OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

(12) (19) (CA) **Demande-Application**

(21) (A1) **2,244,807**
(86) 1997/02/13
(87) 1997/08/15

(72) COHEN, Paul S., US
(72) LAUX, David C., US
(72) FRANKLIN, David P., US
(71) THE BOARD OF GOVERNORS FOR HIGHER EDUCATION, STATE OF RHODE ISLAND AND PROVIDENCE PLANTATIONS, US

(51) Int.Cl.[6] C12N 1/21, C12N 1/36, A61K 39/112
(30) 1996/02/15 (08/601,790) US

(54) **VACCIN PRODUIT A PARTIR DE SALMONELLA TYPHIMURIUM**

(54) **SALMONELLA TYPHIMURIUM VACCINE**

(57) L'invention concerne un vaccin vivant protégeant contre un pathogène microbien, qui est avirulent, essentiellement incapable de se multiplier dans l'hôte et qui ne comporte aucun risque de se reconvertir en souche virulente sauvage.

(57) The present invention is a live vaccine against a microbial pathogen that is avirulent, substantially incapable of multiplication in the host, and has no probability for reverting to a virulent wild strain.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/00, 39/02, 39/112, C12N 1/00, 1/20, 1/21

A1

(11) International Publication Number: WO 97/29768

(43) International Publication Date: 21 August 1997 (21.08.97)

(21) International Application Number: PCT/US97/02074

(22) International Filing Date: 13 February 1997 (13.02.97)

(30) Priority Data:
08/601,790 15 February 1996 (15.02.96) US

(71) Applicant: THE BOARD OF GOVERNORS FOR HIGHER EDUCATION, STATE OF RHODE ISLAND AND PROVIDENCE PLANTATIONS [US/US]; 199 Promenade Street, Providence, RI 02908 (US).

(72) Inventors: COHEN, Paul, S.; 70 Lower College Road, Kingston, RI 02881 (US). LAUX, David, C.; 70 Lower College Road, Kingston, RI 02881 (US). FRANKLIN, David, P.; 60 State Street, Boston, MA 02109 (US).

(74) Agents: STEVENS, Richard, L. et al.; Samuels, Gauthier, Stevens & Reppert, Suite 3300, 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: SALMONELLA TYPHIMURIUM VACCINE

(57) Abstract

The present invention is a live vaccine against a microbial pathogen that is avirulent, substantially incapable of multiplication in the host, and has no probability for reverting to a virulent wild strain.

Title

Salmonella Typhimurium Vaccine

Field of the Invention

This invention relates to vaccines useful for the prevention or modification of microbial pathogenesis. One aspect of this invention relates to the identification and isolation of avirulent mutants of microbial bacteria suitable for such vaccines.

Another aspect of the invention relates to the use of such avirulent mutant bacteria as carriers of antigens derived from the disease causing pathogens, such that the resulting recombinant bacteria may be used as a vaccine against the disease, or diseases, from which the antigens were derived.

Background of the Invention

The means by which a warm blooded animal overcomes microbial pathogenesis is a complex process. Immunity to microbial pathogenesis is one means by which a warm blooded animal avoids pathogenesis, or suffers a less intense pathogenic state. Incomplete immunity to a given pathogen results in morbidity and mortality in a population exposed to a pathogen.

It is generally agreed that in the case of intracellular pathogens vaccines based on live but attenuated micro-organisms (live vaccines) induce a highly effective type of immune response. Such vaccines have the advantage that, once the animal host has been vaccinated, entry of the microbial pathogen into the host induces an accelerated recall of earlier, cell-mediated or humoral immunity which is able to control the further growth of the organism before the infection can assume clinically significant proportions. Vaccines based on a killed pathogen (killed vaccine) are generally conceded to be unable to achieve this type of response. However, vaccines that contain a live pathogen present the danger that the vaccinated host upon vaccination may contract the disease against which protection is being sought.

It would be desirable to have a vaccine that possesses the immunizing attributes of a live vaccine but that is not capable of causing an undesirable infection upon vaccination. To this end, a vaccine based on an avirulent, auxotrophic strain of *Salmonella typhimurium* has been utilized as an experimental immunogen, Hoiseth et al., Nature 291:238-239 (1981).

Curtiss, in United States Patent 5,294,441, teaches an immunogenic composition based on an avirulent derivative of *S. typhimurium*. The avirulent derivative is derived from a virulent wild strain. The derivatives carry the mutations Δ*cya* and/or Δ*crp*. This patent is also a tutorial on the prior art relating to immunogenic compositions based on *S. typhimurium*.

Stocker, in United States Patents 5,201,035; 4,837,151; and 4,735,801, discloses the attenuation of avirulent salmonella strains working with aro A^- and aro C^- including a discussion of heterologous disease antigens as fusion proteins in flagella.

Summary of the Invention

This invention, in one aspect, provides a live vaccine against a microbial pathogen. Because the immune response of the vertebrate host to antigens, in particular surface antigens, of the pathogenic microorganism is the basic mechanism of protection by vaccination, a live vaccine should retain the antigenic complement of the wild-type strain. The live vaccine should be avirulent, substantially incapable of multiplication in the host, and should have substantially no probability for reverting to a virulent wild strain. This vaccine contains, as its immunogenic agent, a live avirulent mutant strain of the microbial pathogen to which an immune reaction is to be induced. The vaccine contains the avirulent mutant strain in an effective amount together with a physiologically tolerable carrier and is free from an infective amount of any virulent strain of the pathogen. The avirulent strain is placed in the carrier for delivery to a warm blooded animal in a dosage amount sufficient to confer

protection against a virulent strain of the same pathogen.

The invention in another aspect comprises a method for preparing a live non-virulent vaccine from a virulent pathogenic microorganism, which vaccine is substantially incapable of reverting to virulence in a vertabrae host subject susceptible to said microorganism which comprises subjecting a virulent strain of said microorganisms to mutating conditions resulting in a mutated microorganism resulting in a block in at least one biosynthetic pathway which renders said organism auxotrophic with a requirement for a metabolite normally unavailable in host susceptible to said microorganism.

Once a pathogenic organism is selected for transformation into an avirulent strain, the desired mutagenesis can be achieved by one of several methods. The method of mutagenesis applied depends on the organism subjected to the procedure. Generally it is preferred that the mutation be as localized as much as possible and further that the reversion to the original genotype of the pathogen be as limited as possible.

Mutagenesis by DNA insertion is preferred and can be accomplished by transposon mutagenesis. This is a relatively non-reverting mutation accomplished at a localized site in the chromosome.

A general transducing phage, such as phage P22, able to adsorb to bacteria of a wide range of genera (if necessary after appropriate genetic modification of their lipopolysaccharide character, to provide the necessary ability to adsorb this phage) can be used to transduce a non-functional biosynthetic gene, inactivated by insertion of a transposon or otherwise, from its original host to a pathogenic bacterial strain of a different species of genus, wherein it will have some probability of incorporation into the chromosome, therefore replacing the homologous wild-type gene, to produce an auxotrophic transductant.

In accordance with the subject invention, the vaccines are produced by introducing a non-reverting mutation in at least one gene, where each mutation is of a sufficient number of bases in tandem to insure a substantially zero probability

of reversion and assurance of the non-expression of each mutated gene, in the sense of its total inability to determine production of an enzymatically active protein. The type and number of genes transduced will result in the likelihood that a host for the vaccine will provide the necessary nutrients for proliferation and will have a probability of reversion approximating zero.

The resulting auxotrophic strain will be an avirulent live vaccine having the desired immunogenicity in that the mutation will not affect the production of the antigens which trigger the natural immune response of the host. At the same time, the mutation results in an avirulent live vaccine incapable of growing in the host.

Those mutants that are determined to be avirulent as compared to the parental strain on the basis of the in vivo assays can then be further tested for their ability to confer protection against challenge with the parental strain by immunizing the host with the mutant strain prior to exposure of the host to the virulent parent strain. Protection is defined as the ability of the host animal to survive the challenge of a lethal dose of the virulent parent strain after immunization with the mutant.

The foregoing procedures of mutant isolation, screening in vivo, and immunization with the selected mutants, provide means by which avirulent strains of pathogenic microorganisms are obtained that are suitable for use in vaccines.

To provide for presentation of antigens of species other than the avirulent bacterial host, one or more genes coding for the desired antigens, or for enzymes for synthesis of the desired antigen(s), may be introduced into the host as expression cassettes. Expression cassettes includes transcriptional and translational initiation and termination regions bordering the structural genes of interest with the structural genes under the regulatory control of such regions.

The expression cassette may be a construct or may be or from part of a naturally-occurring plasmid such as the plasmid encoding the enzymes for production of the O-specific part of the LPS of *Shigella sonnei*. If the expression cassette is a

construct, it may be joined to a replication system for episomal maintenance or may be introduced into the bacterium under conditions for recombination and integration. The construct will normally be joined to a marker, e.g., a structural gene, and regulatory regions providing for antibiotic resistance or complementation in an auxotrophic host, so that the expression vector will usually include a replication system, e.g., plasmid or viral, one or more markers and the expression cassette of the structural gene of interest.

Structural genes of interest come from diverse sources, such as bacteria, viruses, fungi, protozoa, metazoan parasites or the like. The structural genes may encode envelope proteins, capsid proteins, surface proteins, toxins, such as exotoxins or enterotoxins, or the genes of interest may specify proteins, enzymes or other proteins needed for synthesis of a polysaccharide or oligosaccharide antigen or for modification of a saccharide-containing antigen, such as LPS, of the host bacterial strain, or for synthesis of a polypeptide antigen, such as the capsular antigen of *Bacillus anthracis*. These genes may be isolated in conventional ways employing probes where at least a partial amino acid or nucleic acid sequence is known, using Western blots for detection of expression, using λgt11 for expression of fused proteins for obtaining probes, identification of the antigen by the reaction of transconjugant bacterial colonies with antibody and detecting complex formation, e.g., agglutination, etc.

Specific genes of interest include those specifying the heat-labile and heat-stable enterotoxins of enterotoxigenic *E. coli* or *Vibrio chlorea* strains, surface, envelope or capsid proteins of *T. cruzi, B. pertussis,* Streptococci, e.g. *S. pneumoniae,* Haemophilus, e.g., *H. influenzae,* Neisseria, e.g., *N. meningitidis,* Pseudomonas, e.g., *P. aeruginosa,* Pasteurella, Yersinia, Chlamydia, Rickettsia, adenovirus, astrovirus, arenavirus, coronavirus, herpes virus, myxovirus, paramyxovirus, papovavirus, parvovirus, picoranvirus, pxovirus, reovirus,

retrovirus, rhabdovirus, rotavirus, togavirus, etc. or the genes specifying the enzymes needed for synthesis of polysaccharide, or for modification of the oligo- or polysaccharide antigen of the bacterial host strain.

The construct or vector may be introduced into the host strain by any convenient means such as conjugation, e.g., F^+ or Hfr strain, transformation, e.g., Ca precipitated DNA, transfection, transduction, etc; by fusion into fimbriae of flagella or intracellularly.

Among bacteria, the subject invention is particularly applicable to a wide variety of salmonella strains, more particularly of groups A, B, or D, which includes most species which are specific pathogens of particular vertabrae hosts. Illustrative of the salmonella causing disease for which live vaccines can be produced are *S. typhimurium*; *S. typhi*; *S. abortusovi*; *S. abortus-equi*; *S. dublin*; *S. gallinarum*; *S. pullorum*; as well as other which are known or may be discovered to cause infections in mammals.

Other organisms for which the subject invention may also be employed include Shigella, particularly *S. flexneri* and *S. sonnei*; Haemophilus, particularly *N. meningitidis* and *N. gonorrohoeae*; Pasteurella, particularly *P. multocida* and Yersinia, particularly *Y. pestis*.

The subject vaccines may be used in a wide variety of vertebrates. The subject vaccines will find particular use with mammals such as man and domestic animals. Domestic animals include bovine, ovine, porcine, equine, caprine, domestic fowl. Leporidate e.g., rabbits, or other animal which may be held in captivity or may be a vector for a disease affecting a domestic vertebrate.

In a preferred embodiment of the invention, an attenuated strain of S. *typhimurium* is mutated with a selected bacteriophage. A mutant embodying the invention, designated S. *typhimurium* SR-11 Fad^-, is characterized by the inability to utilize fatty acids and citrate for growth. The mutant is avirulent and immunogenic.

DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

Materials and Methods *S. typhimurium* SR-11 was selected as the pathogen. Mutagenesis was performed in *S. typhimurium* TT10427. Selected mutant alleles were bacteriophage P22 HT105 *int* transduced from *S. typhimurium* TT10427 into *S. typhimurium* SR-11.

BALB/C mice (Charles River Laboratories, Wilmington, MA) were selected as the experimental animals because of their sensitivity to *S. typhimurium* SR-11. The LD_{50} for BALB/C mice was found to be 8.2×10^4 Colony Forming Units (CFU) with a mean death time of 8 days following peroral infection.

The *S. typhimurium* strains were routinely grown aerobically in Luria broth (LB) and plated on Luria agar (LA). When platings were done from animal tissues, MacConkey agar was utilized, which is selective for the particular group of organisms of interest. Mutants were isolated on Minimal Broth Davis agar plates containing oleate and citrate as carbon sources. Media utilized was formulated in accordance with the following recipes.

LB

10 grams	Bacto-tryptone
5 grams	Bacto-yeast extract
5 grams	Sodium Chloride
1 liter	Distilled Water

pH adjusted to 7.0; for solid media, 12 grams of agar per liter were added

Minimal Broth Davis

7 grams	Dipotassium Phosphate
2 grams	Monopotassium Phosphate
0.5 grams	Sodium Citrate
0.1 grams	Magnesium Sulfate
1 gram	Ammonium Sulfate
1 liter	Distilled Water

MacConkey Agar

17 grams	Bacto-Peptone
3 grams	Proteose Peptone
10 grams	Bacto-Lactose
1.5 grams	Bacto-Bile Salts No. 3

5 grams	Sodium Chloride
13.5 grams	Bacto-Agar
0.03 grams	Bacto-Neutral Red
0.001 grams	Bacto-Crystal Violet
1 liter	Distilled Water

Plates were supplemented with either oleate (5mM, final concentration) and Brij 58 (5 mg/ml, final concentration) or with glucose (0.1% w/w, final concentration). Difco Noble agar (12 grams per liter) was used to solidify this medium.

Mutagenesis

A wild-type *S. typhimurium* LT2 (attenuated) strain, *S. typhimurium* TT10427, contains the plasmid pNK972, a pBR322 derivative, which carries a functional transposase gene derived from Tn10 (Way et al., Gene 32;369-379). *S. typhimurium* LT7 TT10605 contains an F *lac* plasmid which carries Tn10d Cam, a defective Tn10 which lacks a transposase gene and has chloramphenicol resistance substituted for tetracycline resistance (Elliot and Roth, Mol. Gen. Genet. 213:332-338). Tn10d Cam was introduced into *S. typhimurium* TT10427 from a bacteriophage P22 HT lysate of *S. typhimurium* LT7 TT10605.

Tn10d Cam-induced chloramphenicol resistant mutants were selected on LA containing 30 μg/ml of chloramphenicol as described by Elliot and Roth (Mol. Gen. Genet. 213:332-338). Colonies were toothpicked to Minimal Broth Davis agar plates containing either glucose or both oleate and citrate as carbon sources. Mutants that grew utilizing glucose as the carbon source but not oleate and citrate were bacteriophage P22 HT105 *int* transduced into *S. typhimurium* SR-11 using chloramphenicol for selection. All transductants tested grew utilizing glucose

as the sole carbon source but did not grow with oleate as the carbon source. One mutant, designated *S. typhimurium* SR-11 Fad$^-$ was shown to be free of P22 HT105 *int* by growth on the Green indicator plate described in *Advanced Bacterial Genetics, A Manual for Genetic Engineering,* Cold Spring Laboratory, Cold Spring Harbor, NY 1980.

Characterization of *S. typhimurium* SR-11 Fad$^-$

Both the *S. typhimurium* SR-11 and the *S. typhimurium* SR-11 Fad$^-$ ferment glucose, mannitol, sorbitol, rhamnose, melibiose, maltose, sucrose, and glycerol, suggesting that both strains are *cya*$^+$ and *crp*$^+$. Both strains contain the *S. typhimurium* SR-11 100 kbp virulence plasmid. Neither strain ferments inositol, amylose, arabinose, and saccharose. Both strains are motile in LB and glucose represses motility in both strains, further suggesting that both strains are *cya*$^+$ and *crp*$^+$. Both strains grow identically in Minimal Broth Davis containing glucose, ruling out the possibility that they are *aro*$^-$ or pur A$^-$. The two strains' abilities to grow on oleate, acetate, pyruvate, and tricarboxylic acid cycle intermediate are as follows.

Growth Utilizing Various Carbon Sources

Strain	OLE*	DEC	ACE	PYR	CIT	ICIT	SUC
SR-11	+	+	+	+	+	+	+
SR11 Fad$^-$	-	-	-	+	-	-	+

*Abbreviations: OLE, oleate; DEC, decanoate; ACE, acetate; PYR, pyruvate; CIT, citrate, ICIT, isocitrate; SUC, succinate. Symbols: +, excellent growth; -, no growth.

These results suggest that the mutation in *S. typhimurium* SR-11 Fad$^-$ shuts down part of the tricarboxylic acid cycle as well as the glyoxylate shunt in an as yet unknown way.

Preparation of Vaccine against *Salmonella*

S. typhimurium SR-11 Fad$^-$ is avirulent. *S. typhimurium* SR-11 and *S. typhimurium* SR-11 Fad$^-$ were grown aerobically overnight at 37°C. The cultures were centrifuged, washed twice in HEPES-Hanks buffer, pH 7.4 (Cohen et al., *Infect. Immun.* 40; 62-69) and resuspended in phosphate buffered saline containing 0.1% gelatin such that 20 μl delivered just behind the incisors resulted in administering the proper dose. Prior to inoculation, the BALB/C mice (13-15 grams, female) were starved for food and water for six hours and then fed 50 μl of 10% (W/V) sodium bicarbonate. Thirty minutes after peroral inoculation food and water were returned. The following table shows that whereas *S. typhimurium* SR-11 is highly pathogenic for BALB/C mice, *S. typhimurium* SR-11 Fad$^-$ is completely avirulent. At peroral doses of 10^9 and 10^8 CFU, mice inoculated with *S. typhimurium* SR-11 Fad$^-$ had ruffled hair for two to three days, but otherwise appeared healthy. Mice inoculated with 10^7 CFU of *S. typhimurium* SR-11 Fad$^-$ appeared healthy throughout the course of the experiment.

Table 1

Pathogenicity of *S. typhimurium* SR-11 and *S. typhimurium* SR-11 Fad$^-$ in Balb/c Mice

Peroral	*S. typhimurium* SR-11		*S. typhimurium* SR-11 Fad$^-$	
Inoculum(CFU)[a]	number infected	number alive[b]	number infected	number alive[b]
10^9	6	0	7	7

10^8	11	0	11	11
10^7	11	0	11	11
10^6	8	1	8	8
10^5	8	2	8	8
10^4	8	8	8	8

[a]CFU - Colony Forming Units
[b]All animals that died, died by day 9. All animals that lived, lived indefinitely.

S. typhimurium SR-11 Fad$^-$ protects BALB/C mice against S. typhimurium SR-11.

Three weeks after peroral inoculation, two mice each originally inoculated with 10^9, 10^8, and 10^7 CFU of *S. typhimurium* SR-11 Fad$^-$ were challenged with 10^9 CFU of *S. typhimurium*. None of the mice died and all appeared completely healthy throughout the course of the experiment. In contrast, an age-matched set of six mice which were not inoculated all died by nine days after peroral challenge with 10^9 CFU of *S. typhimurium* SR-11.

Table 2

Protection of Balb/c Mice Vaccinated with *S. typhimurium* SR-11 Fad$^-$ Against Infection by *S. typhimurium* SR-11

Peroral Inoculum (CFU)[a] with SR-11 Fad$^-$	Number infected	Number Alive when infected with 10^9 CFU of SR-11[b]	Number alive after infection with 10^9 CFU of SR-11 at 9 days	30 days
10^9	2	2	2	2
10^8	2	2	2	2
10^7	2	2	2	2
none[7]	6	6	0	0

[a]CFU - Colony Forming Units
[b]Mice were infected with SR-11 (10^9 CFU) at 21 days after infection with SR-11 Fad$^-$

Although a single dose of 10^7 SR-11 Fad$^-$ was protective, viable cells in the liver and spleen 11 days after oral administration (two mice tested) were not found nor was splenomegaly observed. However at single does of 10^8 and 10^9 CFU small numbers of CFU were detected in the liver (about 2

x 10^3 CFU/organ) and spleen about 5 x 10^3 CFU/organ) and splenomegaly was observed (two mice tested, see Table 3 below).

Table 3

Recovery of *S. typhimurium* SR-11 Fad$^-$ from Liver and Spleen Eleven Days Post Peroral Inoculation

Dose (CFU) SR-11 Fad$^-$	Liver Weight (g)	CFU/Liver	Spleen Weight (g)	CFU/Spleen
10^9	1.8	2.0 x 10^3	0.43	1.6 x 10^3
10^8	1.5	3.3 x 10^3	0.35	5.3 x 10^3
10^7	1.3	<10^2	0.09	<10^2

In contrast, mice orally infected with SR-11 at single doses of between 10^7 and 10^9 CFU contained much larger numbers in liver (about 3 x 10^7 CFU per organ) and spleen (about 2 x 10^6 CFU per organ) at day 8 (two mice tested), the time of death (see Table 4).

Table 4

Recovery of S. typhimurium SR-11 Fad- from Liver and Spleen at Death Eight Days After Peroral Inoculation

Dose (CFU) SR-11	Liver Weight (g)	CFU/Liver	Spleen Weight (g)	CFU/Spleen
10^9	1.0	2.0 x 10^7	0.13	1.4 x 10^6
10^7	1.4	6.3 x 10^7	0.10	2.2 x 10^6

In all, more than 40 BALB/c mice have been infected with SR-11 Fad$^-$ at doses that are lethal for mice infected with SR-11 without observing one death or a mouse in ill health.

SR-11 Fad$^-$ is also attenuated relative to SR-11 when administered intraperitoneally. Four out of four BALB/c mice were killed by injection of 50 CFU SR-11 intraperitoneally whereas at the same doses, four out of four mice survived intraperitoneal injection of SR-11 Fad$^-$. However, only two out of five mice survived intraperitoneal injection of 150 CFU of SR-11 Fad$^-$ and only one out of five mice survived intraperitoneal doses of 1.5 x 10^3 and 1.5 x 10^4 CFU (see Table 5). These data suggest that the major defect in SR-11 Fad$^-$ is getting across the intestinal mucosal barrier.

Table 5

Mortality of BALB/c Mice after Intraperitoneal Challenge

Strain	Dose(CFU)	Total Mice	Survivors[1]
SR-11	50	4	0
	120	2	0
SR-11 Fad$^-$	50	4	4
	150	5	2
	1.5×10^3	5	1
	1.5×10^4	5	1
	1.5×10^5	5	0

[1]All survivors lived indefinitely, all mice that died were dead 8 days post challenge.

The serum of three mice orally dosed with SR-11 Fad$^-$, challenged with 10^9 CFU of SR-11 21 days later, and bled 60 days later was assayed. One of these mice originally dosed orally with 10^9 CFU of SR-11 Fad$^-$ had a serum agglutination titer of 1:2 against both SR-11 and SR-11 Fad$^-$. The second mouse originally dosed orally with 10^8 CFU of SR-11 Fad$^-$ had a serum agglutination tier of 1:4 against both strains, and the third mouse had a serum agglutination titer of 1:16 against both strains (see Table 6).

Table 6

Agglutination Testing
Female BALB/c Mice

Serum collected from mice 60 days post SR-11 Challenge
Serum titers on whole bacterial cells

Original SR-11 Fad$^-$ inoculum	SR-11 cells	SR-11 Fad$^-$ cells
10^9	1:2	1:2
10^8	1:4	1:4
10^7	1:16	1:16

Normal mouse serum gave no reaction with either strain of bacterial cells (Female BALB/c mice)

Normal BALB/c mouse serum did not agglutinate either strain. The serum titer against Widal antigens (O-side chain) was determined on the serum taken from one mouse originally dosed with 10^8 CFU of SR-11 Fad$^-$, challenged with 10^9 CFU of SR-11 21 days later, and bled 30 days later. The serum titer

against Widal antigens was 1:320. Normal mouse serum did not show any reactivity.

Restriction analysis of plasmid DNA recovered from S. typhimurium SR-11 WT and SR-11 Fad$^-$ revealed identical digestion profiles when treated with either EcoR1 of PvuII enzymes. Appropriate control stains were used to illustrate presence of absence of 100 kb virulence plasmid:

S. typhimurium SL5319 x 4360 (Vir$^+$)

S. typhimurium SL5319 x 4361 (Vir$^-$)

DEPOSIT

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, deposit of the *S. typhimurium* SR-11 Fad$^-$ was made on Feb. 6, 1997 with the American Type Culture Collection (ATCC) of Rockville, Md., USA, where the deposit was given Accession Number 55928.

The foregoing description has been limited to a specific embodiment of the invention. It will be apparent, however, that variations and modifications can be made to the invention, with the attainment of some or all of the advantages of the invention. Therefore, it is the object of the appended claims to cover all such variations and modifications as come within the true spirit and scope of the invention.

Having described our invention, what we now claim is:

1. An immunogenic composition for the immunization of a vertebrate host which comprises:

a live avirulent *S. typhimurium* obtained from a pathogenic *S. typhimurium* strain, said avirulent *S. typhimurium* being characterized by a mutation which prevents growth on fatty acids and citrate and which mutant is a result of an insertation mutation made to the pathogenic S. *typhimurium* strain.

2. An immunogenic composition according to claim 1, wherein said avirulent S. typhimurium is characterized by an antigen which induces an immune response in said host against said pathogenic *S. typhimurium* strain.

3. A method for stimulating the immune system of a vertebrate host to respond to an immunogenic antigen of *S. typhimurium* comprising administering to said individual an immunogenic composition comprising a live avirulent *S. typhimurium* obtained from a pathogenic *S. typhimurium* strain, said avirulent *S. typhimurium* characterized by a mutation which prevents growth on fatty acids and citrate and which mutant is a result of an insertation mutation made to the pathogenic S. *typhimurium* strain.

4. An isolated avirulent strain of *S. typhimurium* SR-11 Fad$^-$ obtained from a pathogenic strain of *S. typhimurium* said avirulent *S. typhimurium* SR-11 Fad$^-$ characterized by a transposon insertion which prevent reversion to virulence.

5. The isolated avirulent strain of *S. typhimurium* of claim 4 which expresses a mutant gene from an agent pathogenic to a vertebrate host, to produce an antigen which induces an immune response in said host against said pathogen.

6. An immunogenic composition according to claim 1 comprised of a strain according to claim 4.