
<td>CFB</td>
<td>7.0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>0.0001%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

As shown in Table 2 above, only a small number of acid-tolerant *S. typhi* ISP1820 were produced as a result of growth in CFB medium at various pH. However, once again, *S. typhimurium* LT2 proved to acquire greater levels of acid-tolerance and
consequently greater levels of survival at low pH than S. typhi.

L-broth, the pH of which was adjusted to pH 7.2 using 10 M NaOH and to pH 5.0 using 10 M HCl, is a standard laboratory medium used for growth of Salmonella and other enteric bacteria. Thus, L-broth was tested for its capacity to produce cultures highly enriched for acid-tolerant S. typhi. The method employed was identical to that described above for growth in CFB medium. The results are shown in Table 3 below.

**TABLE 3**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MEDIUM</th>
<th>pH</th>
<th>% SURVIVAL AFTER 3 hr AT pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>15.8%</td>
</tr>
<tr>
<td>ISP1820:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.01%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.5%</td>
</tr>
</tbody>
</table>

As shown in Table 3 above, L-broth also failed to produce high levels of acid-tolerant S. typhi.

In order to determine if the low yield of acid-tolerant S. typhi ISP1820 was strain dependent, a variety of other S. typhi strains, i.e., laboratory strains, Ty2 and Quailes, that had been passaged in the laboratory many times since they were originally isolated, were tested in the same manner. The results are shown in Table 4 below.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>MEDIUM</th>
<th>pH</th>
<th>% SURVIVAL AFTER 3 hr AT pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh isolates:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISP1804:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.0054%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.4%</td>
</tr>
<tr>
<td>ISP2822:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.284%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.3%</td>
</tr>
<tr>
<td>ISP2825:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.41%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>3.15%</td>
</tr>
<tr>
<td>Laboratory strains:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ty2:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.000051%</td>
</tr>
<tr>
<td>Quailes:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.012%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.03%</td>
</tr>
<tr>
<td>Strains isolated from different geographical regions:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H28:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.028%</td>
</tr>
<tr>
<td>H29:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.0001%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.038%</td>
</tr>
<tr>
<td>H30:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0.0011%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.0035%</td>
</tr>
<tr>
<td>Previous vaccine strains:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ty21a:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0%</td>
</tr>
<tr>
<td>541Ty:</td>
<td>L-broth</td>
<td>7.2</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.00067%</td>
</tr>
</tbody>
</table>
As shown in Table 4 above, laboratory adapted strains Ty2 and Quailles are inherently less capable of developing acid-tolerance than recently isolated strains of *S. typhi* (ISP1804, ISP1820, ISP2822 and ISP2825) when grown under similar conditions. Furthermore, strains of *S. typhi* isolated from different geographic regions, H28 (Originated from India), H29 (Originated from Malaysia) and H30 (Originated from the Solomon Islands), and previous vaccine strains, Ty2la and 541Ty, proved to be inherently more acid-sensitive. Strains H28, H29 and H30 were provided by J. Morris, Department of Microbiology, University of Melbourne (1986).

The results above indicate that maximum yields of acid-tolerant *S. typhi* is dependent on two key factors. First, the growth conditions under which the *S. typhi* strain is grown. Second, the strain is preferably a recent isolate that is capable of becoming acid-tolerant after growth in the appropriate medium.

The low yield of acid-tolerant Ty2la and 541Ty corroborates with the fact that both these attenuated *S. typhi* strains are poorly immunogenic after a single dose. It is believed in the present invention that live oral attenuated strains of *S. typhi* with greater acid-tolerance will have better immunogenicity.

III. Buffered Medium and Low Oxygen Tension
Produce High Numbers of Acid-Tolerant
*S. typhi*

In order to achieve greater reproducibility, 100 mM MOPS buffer (pH 8.0), and 100 mM citrate buffer (pH 5.0), were added to L-broth. The cultures were then grown with aeration at 37°C and with shaking at 150 rpm, or grown without aeration at 37°C and
standing with 1.0 ml of mineral oil overlaying the culture or in an anaerobic atmosphere by placing the cultures in a GasPak® Jar System (BBL, Cockeysville, MD) with an anaerobic GasPak® (BBL, Cockeysville, MD). The aerated cultures were grown initially for 16 hr, then subcultured 1:10 into fresh medium and grown until the OD₆₀₀ reached 0.5. The freshly grown cells were pelleted by centrifugation at 4000 x g and washed once with 10 ml of L-broth. Finally, the cells were resuspended in 5.0 ml of L-broth (pH 3.0). The cultures grown without aeration were harvested after 20 hr of incubation. At this time, the OD₆₀₀ was typically about 0.5. The cells were pelleted, washed and resuspended in L-broth (pH 3.0) as described for the aerated cultures. Immediately after the cells were resuspended in L-broth (pH 3.0), a 0.1 ml sample was taken and transferred to 0.9 ml of PBS. The pH 3.0 cell suspensions were placed at 37°C, standing with 1.0 ml of mineral oil overlaying the culture or in an anaerobic atmosphere by placing the cultures in a GasPak® Jar System (BBL, Cockeysville, MD) with an anaerobic GasPak® (BBL, Cockeysville, MD). Then, after 90 min, the 1.0 ml samples were taken and mixed with 1.0 ml of PBS. Viability counts were determined for each of the 0 min and 90 min samples as described above. The results are shown in Table 5 below.
**TABLE 5**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>pH</th>
<th>AERATION</th>
<th>%SURVIVAL AFTER 90 min AT pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP1820:</td>
<td>8.0</td>
<td>+</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>+</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>-</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-</td>
<td>75.0-125.0</td>
</tr>
<tr>
<td>CVD906:</td>
<td>8.0</td>
<td>-</td>
<td>0.0037</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-</td>
<td>75.38-95.0</td>
</tr>
<tr>
<td>Ty2:</td>
<td>8.0</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-</td>
<td>0.93-5.0</td>
</tr>
<tr>
<td>LT2:</td>
<td>8.0</td>
<td>-</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-</td>
<td>35.0-67.0</td>
</tr>
</tbody>
</table>

As shown in Table 5 above, the yield of acid-tolerant *S. typhi* is increased by dropping the pH to 5.0. When *S. typhi* is grown without aeration the yield of acid-tolerant *S. typhi* is maximized. This shows that the combination of low oxygen and low pH synergistically enhances acid-tolerance in *S. typhi*. In fact, when *S. typhi*, strain ISP1820, is grown at 37°C in L-broth (pH 5.0) without aeration to an OD<sub>600</sub> of 0.5, 125% of the bacilli are able to survive L-broth (pH 3.0) for 90 min. This demonstrates that some level of acid-tolerance is produced by these growth conditions, thus enabling growth at pH 3.0. Laboratory-adapted strain Ty2, on the other hand, did not display the same level of acid-tolerance under these conditions. Thus, complete
acid-tolerance seems to be restricted to freshly-isolated \textit{S. typhi} that have undergone minimum laboratory passage. Note, \textit{S. typhimurium} LT2 proved to yield less acid-tolerant cells under conditions that maximized the yield for \textit{S. typhi}. This, once again, shows that these strains have different acid-tolerance responses.

Strain CVD906, which is a double \textit{aro} mutant of ISP1820 and was prepared as described in more detail below, gave rise to a higher yield of acid-tolerance when using the maximized conditions described for the parent strain, ISP1820. However, CVD906 is less acid-tolerant than its parent. This difference might the result of the laboratory passage CVD906 underwent during its construction.

Due to the risk of undetected contaminants, solid medium cultures are preferred over liquid cultures when cultures are being prepared for volunteer studies. Thus, the yield of acid-tolerant \textit{S. typhi} strain ISP1820 and CVD906, when harvested from Aro-agar comprising L-agar supplemented with 100 \(\mu\)g/ml of ferric ammonium citrate, 0.01\% (w/v) PABA, 0.01\% (w/v) PHB, 0.25\% (w/v) glucose and 0.2\% (w/v) casamino acids (pH 6.0), was evaluated as follows. Dilutions of cell suspensions of strains ISP1820 and CVD906 were spread evenly onto Aro-agar plates. The plates were incubated overnight at 37\(^\circ\)C. Then, the cells were harvested from the plates that produced well-separated colonies and resuspended in saline. The cells were washed twice and then resuspended in L-broth (pH 3.0) to an \(\text{OD}_{600}\) of 0.2. Samples were taken immediately and after incubation of the suspension for 90 min at 37\(^\circ\)C, and viability counts were determined as described above.
Both ISP1820 and CVD906 yielded high levels of acid-tolerant bacilli under these conditions (75% and 93%, respectively). Thus, while these conditions may not be optimum for the production of acid-tolerant ISP1820, they optimized production of acid-tolerant CVD906.

EXAMPLE 2
Use of Acid-tolerant S. typhi as Live Oral Typhoid Fever Vaccines

I. CVD906, A Double Aro Mutant of Acid-Tolerant S. typhi strain ISP1820

In the past, attenuated candidate vaccine strains of S. typhi have been prepared using classical genetic techniques (Edwards et al, J. Bacteriol., 170:3991-3995 (1988)). The strains described herein were made using recombinant DNA techniques. By introducing deletions in two or more aro genes, e.g., the aroA, aroC, aroB, aroD, and aroE genes, that independently block chorismic acid synthesis, an added level of safety, preventing the chance of in vivo reversion, can be introduced into the attenuated strain. The aro mutations introduced into S. typhi strain ISP1820 to produce strain CVD906 are delaroC1019 and delaroD1013. These aro deletions are defined in molecular terms, they do not extend beyond the limits of the mutant gene and no other foreign DNA (except 30 base pairs of innocuous DNA from transposon Tn1725) was introduced.

II. Construction of Strain CVD906

Attenuated strains of S. typhi, for use as a typhoid fever vaccine and antigen carrier, preferably possess two well-separated and well-defined deletion mutations and preferably are highly immunogenic after a single dose. As discussed above, strains of
Salmonella harboring a single aro mutation are remarkably attenuated but highly immunogenic in animals. In addition, as discussed above, S. typhi strains that carry both aro and pur mutations were shown to be safe but non-immunogenic in volunteers and hence hyperattenuated. The pur mutation, included as a safety factor, has been shown to cause the hyperattenuation in mice given pur and aro, pur strains of S. typhimurium. Because the aro genes on the Salmonella chromosome are widely separated, mutations in two of the aro genes can be exploited to introduce safety against the chance of in vivo reversion, as was attempted with the aro pur double mutants of S. typhi. It is proposed herein to use aro mutations as the primary attenuating marker of S. typhi with the aim of constructing a live oral typhoid fever vaccine that is also suitable as an antigen carrier. To this end, in vitro DNA techniques were used to construct defined deletions in the aroC and aroD genes of acid-tolerant S. typhi strain ISP1820.

A. DNA Methodologies

Restriction endonuclease digestions were carried out at 37°C in the buffers recommended by the manufacturer. End-filling reactions using T4 DNA polymerase, and ligations using T4 DNA ligase were performed using well-known procedures (Sambrook et al, In: Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Electrophoresis of DNA was carried out on 0.5-1.0% (w/v) agarose gels buffered with TAE buffer comprising 0.04 M Tris-acetate and 0.001 M EDTA (pH 7.4) as described by Sambrook et al, In: Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989).
The molecular weight markers employed were the "1.0 kb ladder" (Bethesda Research Laboratories, Gaithersburg, MD). The molecular weight of restriction enzyme generated fragments was determined from a standard curve of the log_{10} molecular weight of the 1.0 kb ladder standard sized fragments versus their mobility in the agarose gel. After electrophoresis, the isolation of restriction enzyme-generated fragments from agarose was achieved using the Gene Clean Kit® (Bio-101, Richmond, CA) as recommended by the manufacturer.

B. Construction of the Defined AroD Deletion Carried on pCVD1013

Plasmid pCVD1001 (see Figure 1) was derived from pAROD-TYPHI (G. Dougan, Wellcome Biotech, Beckenham Kent, England), a 17.6 kb cosmid constructed in the 6.4 kb cosmid vector pHC79. Plasmid pCVD1001 was constructed by digesting pAROD-TYPHI with BglII, which produced 4 fragments sized 8.2, 5.7, 2.1 and 1.6. The largest fragment (8.2 kb) carries sufficient pHC79 DNA (2.7 kb) to enable plasmid replication and expression of ampicillin-resistance, in addition, to 5.5 kb of S. typhii DNA which carries the aroD gene. Thus, digestion of pAROD-TYPHI with BglII followed by recircularization of the 8.2 kb fragment by ligation results in a new plasmid, designated pCVD1001.

Transposon Tn1725 was used to map the location of the aroD gene in pCVD1001 relative to restriction endonuclease sites located in pCVD1001 using the method described by Ubben et al, *Gene*, 41:145-152 (1986).

Two Tn1725 insertions into plasmid pCVD1001, found in plasmids pCVD1006 and pCVD1007, inactivated the aroD gene function as judged by the inability of
these two plasmids to complement the \texttt{aroD} mutation of strain SK2881 \texttt{aroD6} (Kinghorn et al, \textit{Gene}, 14:73-80 (1981)) (see Figure 1). Sequence analysis confirmed the location of an open reading frame associated with the \texttt{aroD} gene function and that the transposon Tn1725 insertions in plasmids pCVD1006 and pCVD1007 were within the \texttt{aroD} open reading frame corresponding the \texttt{aroD} encoding DNA.

Plasmids pCVD1006 and pCVD1007 (see Figure 1) were used to construct an altered \texttt{aroD} gene (see Figure 2). Transposon Tn1725 has \texttt{EcoRI} digestion sites located 15 base pairs from each end. Thus, an insertion using this transposon effectively creates an \texttt{EcoRI} site that can be utilized to create new enzyme-derived fragments (Ubber et al, \textit{Gene}, 41:145-152 (1986)).

More specifically, the 0.75 kb \texttt{HindIII} to \texttt{EcoRI} fragment of pCVD1006, spanning from within pH179 to the \texttt{EcoRI} site of Tn1725 (see Figure 1), was isolated and the \texttt{HindIII}-generated end converted to a \texttt{SalI}-generated end. This was achieved by first digesting pCVD1006 with \texttt{HindIII}. Then, the \texttt{HindIII} ends were end-filled using T4 DNA polymerase. Next, \texttt{SalI} linkers were ligated to the end-filled \texttt{HindIII} ends. The resulting DNA was digested with \texttt{SalI} and \texttt{EcoRI} and the DNA fragments were separated by electrophoresis. The 0.75 kb \texttt{SalI} (formerly \texttt{HindIII}) to \texttt{EcoRI} fragment was then excised from the agarose gel and purified.

Separately, the 0.9 kb \texttt{EcoRI} to \texttt{BamHI} fragment of pCVD1007, spanning from the \texttt{BamHI} site within pCVD1007 and upstream of the \texttt{aroD} gene, to the \texttt{EcoRI} site within Tn1725 (see Figure 1), was isolated and purified. More specifically, pCVD1007 was digested with \texttt{BamHI} and \texttt{EcoRI} simultaneously and the resulting
DNA fragment was electrophoresed without further modification. The 0.9 kb EcoRI to BamHI fragment was excised from the agarose gel after electrophoresis and purified.

The 0.75 kb SalI (formerly HindIII) to EcoRI fragment of pCVD1006 and the 0.9 kb EcoRI to BamHI fragment of pCVD1007 were then joined by ligation and subsequently digested with SalI and BamHI. The resulting 1.65 kb hybrid fragment was ligated to a SalI- and BglII-digested suicide vector pGP704 and transformed into SY327. pGP704 and SY327 were provided by J. Mekalanos, Department of Microbiology and Molecular Genetics, Harvard Medical School (Miller et al., J. Bacteriol., 170:2575-2583 (1988)). The resulting ampicillin-resistance-encoding plasmid, designated pCVD1010 (see Figure 2), contains an aroD gene harboring a 0.35 kb deletion, designated delaroD1013. The EcoRI site present in the precise location of deletion delaroD1013 is flanked by 15 base pairs of non-coding and innocuous DNA from Tn1725 (see Figure 2).

The bla gene of pCVD1010, encoding ampicillin resistance, was then substituted by the cat gene, encoding chloramphenicol resistance, from pKTN701 (Nishibuchi, Department of Microbiology, Faculty of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo, Kyoto 606, Japan) or any other well-known plasmid containing the cat gene, such as pACYC184 (New England Biolabs, Beverly, MA). This was achieved by substituting the 0.7 kb PstI fragment of pCVD1010 with the 3.8 kb PstI fragment of pKTN701 (see Figure 2).

More specifically, plasmids pCVD1010 and pKTN701 were digested with PstI and the enzyme was inactivated by placing the digested DNA at 65°C for 5 min. The digested plasmid DNAs were mixed and ligated. The
resulting ligated DNA was transformed into SY327 and selection for resistance to 20 µg/ml of chloramphenicol was carried out. Chloramphenicol-resistant colonies were screened for the presence of plasmids with the appropriate restriction pattern. One such clone was selected for and the resulting plasmid was designated pCVD1013 (see Figure 2).

Plasmid pCVD1013 was subsequently used to introduce delaroD1013, the deletion-inactivated aroD gene carried by pCVD1013, into the chromosome of S. typhi strain ISP1820.

C. Construction of the Defined ArcC Deletion Carried on pCVD1019

Cosmid pBRD138, obtained from Dr. G. Dougan, carries approximately 17 kb of the S. typhi chromosome and carries a functional arcC gene. An 8.0 kb BglII-generated fragment of pBRD138 was subcloned into BamHI-digested pUC9 (Yanisch-Perron et al, Gene, 33:103-107 (1985)) to create pCVD1003 (see Figure 3).

More specifically, pBRD138 was digested with BglII and pUC9 was digested with BamHI. Then, the restriction enzymes were inactivated as described above. The two digested plasmids were mixed and ligated and the ligated DNA was transformed into BRD250 (Dougan et al, J. Infect. Dis., 158:1329-1335 (1988)). The resulting plasmid, designated pCVD1003 (see Figure 3), was able to complement the well-characterized aroC mutant strain, BRD250.

For construction of the deletion-inactivated aroC gene, conveniently located PstI and NruI restriction sites, both located within the aroC coding region, were employed (see Figure 3).
More specifically, plasmid pCVD1003 was digested with SalI, circularized by ligation and transformed into DH5-α (Bethesda Research Laboratories, Gaithersburg, MD). The resulting plasmid, designated pCVD1015, was found to lack the 1.0 kb SalI fragment and had a unique NruI site. Plasmid pCVD1015 was digested with NruI and the NruI-generated ends were converted to PstI ends by ligation to PstI linkers (Bethesda Research Laboratories, Gaithersburg, MD). This modified plasmid DNA was then digested with PstI, circularized by ligation and transformed into DH5-α. The resulting plasmid, designated pCVD1016, was found to have lost the 0.654 kb NruI to PstI fragment located within the aroC gene. This fragment was replaced by a PstI site. Next, plasmid pCVD1016 was digested with EcoRI and SalI and ligated to EcoRI- and SalI-digested pGP704. The resulting plasmid, designated pCVD1011, carries a 1.6 kb SalI to EcoRI insert which in turn carries the deletion inactivated aroC gene designated delaroC1019 (see Figure 4), which is missing the 0.654 kb NruI to PstI fragment.

The bla gene of pCVD1011 was substituted by the cat gene of pKTN701 by ligating the 4.6 kb SalI to BamHI fragment of pKTN701 to the 2.1 kb SalI to BamHI fragment of pCVD1011 (see Figure 4).

More specifically, pCVD1011 was digested with SalI and BamHI. Then, the 2.1 kb fragment was excised from an agarose gel after electrophoresis and purified. Plasmid pKTN701 also was digested with SalI and BamHI and the enzymes were inactivate as described above. These two DNA preparations were mixed, then ligated and transformed into SY327 and selection for resistance to 20 μg/ml of chloramphenicol was carried out. Chloramphenicol-resistant colonies were screened for the presence of plasmids with the appropriate
restriction pattern. One such clone was selected for and the resulting plasmid was designated pCVD1019 (see Figure 4).

Plasmid pCVD1019 was subsequently used to introduce delaroC1019, the deletion-inactivated aroc gene carried by pCVD1019, into the chromosome of S. typhi strain ISP1820.

D. Construction of pCVD1017 and pCVD1018

Plasmid pCVD1017 carries a 0.654 kb PstI to NruI fragment of pCVD1003 which is located within the aroc coding region and which is deleted in delaroC1019-mutant strains. pCVD1017 was constructed by digesting plasmid pCVD1003 with PstI and NruI. The 0.6 kb fragment flanked by PstI- and NruI-generated ends was then inserted by ligation into Smal- and PstI-digested pUC19 (Yanisch-Perron et al., Gene, 33:103-107 (1985)) so as to obtain plasmid pCVD1017. Plasmid pCVD1017 is useful as a probe because it does not hybridize with delaroC1019-mutant strains.

Plasmid pCVD1018 carries the 0.3 kb EcoRI to EcoRV fragment of pCVD1006 which is located within the arpD coding region and which is deleted in delaroD1013-mutant strains. pCVD1018 was constructed by digesting pCVD1006 with EcoRI and EcoRV. The 0.3 kb EcoRI to EcoRV fragment was isolated and ligated to EcoRI- and SmaI-digested pUC19 DNA so as to obtain plasmid pCVD1018. Plasmid pCVD1018 is useful as a probe because it does not hybridize with delaroD1013-mutant strains.
E. Introduction of delaroCl019 and delaroD1013 into the Chromosome of S. typhi ISP1820

Plasmids pCVD1013 and pCVD1019 cannot replicate in S. typhi. When either pCVD1013 or pCVD1019 are introduced by electroporation into S. typhi they form stable cointegrates as a result of a single homologous recombination event between S. typhi DNA carried on the plasmids and the S. typhi chromosome. Such cointegrates can be cured using a chloramphenicol-sensitive enrichment process. A significant proportion of the chloramphenicol-sensitive derivatives of the cointegrate strains S. typhi::pCVD1019 or S. typhi::pCVD1013 carry the delaroCl019 mutation and the delaroD1013 mutation, respectively.

To create a strain that carries delaroCl019, S. typhi strain ISP1820 (ISP1820 has been deposited at the American Culture Type Collection on May 15, 1990, under ATCC No. 55047) was transformed by electroporation with pCVD1019 (see Figure 5). The electroporation procedure was a modification of the procedure described by Sambrook et al, In: Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989).

More specifically, about 10^6 c.f.u. of strain ISP1820 were taken from a fresh L-agar plate, placed into 10 ml of L-broth and incubated for 16 hr at 37°C with aeration and with shaking at 150 opm. Then, the 16 hr culture was diluted 1:100 into 250 ml of fresh L-broth in a 1.0 liter baffled flask and grown at 37°C with aeration and with shaking at 250 opm until the OD_600 reached 0.3 relative to an L-broth control. The cells were pelleted by centrifugation at 4000 x g at 4°C and then gently resuspended in 100 ml of cold, sterile, and ultra-pure H_2O (Millipore milli-Q system).
The cells were pelleted again as described above and
resuspended in another 100 ml of H₂O. The 100 ml-H₂O
wash was repeated one more time. After one final
pelleting, the cells were resuspended in 10% (v/v)
glycerol to a cell density of 2 x 10¹⁰/ml. To a 400 µl
aliquot of the cell suspension, 2.5 µg of plasmid
pCVD1019 DNA, suspended in 10 µl of H₂O, was added and
the DNA/cell mixture was placed into a 0.2 cm cuvette
(Bio-Rad, Richmond, CA). Using a Bio-Rad gene pulser
set at 2.5 kV, 25 µF and 800 ohms, the DNA/cell
mixture was pulsed, typically with time decay factors
of between 9.0 to 11.0 msec. The cells were
immediately transferred to 10 ml of SOC recovery
medium comprising 1.2% (w/v) Bactone-tryptone (Difco,
Detroit, MI), 0.5% (w/v) Yeast extract (Sigma, St.
Louis, MO), 0.05% (w/v) NaCl (Sigma, St. Louis, MO)
and 20 mM glucose (Sigma, St. Louis, MO) (Sambrook et
al, In: Molecular Cloning a Laboratory Manual, Cold
Spring Harbor Laboratory Press (1989)) and placed on
ice for 30 min, followed by incubation for 30 min at
37°C with shaking at 150 opm. Then, the cells were
pelleted by centrifugation at 4000 x g, resuspended in
200 µl of SOC recovery medium and spread evenly onto
4 L-agar plates containing 20 µg/ml of
chloramphenicol. The frequency that plasmid pCVD1019
was introduced into ISP1820 using this method was
about 150 transformants/µg of DNA. This was about
3 x 10³-fold lower than if chloramphenicol-resistant
control plasmid DNA, pACYC184 (New England Biolabs,
Beverly, MA; and Chang et al, J. Bacteriol.,
134:1141-1156 (1978)), was introduced into in ISP1820.
Chloramphenicol-resistant derivatives were the result
of pCVD1019 forming a cointegrate with the chromosome
of ISP1820 at the aroC locus. One such
chloramphenicol-resistant cointegrate of ISP1820 was
chosen for further derivation and was designated H236.1 (see Figure 5). To confirm that pCVD1019 had inserted into the *argC* locus, chromosomal DNA, isolated from H236.1, was purified and probed with [*P*-labelled pCVD1003. This demonstrates that H236.1 had an altered chromosomal restriction pattern consistent with pCVD1019 forming a cointegrate with the chromosome. Next, colonies of H236.1 which were spontaneously cured of the cointegrate by homologous recombination, were isolated using a chloramphenicol-sensitive enrichment procedure which is a modification of the method described by Miller, *In: Experiments in Molecular Biology*, Cold Spring Harbor Press (1979). More specifically, strain H236.1 was grown on L-agar containing 20 μg/ml of chloramphenicol at 37°C for 16 hr. Then, about 1 x 10^6 cells from this culture were transferred to Aro-broth comprising L-broth supplemented with 100 μg/ml of ferric ammonium citrate, 0.01% (w/v) PABA, 0.01% (w/v) PHB, 0.25% (w/v) glucose and 0.2% (w/v) casamino acids (pH 6.0), and incubated at 37°C with aeration and shaking at 150 opm. After 16 hr, the cells were diluted to an OD_{600} of 0.05 relative to an Aro-broth control. Then, chloramphenicol was added to a concentration of 20 μg/ml and the culture was incubated at 37°C with aeration and with shaking at 250 opm until the OD_{600} reached 0.2 relative to an Aro-broth control. At this point, D-cycloserine (Sigma, St. Louis, MO) was added to a final concentration of 200 μg/ml and the culture was immediately returned to the 37°C incubator and incubated with aeration and with shaking at 250 opm for an additional hour. During this incubation in the presence of D-cycloserine, the chloramphenicol-resistant bacteria, which were rapidly
dividing, were lysed. On the other hand, the chloramphenicol-sensitive bacteria, which were static due to the chloramphenicol added at the beginning, remained unaffected by the D-cycloserine. Lysis was accompanied by a drop in OD$_{600}$. Cells were harvested by centrifugation at 4000 x g and gently resuspended in 10 ml of saline. The pelleting resuspension steps were repeated two more times. Then, 10-fold serial dilutions were made of this final cell suspension and 100 µl aliquots were spread evenly onto Aro-agar plates. After incubation for 16 hr at 37°C, individual colonies were picked and screened for their chloramphenicol-resistance phenotype and their growth factor requirements. After enrichment of strain H236.1, 20% of the colonies were found to be chloramphenicol-sensitive. Of these chloramphenicol-sensitive derivatives, 30% of the colonies were aromatic amino acid-dependent. These aromatic amino acid-dependent mutants did not hybridize with [³²P]-labelled pCVD1017 and Southern hybridization analysis of chromosomal DNA extracted from one such strain, H238.1, showed that the delaroC1019 was introduced into the chromosome (see Figure 5). At this point delaroD1013 could have been introduced into strain H238.1, however, this strain was not used but, rather, the following procedures were carried out.

The procedure described above (except that pCVD1013 was used in place of pCVD1019) was used to isolate a delaroD1013 mutant of ISP1820 which was designated H233.2. Strain H233.2 was then transformed with pCVD1019 using the electroporation procedure to produce chloramphenicol-resistant strain H237.1. After passage of strain H237.1 through the chloramphenicol-sensitive enrichment procedure (40% of
the colonies were chloramphenicol-sensitive), colonies were picked onto duplicate Aro-agar plates and one set was transferred to nitrocellulose and probed with [32P]-labelled pCVD1017 (Sambrook et al, In: Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Of the chloramphenicol-sensitive derivatives, 40% of the colonies did not hybridize with pCVD1017. Analysis of the chromosome of one of these probe-negative derivatives, designated strain CVD906, by Southern hybridization analysis showed that the mutation delaroC1019 was introduced into its chromosomes. CVD906 has been deposited at the American Culture Type Collection on May 15, 1990, under ATCC No. 55048.

EXAMPLE 3
S. typhi Strains and Their LD₅₀ Values
S. typhi strain ISP1820 and the mutants thereof were grown in Aro-broth at 37°C to an OD₆₀₀ of 0.6. Then, the cells were harvested by centrifugation at 4000 x g and resuspended in 10 ml of saline. After repeating the centrifugation step as described above, the cells were resuspended in saline. Then, 10-fold serial dilutions of the cell suspensions were mixed with 5.0% (w/v) hog gastric mucin (Wilson Laboratories, lot #0347A001) as described by Hone et al, Infect. Immun., 56:1326-1333 (1988). The bacterial dilutions suspended in 5.0% (w/v) hog gastric mucin were injected intraperitoneally into 18-20 g female CD-1 mice (Charles River, PA). The LD₅₀ values were calculated as described by Hone et al, Infect. Immun., 56:1326-1333 (1988). The results are shown in Table 6 below.
TABLE 6

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP1820</td>
<td>Wild-type parent</td>
<td>&lt;150</td>
</tr>
<tr>
<td>H233.2</td>
<td>delaroD1013</td>
<td>3.3 x 10⁷</td>
</tr>
<tr>
<td>H238.1</td>
<td>delaroC1019</td>
<td>3.0 x 10⁷</td>
</tr>
<tr>
<td>CVD906</td>
<td>delaroC1019, delaroD1013</td>
<td>2.9 x 10⁷</td>
</tr>
</tbody>
</table>

As shown in Table 6 above, using this method, the single aro mutants, strain H233.2 (delaroD1013) and strain H238.1 (delaroC1019) and the double aro mutant, strain CVD906 (delaroD1013 and delaroC1019), are safely attenuated in mice.

EXAMPLE 4
Strain Characterization

S. typhi strain ISP1820 and its aro derivatives were tested for the presence of capsular, somatic and flagellar antigens. These antigens included the Vi antigen, the O9 and O12 O-polysaccharide antigenic determinants and the flagella antigen H-d. The biochemical characteristics were also assessed by using the API 20E biochemical profile (Analytab of Sherwood Medicals, Plainview, NY), which assays for the presence of β-galactosidase activity, arginine dihydrolase activity, lysine decarboxylase activity, ornithine decarboxylase activity, citrate utilization, urease activity, tryptophan deaminase activity, indole production from tryptophan, acetoin production from creatine and pyruvate, liquefaction of gelatin,
utilization of carbohydrates (glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and (L+)-arabinose), conversion of nitrate to nitrate, H$_2$S production and catalase activity, their ability to produce H$_2$S on triple sugar iron (TSI) agar slopes (Gerhardt et al, eds., In: Manual for Methods for General Bacteriology, American Society for Microbiology, Washington, D.C. (1981)), made according to the recommendations of the manufacturer (BBL, Cockeysville, MD); and the ability to grow on minimal M9 medium supplemented with 0.01% (w/v) tryptophan, 0.1% (w/v) cysteine, and 100 μg/ml of ferric ammonium citrate (hereinafter "Typhi-M9 medium") or Typhi-M9 medium supplemented with 0.01% (w/v) PABA, 0.01% (w/v) DHB, 0.01% (w/v) p-hydroxybenzoate, 10 μg/ml of ferric ammonium citrate, 0.01% (w/v) tyrosine and 0.01% (w/v) phenylalanine (hereinafter "M9+ARO medium"). The results are shown in Table 7 below.
### TABLE 7

**MUTANT STRAIN PHENOTYPIC CHARACTERIZATION**

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>Vi</th>
<th>O9</th>
<th>O12</th>
<th>H-d</th>
<th>API</th>
<th>H$_2$S</th>
<th>M9</th>
<th>M9+ARO</th>
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<tbody>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>St</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H233.2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>St</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>H238.1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>St</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CVD906</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>St</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ denotes positive; - denotes negative; S denotes strong agglutination; St denotes profile identified strain as *S. typhi*.

As shown in Table 7 above, no changes in the antigen characteristics were detected between the parent strain and the mutants thereof. In addition, the aro mutants displayed the same API 20E biochemical profile as the parent strain, except that they could not produce H$_2$S. This lack of H$_2$S production by the aro mutants has been previously described by Pittard, *In: Escherichia coli and Salmonella typhimurium*, Neidhardt, ed., American Society for Microbiology, Washington, D.C. (1984).

While the parent strain grew well on Typhi-M9 medium, as expected, the aro mutants did not grow on this medium because this medium does not contain the nutrients required to complement the aro mutations.

No revertants of strain CVD906 to aromatic-independence were detected when $5 \times 10^{10}$ viable organisms were spread on Typhi-M9 medium containing 1.5% (w/v) agar (Difco, Detroit, MI).
Strain CVD906 only underwent 3 generations when placed into Typhi-M9 medium whereas ISP1820 grew through about 20 generations in this medium. CVD906 also could not undergo more than 3 generations in human serum. This was in contrast to the parent, ISP1820, which grew well in human serum.

On the other hand, as expected, strain CVD906 could mimic the growth of parent strain, ISP1820, in Typhi-M9 medium and human serum, but only if supplemented with 0.01% (w/v) PABA, 0.01% (w/v) DHB, 0.01% (w/v) p-hydroxybenzoate, 10 µg/ml of ferric ammonium citrate (serum only), 0.01% (w/v) tyrosine and 0.01% (w/v) phenylalanine; i.e., the nutrients required to complement the aro mutations.

No changes in the antibiotic resistance profile, e.g., amp, chlor, tet and cipro were observed in the aro mutants of ISP1820.

EXAMPLE 5
Volunteer Studies

An initial clinical study with S. typhi candidate vaccine strain CVD906 was carried out with approval of the United States Food and Drug Administration. Nine informed volunteers received a single dose of 3 x 10^7 viable CVD906 organisms with bicarbonate buffer. The organisms were grown on Aro-agar plates and acid-tolerant CVD906 were harvested. As discussed above Aro-agar plates have been shown herein to increase the yield of acid-tolerant S. typhi bacilli to around 95%.

The clinical acceptability, infectivity and immunogenicity of the candidate S. typhi oral vaccine strain at a dose of 3 x 10^7 viable organisms was evaluated.
A. Clinical Acceptability and Infectivity

At a dose of $3 \times 10^7$ viable organisms, acid-tolerant strain CVD906 impressively survived passage through the gastric barrier and was excreted in high titer for 2 to 4 days by all of the 9 volunteers and in 2 volunteers, excreted organisms reappeared again at day 10 or day 11 post-vaccination. Also, 5 of the volunteers produced positive blood cultures 6 to 7 days after vaccination. These two observations, taken together, indicate that the vaccine strain colonized the intestine far better than expected, thus enabling larger numbers of acid-tolerant strain CVD906 to invade through the mucosa and enter the systemic circulation. Eight of the volunteers tolerated the vaccine without fever, while 1 volunteer developed fever, anorexia, headache, bronchitic cough and abdominal discomfort. All of the isolates from the volunteers demonstrated the phenotypic characteristics of the vaccine strain. These include a negative H$_2$S reaction and an inability to grow on Typhi-M9 medium that was not supplemented with aromatic amino acids, PABA and DHB.

B. Immunogenicity of CVD906 When Given to Volunteers

Intestinal immunological priming by acid-tolerant strain CVD906 was measured using the antibody secreting cell (ASC) assay, carried out as described by Czerkinsky et al, J. Immunol. Meth., 65:109-121 (1983); and Czerkinsky et al, Proc. Natl. Acad. Sci. USA, 84:2449-2453 (1987). The results are shown in Table 8 below. For purposes of comparison, Table 8 below also shows the ASC responses to S. typhi lipopolysaccharide antigen in the 9 volunteers who have received a single $5 \times 10^7$ organism dose of
acid-tolerant strain CVD906 versus the 9 volunteers who received three $10^9$ organism doses of acid-sensitive strain Ty21a-Vi+.

**TABLE 8**

ANTIBODY SECRETING CELL RESPONSES AMONG TYPHOID VACCINEES

<table>
<thead>
<tr>
<th></th>
<th>CVD906</th>
<th>CVD906</th>
<th>Ty21a-Vi+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose</strong></td>
<td>$5 \times 10^3$</td>
<td>$3 \times 10^7$</td>
<td>$1 \times 10^9$</td>
</tr>
<tr>
<td><strong>Number of Doses</strong></td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><strong>ASC to Lipopolysaccharide</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG seroconversion rate</td>
<td>3/11</td>
<td>8/9</td>
<td>3/9</td>
</tr>
<tr>
<td>Mean net OD$_{A50}^*$</td>
<td>0.71</td>
<td>1.48</td>
<td>0.31*</td>
</tr>
<tr>
<td>IgM seroconversion rate</td>
<td>ND</td>
<td>7/9</td>
<td>1/9</td>
</tr>
<tr>
<td>Mean net OD$_{A50}^*$</td>
<td>ND</td>
<td>1.54</td>
<td>0.35</td>
</tr>
<tr>
<td>IgA seroconversion rate</td>
<td>3/11</td>
<td>8/9</td>
<td>5/9</td>
</tr>
<tr>
<td>Mean net OD$_{A50}^*$</td>
<td>1.11</td>
<td>1.92</td>
<td>0.62b</td>
</tr>
</tbody>
</table>

* Among responders
* p<0.05, t test
* p<0.001, t test
ND No data

As shown in Table 8 above, the difference in immunogenicity between acid-tolerant strain CVD906 and acid-sensitive strain Ty21a-Vi+ is marked. This supports the belief in the present invention that acid-tolerance enhances the immunogenicity of live oral typhoid fever vaccine strains.

Serological responses of acid-tolerant strain CVD906 to lipopolysaccharide and H-d antigen are shown in Tables 9 and 10 below.
TABLE 9
SERUM ANTIBODY RESPONSES TO LIPOPOLYSACCHARIDE

<table>
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<tr>
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<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion rate</td>
<td>9/9</td>
<td>6/9</td>
<td>8/9</td>
</tr>
<tr>
<td>Mean fold-rise</td>
<td>6.2*</td>
<td>8.25*</td>
<td>13.14#</td>
</tr>
</tbody>
</table>

* among responders day 21.
# among responders day 7.

TABLE 10
SERUM ANTIBODY RESPONSES TO H-d ANTIGEN

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seroconversion rate</td>
<td>7/9</td>
<td>8/9</td>
</tr>
<tr>
<td>Mean fold-rise</td>
<td>28.6*</td>
<td>43.8#</td>
</tr>
</tbody>
</table>

* among responders day 21.
# among responders day 7.

As shown in Tables 9 and 10 above, all 9 of the volunteers seroconverted, indicating that acid-tolerant strain CVD906 has remarkable, colonization and immunological properties.

5

In addition to the serum responses, the antibody-dependent cell-mediated cytotoxic (hereinafter "ADCC") response in peripheral mononuclear cells was assayed as described by Levine et al., J. Clin. Invest., 79:888-902 (1987). Significant ADCC responses were detected in all of the 5 volunteers assayed. High levels of non-specific mononuclear-mediated bacterial inactivation was noted.
This suggests that acid-tolerant strain CVD906 activated peripheral blood macrophages. These non-specific responses elicited by acid-tolerant strain CVD906 were in marked contrast to the ADCC responses seen after immunization with 541Ty and 543Ty, where very little non-specific activity was detected. A much lower dose, such as $5 \times 10^3$ organisms, acid-tolerant strain CVD906 was also found to well-tolerated and immunogenic in volunteers.

C. Summary of Clinical Investigation

In summary, the observations made in the above study of acid-tolerant strain CVD906 demonstrated that acid-tolerant strain CV906 is attenuated, albeit not sufficiently attenuated at the $3 \times 10^7$ dosage level administered with bicarbonate buffer. With a fully pathogenic wild-type $S. typhi$ strain, given at a dose of $3 \times 10^7$ without buffer, one would anticipate that about 50% of the volunteers would develop full-blown typhoid fever, based on studies carried out by Woodward and Hornick in the 1960s. If a dose of $3 \times 10^7$ pathogenic $S. typhi$ organisms were administered with bicarbonate buffer, one would anticipate a 100% attack rate of full-blown typhoid fever. Thus, acid-tolerant strain CVD906 appears markedly attenuated compared with wild-type $S. typhi$ strains.

While this invention has been described in detail and with to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.
International Application No: PCT/ 

**MICROORGANISMS**

Optional Sheet in connection with the microorganisms referred to on page____ line____ of the description 1

A. IDENTIFICATION OF DEPOSIT 1

Further deposits are identified on an additional sheet 2.

Name of depository institution 4

**AMERICAN TYPE CULTURE COLLECTION (ATCC)**

Address of depository institution (including postal code and country) 4

12301 Parklawn Drive
Rockville, MD 20852, US

Date of deposit 1 Accession Number 6

15 May 1990 55047

B. ADDITIONAL INDICATIONS 1 (leave blank if not applicable). This information is continued on a separate attached sheet 0

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE 1 (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS 1 (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later 4 (Specify the general nature of the indications e.g., "Accession Number of Deposit 4")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

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was

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Form PCT/RD/134 (January 1985)
International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 70, line 14 of the description.

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet.

Name of depository institution:

AMERICAN TYPE CULTURE COLLECTION (ATCC)

Address of depository institution (including postal code and country):

12301 Parklawn Drive
Rockville, MD 20852, US

Date of deposit

15 May 1990

Accession Number

55048

B. ADDITIONAL INDICATIONS (Leave blank if not applicable). This information is continued on a separate attached sheet.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States).

D. SEPARATE FURNISHERS OF INDICATIONS (Leave blank if not applicable).

The indications listed below will be submitted to 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 Officer:

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

Authorized Officer:

Form PCT/AO/134 (January 1985)
WHAT IS CLAIMED IS:

Claim 1. Substantially pure acid-tolerant Salmonella typhi.

Claim 2. The substantially pure acid-tolerant Salmonella typhi as claimed in Claim 1, wherein said acid-tolerant Salmonella typhi is derived from a recently isolated strain selected from the group consisting of ISP1804, ISP1820, ISP2822, ISP2825, a Chilean strain, a Thai strain and a Peruvian strain.

Claim 3. The substantially pure acid-tolerant Salmonella typhi as claimed in Claim 2, wherein said acid-tolerant Salmonella typhi is derived from ISP1820.

Claim 4. The substantially pure acid-tolerant as Salmonella typhi claimed in Claim 1, wherein said acid-tolerant Salmonella typhi is derived from a vaccine strain selected from the group consisting of Ty21a, Ty21a-Vi*, 541Ty, 543Ty, 27V and CDV906 having the identifying characteristics of ATCC No. 55048.

Claim 5. The substantially pure acid-tolerant as Salmonella typhi as claimed in Claim 4, wherein said acid-tolerant Salmonella typhi is derived from vaccine strain CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 6. The substantially pure acid-tolerant Salmonella typhi as claimed in Claim 1, wherein said acid-tolerant Salmonella typhi is lyophilized.

Claim 7. A vaccine against typhoid fever comprising:

(A) a pharmaceutically effective amount of an acid-tolerant vaccine strain of Salmonella typhi; and

(B) a pharmaceutically acceptable carrier or diluent.
Claim 8. The vaccine as claimed in Claim 7, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from a recently isolated strain selected from the group consisting of ISP1804, ISP1820, ISP2822, ISP2825, a Chilean strain, a Thai strain and a Peruvian strain.

Claim 9. The vaccine as claimed in Claim 8, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from ISP1820.

Claim 10. The vaccine as claimed in Claim 7, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from a vaccine strain selected from the group consisting of Ty21a, Ty21a-Vi\(^t\), 541Ty, 543Ty, 27V and CDV906 having the identifying characteristics of ATCC No. 55048.

Claim 11. The vaccine as claimed in Claim 10, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from vaccine strain CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 12. The vaccine as claimed in Claim 7, wherein said vaccine strain is a carrier of a gene expressing a protective antigen cloned from another pathogen.

Claim 13. The vaccine as claimed in Claim 12, wherein said protective antigen is from a protozoan pathogen.

Claim 14. The vaccine as claimed in Claim 13, wherein said protective antigen is selected from the group consisting of a circumsporozoite antigen of *Plasmodium* spp. and gp63 of *Leishmania* spp.

Claim 15. The vaccine as claimed in Claim 14, wherein said circumsporozoite antigen of *Plasmodium* spp. is selected from the group consisting of the circumsporozoite antigen of *P. bergeri* and the circumsporozoite antigen of *P. falciparum*. 
Claim 16. The vaccine as claimed in Claim 12, wherein said protective antigen is from a viral pathogen.

Claim 17. The vaccine as claimed in Claim 16, wherein said protective antigen is selected from the group consisting of hepatitis B surface antigen; a human immunodeficiency virus antigen; and a rotavirus antigen.

Claim 18. The vaccine as claimed in Claim 17, wherein said human immunodeficiency virus antigen is selected from the group consisting of gp120, gp41, T cell epitope of gp160 and B cell epitope of gp120; and said rotavirus antigen is selected from the group consisting of VP4 and VP7.

Claim 19. The vaccine as claimed in Claim 12, wherein said protective antigen is from a bacterial pathogen.

Claim 20. The vaccine as claimed in Claim 19, wherein said protective antigen is selected from the group consisting of the Shigella sonnei form 1 antigen; the O-antigen of V. cholerae Inaba strain 569B; a protective antigen of enterotoxigenic E. coli; and the fragment C of tetanus toxin of Clostridium tetani.

Claim 21. The vaccine as claimed in Claim 20, wherein said protective antigen of enterotoxigenic E. coli is selected from the group consisting of the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin.

Claim 22. The vaccine as claimed in Claim 7, wherein said pharmaceutically effective amount is about $5.0 \times 10^3$ to $5.0 \times 10^{11}$ viable organisms.

Claim 23. The vaccine as claimed in Claim 22, wherein said pharmaceutically effective amount is about $1.0 \times 10^5$ to $5.0 \times 10^9$ viable organisms.
Claim 24. A method of immunizing a subject against typhoid fever comprising orally administering a pharmaceutically effective amount of an acid-tolerant vaccine strain of *Salmonella typhi*.

Claim 25. The method as claimed in Claim 24, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from a recently isolated strain selected from the group consisting of ISP1804, ISP1820, ISP2822, ISP2825, a Chilean strain, a Thai strain and a Peruvian strain.

Claim 26. The method as claimed in Claim 25, wherein said acid-tolerant *Salmonella typhi* typhoid fever vaccine strain is derived from ISP1820.

Claim 27. The method as claimed in Claim 24, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from a vaccine strain selected from the group consisting of Ty21a, Ty21a-Vi+, 541Ty, 543Ty, 27V and CDV906 having the identifying characteristics of ATCC No. 55048.

Claim 28. The method as claimed in Claim 27, wherein said acid-tolerant *Salmonella typhi* vaccine strain is derived from vaccine strain CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 29. The method as claimed in Claim 24, wherein said vaccine strain is a carrier of a gene expressing a protective antigen cloned from another pathogen.

Claim 30. The method as claimed in Claim 29, wherein said protective antigen is from a protozoan pathogen.

Claim 31. The method as claimed in Claim 30, wherein said protective antigen is selected from the group consisting of a circumsporozoite antigen of *Plasmodium spp.* and gp63 of *Leishmania spp.*
Claim 32. The method as claimed in Claim 31, wherein said circumsporozoite antigen of *Plasmodium spp.* is selected from the group consisting of the circumsporozoite antigen of *P. bergerii* and the circumsporozoite antigen of *P. falciparum*.

Claim 33. The method as claimed in Claim 29, wherein said protective antigen is from a viral pathogen.

Claim 34. The method as claimed in Claim 33, wherein said protective antigen is selected from the group consisting of hepatitis B surface antigen; a human immunodeficiency virus antigen; and a rotavirus antigen.

Claim 35. The method as claimed in Claim 34, wherein said human immunodeficiency virus antigen is selected from the group consisting of gp120, gp41, T cell epitope of gp160 and B cell epitope of gp120; and said rotavirus antigen is selected from the group consisting of VP4 and VP7.

Claim 36. The method as claimed in Claim 29, wherein said protective antigen is from a bacterial pathogen.

Claim 37. The method as claimed in Claim 36, wherein said protective antigen is selected from the group consisting of the *Shigella sonnei* form 1 antigen; the O-antigen of *V. cholerae* Inaba strain 569B; a protective antigen of enterotoxigenic *E. coli*; and the fragment C of tetanus toxin of *Clostridium tetani*.

Claim 38. The method as claimed in Claim 37, wherein said protective antigen of enterotoxigenic *E. coli* is selected from the group consisting of the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin.
Claim 39. The method as claimed in Claim 29, wherein said pharmaceutically effective amount is about $5.0 \times 10^3$ to $5.0 \times 10^{11}$ viable organisms.

Claim 40. The method as claimed in Claim 39, wherein said pharmaceutically effective amount is about $1.0 \times 10^5$ to $5.0 \times 10^9$ viable organisms.

Claim 41. A method for enrichment of acid-tolerant *Salmonella typhi* comprising the steps of:

(A) culturing *Salmonella typhi* at an adaptive pH of about 4.5 to 5.5, at about 20 to 42°C and without aeration;

(B) screening for viable acid-tolerant *Salmonella typhi* by:

(i) culturing an aliquot of the resulting *Salmonella typhi* of step (A) at a selective pH of about 2.0 to 3.5 at about 30 to 40°C; and

(ii) determining viability of the cultured *Salmonella typhi* of step (i), wherein if the cultured *Salmonella typhi* of step (i) is viable;

(C) harvesting the resulting *Salmonella typhi* of step (A), so as to isolate and enrich for acid-tolerant *Salmonella typhi*.

Claim 42. The method as claimed in Claim 41, wherein said acid-tolerant *Salmonella typhi* is derived from a recently isolated strain selected from the group consisting of ISP1804, ISP1820, ISP2822, ISP2825, a Chilean strain, a Thai strain and a Peruvian strain.

Claim 43. The method as claimed in Claim 42, wherein said acid-tolerant *Salmonella typhi* is derived from ISP1820.
Claim 44. The method as claimed in Claim 41, wherein, in step (A) the adaptive pH is about pH 4.9 to 5.1, and the temperature is about 35 to 39°C.

Claim 45. The method as claimed in Claim 44, wherein, in step (A) the adaptive pH is about pH 5.0.

Claim 46. The method as claimed in Claim 41, wherein, in step (B) the selective pH is about pH 2.9 to 3.1, and the temperature is about 35 to 39°C.

Claim 47. An aroC, aroD double mutant of Salmonella typhi.

Claim 48. The aroC, aroD double mutant of Salmonella typhi as claimed in Claim 47, wherein said double mutant contains a deletion in both the aroC gene and the aroD gene.

Claim 49. The aroC, aroD double mutant of Salmonella typhi as claimed in Claim 47, wherein said mutant is CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 50. The aroC, aroD double mutant of Salmonella typhi as claimed in Claim 47, wherein said mutant is lyophilized.

Claim 51. A vaccine against typhoid fever comprising:

(A) a pharmaceutically effective amount of an aroC, aroD double mutant of Salmonella typhi; and

(B) a pharmaceutically acceptable carrier or diluent.

Claim 52. The vaccine as claimed in Claim 51, wherein said double mutant contains a deletion in both the aroC gene and the aroD gene.

Claim 53. The vaccine as claimed in Claim 51, wherein said S. typhi is a carrier of a gene
expressing a protective antigen cloned from another pathogen.

Claim 54. The vaccine as claimed in Claim 53, wherein said protective antigen is from a protozoan pathogen.

Claim 55. The vaccine as claimed in Claim 54, wherein said protective antigen is selected from the group consisting of a circumsporozoite antigen of *Plasmodium* spp. and gp63 of *Leishmania* spp.

Claim 56. The vaccine as claimed in Claim 55, wherein said circumsporozoite antigen of *Plasmodium* spp. is selected from the group consisting of the circumsporozoite antigen of *P. bergerii* and the circumsporozoite antigen of *P. falciparum*.

Claim 57. The vaccine as claimed in Claim 53, wherein said protective antigen is from a viral pathogen.

Claim 58. The vaccine as claimed in Claim 57, wherein said protective antigen is selected from the group consisting of hepatitis B surface antigen; a human immunodeficiency virus antigen; and a rotavirus antigen.

Claim 59. The vaccine as claimed in Claim 58, wherein said human immunodeficiency virus antigen is selected from the group consisting of gp120, gp41, T cell epitope of gp160 and B cell epitope of gp120; and said rotavirus antigen is selected from the group consisting of VP4 and VP7.

Claim 60. The vaccine as claimed in Claim 53, wherein said protective antigen is from a bacterial pathogen.

Claim 61. The vaccine as claimed in Claim 60, wherein said protective antigen is selected from the group consisting of the *Shigella sonnei* form 1 antigen; the O-antigen of *V. cholerae* Inaba strain
569B; a protective antigen of enterotoxigenic E. coli; and the fragment C of tetanus toxin of Clostridium tetani.

Claim 62. The vaccine as claimed in Claim 61, wherein said protective antigen of enterotoxigenic E. coli is selected from the group consisting of the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin.

Claim 63. The vaccine as claimed in Claim 51, wherein said mutant is CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 64. The vaccine as claimed in Claim 51, wherein said pharmaceutically effective amount is about $5.0 \times 10^3$ to $5.0 \times 10^9$ viable organisms.

Claim 65. The vaccine as claimed in Claim 64, wherein said pharmaceutically effective amount is about $1.0 \times 10^5$ to $5.0 \times 10^9$ viable organisms.

Claim 66. A method of immunizing a subject against typhoid fever comprising orally administering a pharmaceutically effective amount of an aroC, aroD double mutant of Salmonella typhi.

Claim 67. The method as claimed in Claim 66, wherein said double mutant contains a deletion in both the aroC gene and the aroD gene.

Claim 68. The method as claimed in Claim 66, wherein said S. typhi is a carrier of a gene expressing a protective antigen cloned from another pathogen.

Claim 69. The method as claimed in Claim 68, wherein said protective antigen is from a protozoan pathogen.

Claim 70. The method as claimed in Claim 69, wherein said protective antigen is selected from the group consisting of a circumsporozoite antigen of Plasmodium spp. and gp63 of Leishmania spp.
Claim 71. The method as claimed in Claim 70, wherein said circumsporozoite antigen of *Plasmodium spp.* is selected from the group consisting of the circumsporozoite antigen of *P. berghei* and the circumsporozoite antigen of *P. falciparum*.

Claim 72. The method as claimed in Claim 68, wherein said protective antigen is from a viral pathogen.

Claim 73. The method as claimed in Claim 72, wherein said protective antigen is selected from the group consisting of hepatitis B surface antigen; a human immunodeficiency virus antigen; and a rotavirus antigen.

Claim 74. The method as claimed in Claim 73, wherein said human immunodeficiency virus antigen is selected from the group consisting of gp120, gp41, T cell epitope of gp160 and B cell epitope of gp120; and said rotavirus antigen is selected from the group consisting of as VP4 and VP7.

Claim 75. The method as claimed in Claim 68, wherein said protective antigen is from a bacterial pathogen.

Claim 76. The method as claimed in Claim 75, wherein said protective antigen is selected from the group consisting of the *Shigella sonnei* form 1 antigen; the O-antigen of *V. cholerae* Inaba strain 569B; a protective antigen of enterotoxigenic *E. coli*; and the fragment C of tetanus toxin of *Clostridium tetani*.

Claim 77. The method as claimed in Claim 76, wherein said protective antigen of enterotoxigenic *E. coli* is selected from the group consisting of the CFA/I fimbrial antigen and the nontoxic B-subunit of the heat-labile toxin.
Claim 78. The method as claimed in Claim 66, wherein said mutant is CVD906 having the identifying characteristics of ATCC No. 55048.

Claim 79. The method as claimed in Claim 66, wherein said pharmaceutically effective amount is about $5.0 \times 10^3$ to $5.0 \times 10^9$ viable organisms.

Claim 80. The method as claimed in Claim 79, wherein said pharmaceutically effective amount is about $1.0 \times 10^5$ to $5.0 \times 10^9$ viable organisms.
FIG. 5

S. typhi chromosome

aroC

delaroC1019

cat

pcVD1019

aroC

delaroC1019

cat

H236.1 chromosome

aroC

H239.1 chromosome

delaroC1019

cat

H238.1 chromosome

1.

2.

SUBSTITUTE SHEET
ACAGGACACGC A1TG AAA ACC GTC ACC GTA AAA AAT CTT ATC ATT GGC GAA
GGG ATG CCC AAA ATT ATC GTG TCG TTG ATG GGA AGA GAC ATC AAT AGC GTG
AAA GCC GAG GCG CTG GCC TAC CGC GAA GCT ACA TTC GAT ATT CTT GAG TGG
GCG GTC GAT CAC TTT ATG GAT ATC GCA TCG ACT CAA TCC GTT CTT ACC
GCT GCG CGT GTT ATC CGC GAT GCG ATG CCT GAC ATT CCG TTA CTG TTT ACT
TTC CGC AGC GCC AAA GAA GGC GGC GAG CAG ACA ATA ACC ACT CAG CAT TAT
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CGT CCG GTA ATT ACT ATG TCA ATG GCG AAA GAG GGT GTC ATT TCA CGT CTG
GCA GGG GAA GTG TTT GCC TCT GCC GCC ACG TTT GCC GCG GTG AAG CAG GCT
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CTG CAC AAC GCC T755 TGA TCCACAGGACGGGTGCTGGTCGACATC

FIG. 7

SUBSTITUTE SHEET
**INTERNATIONAL SEARCH REPORT**

**Classification of Subject Matter**

Accordng to International Patent Classification (IPC) or to both National Classification and IPC

IPC(S): C12N 15/00; A61K 39/112

U.S.Cl.: 424/92:435/172.3

**Fields Searched**

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Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched

Biosis, Medicine, APS

**Documents Considered to Be Relevant**

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<td>US A 4,837,151 (STOCKFR) 06 June 1989, see entire document. 1-80</td>
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<td>Y</td>
<td>The Journal of Infectious Diseases, volume 155, no. 1, issued January 1987, A. Brown et al., &quot;An attenuated aroA Salmonella typhimurium Vaccine Flicits Hormonal and Cellular Immunity to Cloned B-Galactosidase in mice&quot; pages 86-92, see entire article. 13,29, 51,68</td>
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**Certification**

- Date of the Actual Completion of the International Search: 16 August 1991
- Date of Mailing of this International Search Report: 12 SEP 1991

International Searching Authority: ISA/US

Signature of Authorized Officer: H. Sidberry


X Infection and Immunity, volume 53, no. 6, issued September 1986, J.D. Clements, "Oral Immunization of Mice with Attenuated Salmonella enteritidis Containing a Recombinant Plasmid Which Codes for Production of the B Subunit of Heat-Labile Escherichia coli Enterotoxin." pages 685-692, see entire article.

X The Journal of Infectious Diseases, volume 158, no. 6, issued December 1988, G. Dougan et al., "Construction and Characterization of Vaccine Strains of Salmonella harboring mutations in two different aro Genes" pages 1329-1335, see entire article.


V. □ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claim numbers , because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6(4)(a).

VI. □ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

see attached sheet

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. □ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest
□ The additional search fees were accompanied by applicant's protest.
□ No protest accompanied the payment of additional search fees.
I. Claims 1-40, drawn to acid-tolerant bacteria, vaccines comprising them and a method of immunizing with the vaccines, classified in classes 435 and 424 subclasses 252.3, 253.8 and 92.

Claims 41-46, drawn to a method of enrichment for acid tolerant bacteria, classified in class 435 subclasses 245 and 252.3.

II. Claims 47-65, drawn to aroC, aroD double mutants, vaccine comprising the mutants, classified in Classes 435 and 424, subclasses 252.3 and 92.

III. Claims 66-80, drawn to methods of immunizing with the mutant bacteria classified in Class 424, subclass 92.

Groups I and II contain different products. The groups are drawn to mutant or adaptive microorganisms with adaptations, genetic deletions and/or substitutions, resulting genotypically distinct microorganisms.

The application contains claims directed to the following distinct species of the claimed invention with regards to Group I, II and III.

1. Species of antigen the bacteria will express, when used as a carrier:
   a. protozoan. claims 12, 30 and 54, 64
   b. viral. claims 16, 33 and 57, 72
   c. bacterial. claims 19, 36 and 65, 75

With regard to Group II

1. Species of mutant bacteria
   a. Double aroC, aroD mutant with deletions. claim 48
   b. Double aroC, aroD mutant. claim 47.