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(54) **AVIRULENT SALMONELLA GALLINARUM VARIANTS AND PHARMACEUTICAL COMPOSITION USING THE SAME**

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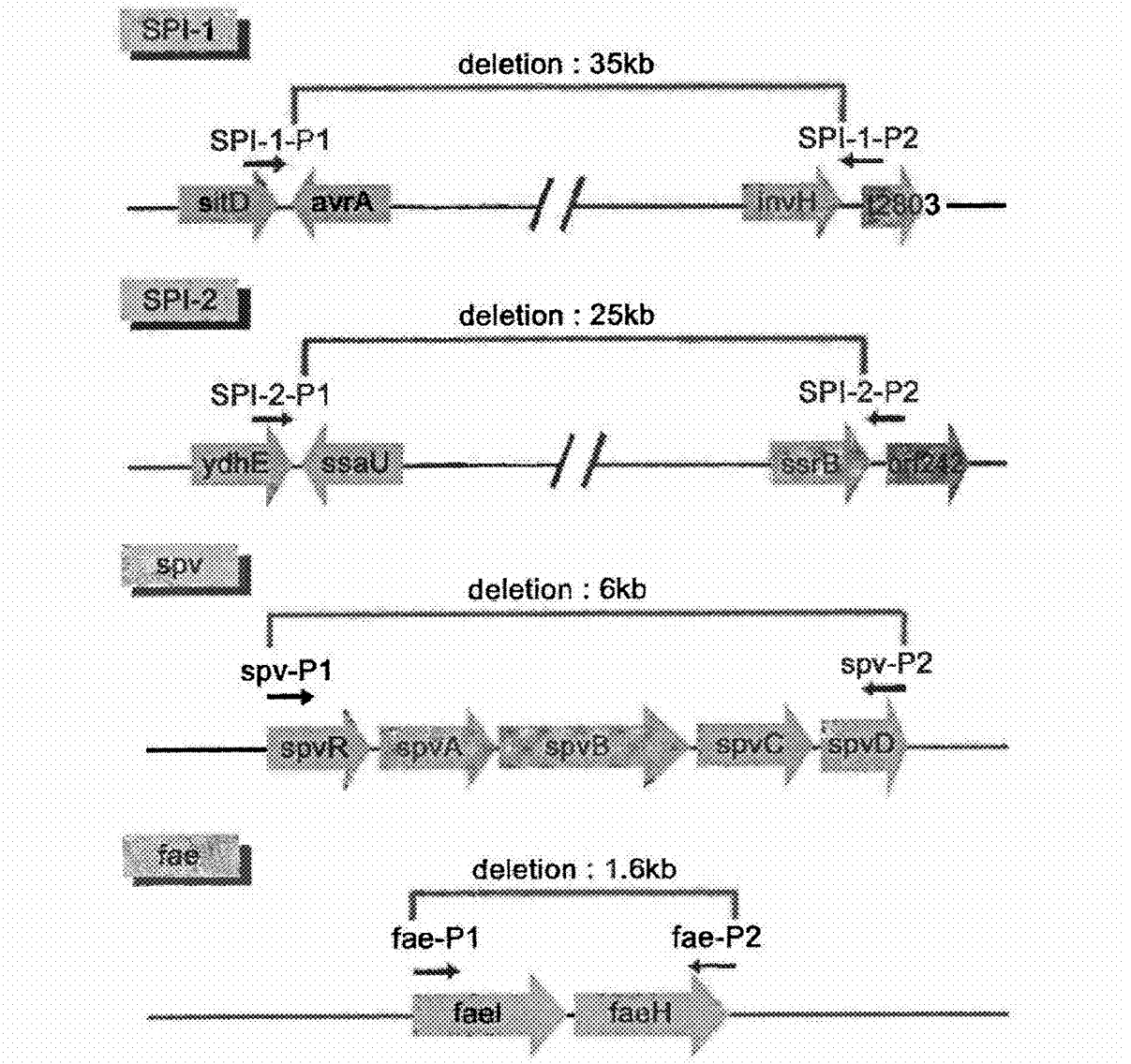
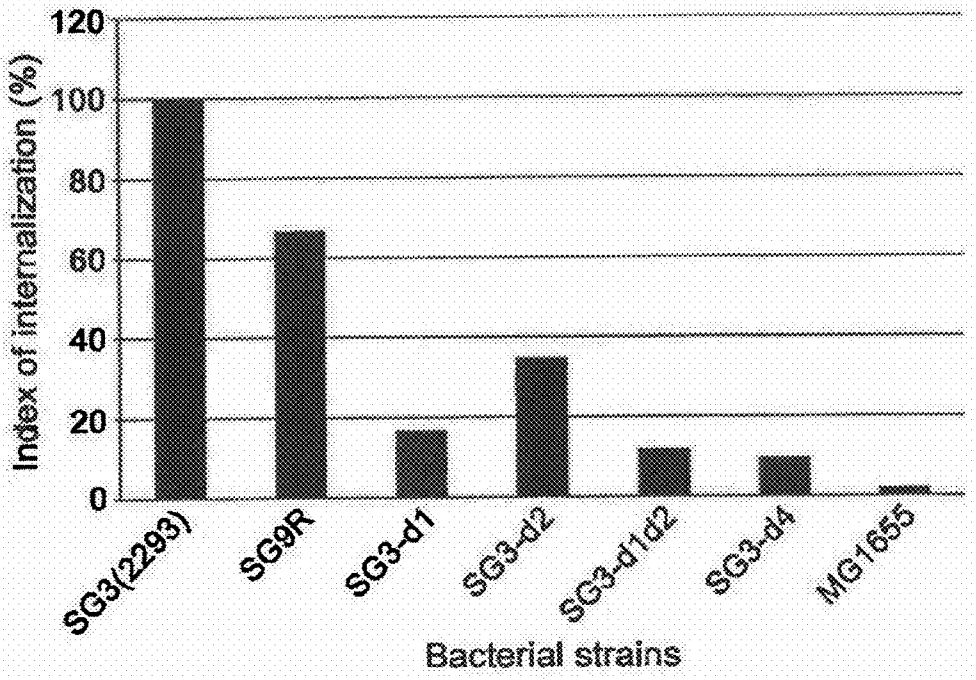
(57) **ABSTRACT**

The present invention relates to avirulent *Salmonella Gallinarum* variants by inactivating virulence gene clusters of *Salmonella Gallinarum* (SG), a main pathogen of avian salmonellosis, and various uses thereof notably in the production of *Salmonella*-specific lytic bacteriophages, pharmaceutical compositions and feed additives.

[Figure 1]


SPI-1
deletion : 35kb
SPI-1-P1
SPI-1-P2
sitD
avrA
invH
SPI-2
deletion : 25kb
SPI-2-P1
SPI-2-P2
ydhE
ssaU
ssrB
spv
deletion : 6kb
spv-P1
spv-P2
spvR
spvA
spvB
spvC
spvD
fae
deletion : 1.6kb
fae-P1
fae-P2
faeI
faeH

[Figure 2]


120
100
80
60
40
20
0
Index of internalization (%)
SG3(2293)
SG9R
SG3-d1
SG3-d2
SG3-d1d2
SG3-d4
MG1655
Bacterial strains

# AVIRULENT SALMONELLA GALLINARUM VARIANTS AND PHARMACEUTICAL COMPOSITION USING THE SAME

## REFERENCE TO A SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

**[0001]** The content of the electronically submitted sequence listing, file name: 2511_0120002_SEQ_ID_Listing.ascii.txt; size: 95,695 bytes; and date of creation: Sep. 14, 2012, filed herewith, is incorporated herein by reference in its entirety.

## BACKGROUND OF THE INVENTION

**[0002]** 1. Field of the Invention

**[0003]** The present invention provides avirulent *Salmonella* variants and various uses thereof, particularly in the production of *Salmonella*-specific lytic bacteriophages, pharmaceutical compositions, and feed additives.

**[0004]** 2. Description of the Related Art

**[0005]** Currently over 2,000 *Salmonella* strains are generally classified into host-specific serotypes, and non-host-specific serotypes pathogenic for both animals and humans. Representative among fowl-adapted pathogens are *Salmonella Gallinarum* (SG) and *Salmonella Pullorum* (SP) which are known to cause fowl typhoid and *pullorum* disease, respectively. These *Salmonella*-caused fowl diseases occur at low frequency in advanced countries, but have inflicted tremendous economic damage on the poultry farming in developing countries.

**[0006]** *Salmonella Gallinarum* strains have serologically the same somatic antigen (O-antigen) structures and are classified as being non-motile because they have no flagella. When entering into a host animal via contaminated feed or a contaminated environment, *Salmonella* pass through the gastrointestinal tract, and invade intestinal epithelial cells by interaction with Peyer's patch M (microfold) cells and penetrate into the intestinal membrane. *Salmonella* are transported by the M cells to macrophages in adjacent intestinal membranes, and then *Salmonella* infection develops into a systemic disease.

**[0007]** The type III secretion system (TTSS) is a protein appendage found in Gram-negative bacteria, which consists of a needle-like protein complex structure through which virulence effector proteins pass from the bacterial cytoplasm directly into the host cytoplasm (Mota L J et al., Ann Med. (2005);37(4):234-249). The type III secretion system is essential for the delivery of the pathogenicity of *Salmonella* (Schlumberger M C et al., Curr Opin Microbiol. (2006);9(1): 46-54). Wild-type *Salmonella* take advantage of TTSS when adhering to and invading host cells, and then survives during the phagocytosis of macrophages and circulates throughout the body via the bloodstream, causing a systemic infection. Hence, *Salmonella* infection cannot proceed without the normal operation of TTSS. *Salmonella* pathogenicity island-1 (hereinafter referred to as "SPI-1") is a discrete region of the *Salmonella* chromosome encoding the type III secretion system and virulent effector proteins which are necessary for invasion into intestinal epithelial cells in the early stage of infection (Kimbrough T G et al., Microbes Infect, (2002);4 (1):75-82). *Salmonella* pathogenicity island-2 (hereinafter referred to as "SPI-2") is also a discrete region of the *Salmonella* chromosome encoding the type III secretion system and effector proteins which involved in survival and proliferation during phagocytosis by macrophages in intestinal immune organs or immune organs such as the spleen and the liver after translocation across epithelial cells (Waterman S R et al., Cell Microbiol, (2003);5(8):501~511, Abrahams G L, Cell Microbiol, (2006);8(5):728-737). Genes within SPI-1 and SPI-2 and their functions are summarized in Table 1, below.

TABLE 1

| | Gene | Characteristics |
|---|---|---|
| SPI-1 | avrA | putative inner membrane protein |
| | sprB | transcriptional regulator |
| | hilC | bacterial regulatory helix-turn-helix proteins, araC family |
| | orgA | putative flagellar biosynthesis/type III secretory pathway protein |
| | prgK | cell invasion protein; lipoprotein, may link inner and outer membranes |
| | prgJIH | cell invasion protein |
| | hilD | regulatory helix-turn-helix proteins, araC family |
| | hilA | invasion genes transcription activator |
| | iagB | cell invasion protein |
| | sptP | protein tyrosine phosphate |
| | sicP | chaperone, related to virulence |
| | iacP | putative acyl carrier protein |
| | sipADCB | cell invasion protein |
| | sicA | surface presentation of antigens; secretory proteins |
| | spaSRQPO | surface presentation of antigens; secretory proteins |
| | invJICB | surface presentation of antigens; secretory proteins |
| | invAEGFH | invasion protein |
| SPI-2 | ssaUTSRQPON VMLKJIHG | Secretion system apparatus |
| | sseGF | Secretion system effector |
| | sscB | Secretion system chaperone |
| | sseEDC | Secretion system effector |
| | sscA | Secretion system chaperone |
| | sseBA | Secretion system effector |
| | ssaE | Secretion system effector |
| | ssaDCB | Secretion system apparatus |
| | ssrA | Secretion system regulator: Sensor component |
| | ssrB | Secretion system regulator: transcriptional activator, homologous with degU/uvrY/bvgA |

**[0008]** In addition to these type III secretion systems, fimbriae gene (faeHI) (Edwards R A et al., PNAS (2000); 97 (3):1258-1262) and the virulent factor (spvRABCD operon) present in virulent plasmids of *Salmonella* are implicated in the virulence of *Salmonella* (Gulig P A et al., Mol Microbiol (1993);7(6):825-830).

**[0009]** *Salmonella*-caused fowl diseases are difficult to control because they are transmitted in various ways including egg transmission, and feed or environmental infection, and show high recurrence rates even after post-infectious treatment with antibiotics. Therefore, it is importance of preventing the onset of disease by using a vaccine as well as sanitizing breeding farms and feed. In the poultry industry, a lot of effort has been poured into the use of live vaccines (attenuated *Salmonella Gallinarum* strains—SG9S, SG9R) and dead vaccines (gel vaccines, oil vaccines, etc.) to prevent the onset of fowl typhoid. However, the effects of the vaccine vary with the concentration of the vaccine used, the condition of the fowl vaccinated, and the environment of chicken houses. And, the efficacy of these vaccines is reported to be significantly lower than that of the vaccines for other diseases. Treatment with antibiotics, although reducing the lesion, con-

verts infected fowls into chronic carriers (See: Incidence and Prevention of Hen Salmonellosis, the National Veterinary Research & Quarantine Service, Korea).

**[0010]** Therefore, new *Salmonella*-controlling approaches that are better than conventional vaccines or antibiotics are being demanded. Many scientists have recently paid attention to bacteriophages, which infect and lyse bacteria specifically and are safe to humans, as a potent alternative to antibiotics. There are many reports concerning the use of bacteriophages being used in the prevention or therapy of *Salmonella* diseases (Atterbury R J et al., Appl Environ Microbiol, (2007); 73 (14):4543-4549) and as disinfectants or detergents to prevent the putrefaction of foods (PCT 1998-08944, PCT 1995-31562, EP 1990-202169, PCT 1990-03122), and concerning phage display techniques for diagnosis (Ripp S et al., J Appl Microbiol, (2006);100(3):488-499), *Salmonella* vaccines prepared by deleting or modifying one or two genes within SPI-2 gene cluster have recently been disclosed (U.S. Pat. No. 6,923,957, U.S. Pat. No. 7,211,264, U.S. Pat. No. 7,887,816).

**[0011]** For industrial use, bacteriophages are produced by separating the phage progenies from the host cells lysed during the proliferation of bacteriophages which have been inoculated into the host cells cultured on a mass scale. As for bacteriophages specific for pathogenic bacteria, however, their lysates may contain the pathogenic host cells being not removed, and/or virulent materials such as pathogenic proteins of the host. This likelihood acts as a great risk factor to the safety of bacteriophages produced on the basis of pathogenic host cells.

**[0012]** Many bacteria have lysogenic phages on their chromosomes; however, most of the phages are cryptic and cannot produce progeny because of the accumulation of many mutations as ancestral remnants. Lysogenic phages, although inactive, may help the survival capacity of *Salmonella* upon host infection because they contain the genes necessary for lytic and lysogenic growth and some of the genes encode pathogenic factors. However, these genes are likely to undergo homologous recombination with the viral genome of other similar phages which newly infect animals, thus producing genetically modified phages. As for the typical *Salmonella typhimurium*, it has fels-1, fels-2, gifsy-1, and gifsy-2 prophages and two cryptic phages. In contrast, *Salmonella Gallinarum* could be used as a phage-producing host since *Salmonella Gallinarum* have neither prophages nor cryptic phages, and then are not genetically modified by recombination, (Edwards P A et al, Trends Microbiol, (2002); 10(2):94-99).

**[0013]** For the purpose of minimizing toxic remnants during progeny production and phage's opportunity for mutation, the present inventors designed the idea that the virulence gene clusters of *Salmonella Gallinarum* could be inactivated for producing bacteriophages. There have no precedent cases wherein avirulent bacteria, which had been converted from virulent bacteria by inactivating a virulence gene cluster, were used as a bacteriophage host cell.

**[0014]** In addition to the production of bacteriophages, the *Salmonella* deprived of virulence by inactivating virulence gene clusters are themselves used for developing attenuated live vaccines for controlling *Salmonella* or applied to the bioindustry, guaranteeing significant added values.

**[0015]** In the present invention, avirulent *Salmonella Gallinarum* variants obtained by inactivating at least one of the main *Salmonella* virulence gene clusters (SPI-1, SPI-2, spvRABCD and faeHI operons) are used as a bacteriophage-producing host cell and applied to various uses.

## SUMMARY OF THE INVENTION

**[0016]** With the aim of solving the problems with the recombinational modification of progeny phages and the toxic bacterial remnants in the course of bacteriophage production on the basis of the above-described facts, the present inventors developed avirulent *Salmonella Gallinarum* variants as a host cell for bacteriophage-producing by inactivating at least one of the four main *Salmonella*. Gallinarum gene clusters (SPI-1, SPI-2, spvRABCD and faeHI operons). In addition, the present inventors primarily confirmed reduced virulence by measuring the efficiency of the invasion of *Salmonella Gallinarum* into avian epithelial cells, and reconfirmed by measuring the mortality of hens infected with avirulent *Salmonella Gallinarum* variants. On the other hand, the present inventors approve the use of bacteriophage-producing host, the use of the pharmaceutical compositions and feed additives for the prevention or treatment of avian salmonellosis through comparison of the productivity of bacteriophages between wild-type and the avirulent *Salmonella Gallinarum* variants.

**[0017]** It is therefore a primary object of the present invention to provide a *Salmonella Gallinarum* variant in which the SPI-2 gene cluster is inactivated, a *Salmonella Gallinarum* variant in which both SPI-1 and SPI-2 gene clusters are inactivated, and an avirulent *Salmonella Gallinarum* variant in which at least one of the four main virulence gene clusters (SPI-1, SPI-2, spvRABCD, and faeHI operon) has been inactivated.

**[0018]** It is another object of the present invention to provide the use of the avirulent *Salmonella Gallinarum* variant in the production of *Salmonella*-specific bacteriophages or a method for producing phages using the avirulent *Salmonella Gallinarum* variant. The avirulent *Salmonella Gallinarum* variants according to the present invention can be used for the mass-production of *Salmonella*-specific lytic bacteriophages free of remnant toxicity and applied to the development of a novel concept of antibiotic substitutes which have high industrial utility value and guarantee significant added value.

**[0019]** It is a further object of the present invention to provide a pharmaceutical composition comprising avirulent *Salmonella Gallinarum* variants as an active ingredient, preferably a live vaccine and a feed additive. The SPI-1 gene cluster encodes type III secretion system proteins which remain on cell surfaces, acting as an antigen while the SPI-2 gene cluster encodes proteins which are involved in survival in the phagosomes after passage across epithelial cells. Hence, the inactivation of the SPI-2 gene cluster alone, with SPI-1 gene cluster remaining intact, leaves the antigen necessary for the production of an antibody inducing an immune response, but does not allow the bacteria to survive during phagocytosis, which does not result in a systemic disease. Thus, the SPI-2 gene cluster-inactivated *Salmonella Gallinarum* variant might be used as a live vaccine.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0020]** The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

**[0021]** FIG. **1** is a schematic diagram showing virulence genes of avian *Salmonella* (Salmonella pathogenicity island-1, *Salmonella* pathogenicity island-2, spvRABCD, faeHI) and sites corresponding to primers for inactivating the virulence genes; and

**[0022]** FIG. **2** is a graph showing the efficiency of the in vitro invasion into avian epithelial cells of the virulence gene-inactivated *Salmonella Gallinarum* variants (SG3-d1, SG3-d2, SG3-d1d2, SG3-d4), together with controls wild-type *Salmonella Gallinarum* SG2293), *Salmonella Gallinarum* live vaccine (SG9R), and non-pathogenic *E. coli* (MG1655). Invasion efficiency is expressed as a percentage of the count of microorganisms within cells divided with the count of microorganisms within a culture medium. The microorganisms were used at a concentration of $8.0\times10^7$ cfu per well.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

**[0023]** In order to accomplish the above objects, an aspect of the present invention provides the avirulent *Salmonella Gallinarum* variants which are remarkably decreased in pathogenicity.

**[0024]** The *Salmonella Gallinarum* variants are rendered avirulent by inactivating at least one of the virulence gene clusters *Salmonella* pathogenicity island-1, *Salmonella* Pathogenicity Island-2, spvRABCDf and faeHI.

**[0025]** As used herein, the term "virulence gene clusters of *Salmonella*" refers to the four gene clusters involved in the virulence of *Salmonella Gallinarum*, including the *Salmonella* Pathogenicity Island-1 (hereinafter referred to as "SPI-1") operon coding for the structural proteins and toxic effector proteins of type III secretion system, the *Salmonella* Pathogenicity Island-2 (hereinafter referred to as "SPI-2") operon coding for the structural proteins and toxic effector proteins of type III secretion system, the spvRABCD operon coding for pathogenically active proteins on avian *Salmonella*-specific virulent plasmids, and the faeHI operon coding for fimbriae. So long as it functionally works in *Salmonella Gallinarum*, any gene cluster may be used.

**[0026]** The term "gene cluster," as used herein, refers to a population of adjacent genes on a chromosome or a plasmid that are commonly responsible for the same products. The genes in one cluster are under the regulation of common regulatory genes.

**[0027]** The inactivation of genes in bacteria can be achieved using various methods. For example, single or multiple nucleotides of an active site within a gene may be modified to decrease the activity of the protein expressed. Alternatively, an antibiotic-resistant gene or other gene(s) may be inserted into the gene of interest to prevent the expression of intact proteins. The most reliable method is to delete the entire sequence of a gene from the genome (Russell C B et al., J. Bacteriol. (1989); 171:2609-2613, Hamilton C M et al., J. Bacteriol. (1989); 171:4617-4622, Link A J et al., J. Bacteriol. (1997); 179:6228-6237). In the present invention, entire sequences of the genes of interest are deleted to effectively promise the inactivation of the genes. For this, the one-step deletion method using lambda Red recombinase, known as a method of deleting gene clusters, developed by Datsenk K A et al., may be employed (Datsenko K A et al., PNAS, (2000); 97 (12):6640-6645).

**[0028]** With regard to the information of virulence genes to be deleted, nucleotide sequences of SPI-1 and SPI-2 were, obtained referring to the virulence gene sequences within the *Salmonella Gallinarum* chromosome (*Salmonella enterica* subsp. *enterica* serovar *Gallinarum* str. 287/91, NC 011274), disclosed by the NCBI. For the faeHI operon sequence, reference was made to the sequence of the *Salmonella Gallinarum* virulence plasmid gene (*Salmonella Gallinarum* virulence plasmid minor fimbrial subunit genes, AF005899). For the spvRABCD operon, the sequence of the same name gene of *Salmonella Typhimurium* LT2, which has highly homology with *Salmonella Gallinarum*, was consulted because its sequence is not disclosed in the NCBI. The sequencing of the spvRABCD operon of *Salmonella Gallinarum* was also performed with reference to the sequence of the corresponding gene of *Salmonella Typhimurium*.

**[0029]** Examples of the *Salmonella* virulence genes clusters include the SPI-1 gene cluster (SEQ ID NO: 1), the SPI-2 (SEQ ID NO: 2), the spvRABCD operon (SEQ ID NO: 3), and the faeHI operon (SEQ ID NO: 4) of *Salmonella Gallinarum* 287/91.

**[0030]** To prepare strains that, had definitely been rendered avirulent, all of the plural virulence gene clusters were deleted. To inactivate many gene clusters in one strain, the gene clusters may have been deleted sequentially.

**[0031]** In the present invention, a *Salmonella Gallinarum* strain in which only the SPI-2 gene cluster is inactivated (SG3-d2), a *Salmonella Gallinarum* strain in which both SPI-1 and SPI-2 gene clusters are integrally inactivated (SG3-d1d2) and a *Salmonella Gallinarum* strain in which all of the four virulence gene clusters (SPI-1, SPI-2, spvRABCD, faeHI) are integrally inactivated (SG3-d4). SG3-d2 is deposited under accession No. KCCM 11009P, SG3-d1d2 under accession No. KCCM 11010P, and SG3-d4 under accession No. KCCM 11011P.

**[0032]** Studies on the independent deletion of individual genes of the gene clusters have been reported (Hapfelmeier S et al., J Immunol, (2005); 174(3): 1675-1685, Brumme S et al., Vet Microbiol, (2007); 124(3-4):274-285, Desin T S et al., Infect Immun, July (2009); 2866-2875), but avirulent *Salmonella* strains developed by integrally inactivating two or more entire gene clusters had not been disclosed prior to the study of the present inventors. The *Salmonella Gallinarum* strain was named *Salmonella Gallinarum* SG2293-d2 when only the SPI-2 gene cluster is inactivated, and SG2293-d1d2 when both SPI-1 and SPI-2 were integrally inactivated. Further, it was named SG2293-d4 upon the inactivation of all of SPI-1, SPI-2, spvRABCD, and faeHI.

**[0033]** To ascertain the avirulence thereof, the strains prepared by inactivating virulence gene clusters according to the present invention were assayed for the efficiency of invasion into avian epithelial cells and for disease outbreak and mortality (%) upon infection into poultry. Preferably, the *Salmonella Gallinarum* strains in which the virulence gene clusters had been inactivated by transformation were allowed to invade avian epithelial cells so that invasion efficiency could be measured. Also, the strains were injected into brown egg layers to measure mortality.

**[0034]** In accordance with another aspect thereof, the present invention provides an avirulent *Salmonella*, strain for use in producing *Salmonella*-specific lytic bacteriophages and a method for producing phages using the same.

**[0035]** ΦCJ1 (US 20100135962), a *Salmonella*-specific phage, was used to examine the bacteriophage productivity of the avirulent *Salmonella Gallinarum* variants. The phage shows a specific bactericidal activity against *Salmonella Gallinarum* and *Salmonella pullorum*, belongs to the morphotype group of the family Siphoviridae B1, characterised by isometric capsid and long non-contractile tail, and has a total genome size of 61 kb and major structural proteins with a size of 38 kDa and 49 kDa.

**[0036]** The method for producing a bacteriophage in accordance with the present invention comprises culturing the avirulent *Salmonella Gallinarum* variants in a medium, inoculating a bacteriophage into the medium, and recovering the bacteriophage. In this regard, the phage may be produced briefly using a plate or on a mass scale using broth. In the case of production using a plate, a bacteriophage is inoculated at a suitable ratio into bacteria when the bacteria enter a log phase, mixed with top agar, and poured onto a plate. When phage plaques appear, the top agar fractions are collected and centrifuged, followed by filtering the supernatant to afford a phage stock. For mass production as a broth, a mixture of phages and bacteria is prepared in the same manner as in plate production, and incubated for 5 hours in fresh broth, instead of in top agar.

**[0037]** In accordance with a further aspect thereof, the present invention provides a pharmaceutical composition for the prevention of fowl typhoid, comprising the avirulent *Salmonella*, strain as an active ingredient and optionally a pharmaceutically acceptable vehicle, and preferably a vaccine for the prevention of fowl typhoid, formulated with the avirulent *Salmonella* strain and optionally a pharmaceutically acceptable vehicle.

**[0038]** The term "pharmaceutically acceptable vehicle," as used herein, refers to a carrier or diluent which does not deteriorate the biological activity and property of the active ingredient and which does not irritate the subject. Preparations intended for oral administration may take the form of tablets, troches, lozenges, aqueous or oily suspensions, powders, granules, emulsions, hard or soft capsules, syrups, elixirs, etc. In regards to the oral forms such as tablets and capsules, the active ingredient may be formulated in combination with a binder such as lactose, saccharose, sorbitol, mannitol, starch, amylpectin, conjugate such as cellulose or gelatin, an excipient such as dicalcium phosphate, a disintegrant such as corn starch or sweet potato starch, or a lubricant such as magnesium stearate, calcium stearate, sodium stearylfumarate or polyethylene glycol wax. As for capsules, they may further comprise a liquid carrier such as fatty oil.

**[0039]** The composition of the present invention may be formulated into preparations for non-oral administration, such as subcutaneous injections, intravenous injections, or intradermal injections. For this, the composition of the present invention may be mixed with a stabilizer or buffer in water to give a solution or a suspension which is then formulated into unit doses such as ampules or vials.

**[0040]** As used herein, the term "vaccine" refers to a biological preparation that improves immunity to a particular disease by inducing the formation of an antibody upon injection into the body, a preparation containing an antigen, e.g., killed or attenuated forms of a disease-causing microorganism. Vaccines may be prepared from killed pathogens. There are also live vaccines, but with the virulence thereof attenuated. The *Salmonella Gallinarum* variants of the present invention have the same antigenic proteins as those of the wild-type, but are greatly decreased in virulence compared to the wild-type, so that they can be used as live vaccines prophylactic of fowl typhoid.

**[0041]** In accordance with still another aspect thereof, the present invention provides a feedstuff containing the avirulent *Salmonella Gallinarum*, and preferably a feed additive containing the avirulent *Salmonella Gallinarum*. When applied to poultry, the feed additive of the present invention serves as a live vaccine that prevents fowl typhoid.

**[0042]** The feedstuff of the present invention may foe prepared by mixing feedstuff with the *Salmonella Gallinarum* variant as it is or in the form of a feed additive. In the feedstuff, the *Salmonella Gallinarum* variant may be in a liquid or dry state. The dry state can be accomplished by various drying methods including, but not limited thereto, pneumatic drying, spontaneous drying, spray drying and freeze drying. In addition to the *Salmonella Gallinarum* variant of the present invention, the feedstuff of the present invention may further comprise a typical additive useful for improving the preservation of the feedstuff.

**[0043]** The feedstuff comprising the *Salmonella Gallinarum* variant of the present invention may be vegetable matter such as a cereal, nut, a by-product of food processing, millet, fiber, pharmaceutical by-product, a vegetable oil, starch, oil seed meals and cereal remnants, or animal matter such as proteins, minerals, fats, mineral oils, unicellular proteins, animal planktons and leftover food etc.

**[0044]** Examples of the feed additive comprising the *Salmonella Gallinarum* variant of the present invention include, but are not limited to, various agents for preventing quality deterioration and improving utility, such as binders, emulsifiers, preservatives, amino acids, vitamins, enzymes, probiotics, flavoring agents, non-protein nitrogen compounds, silicates, buffer, colorants, extracts, oligosaccharides, etc. Also, a mixing agent may be within the scope of the feed additive.

**[0045]** In accordance with still a further aspect thereof, the present invention provides a method for treating the *Salmonella Gallinarum* infectious disease fowl typhoid using the pharmaceutical composition.

**[0046]** The composition of the present invention may be administered to animals in the form of a pharmaceutical preparation to animals, or in the form of being mixed with feedstuff or water. Preferably, it is mixed in the form of a feed additive with feedstuff before administration.

**[0047]** So long as it allows the composition of the present invention to reach tissues or cells of interest, any administration route, such as non-oral, intraartery, intradermal, transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral or intranasal route, may be taken.

**[0048]** The treating method of the present invention comprises administering the composition of the present invention in a pharmaceutically effective amount. It will be apparent to those skilled in the art that the suitable, total daily dose may be determined by an attending physician within the scope of medical judgment. The specific therapeutically effective dose level for any particular patient may vary depending on a variety of factors, including the kind and degree of desired reaction, the specific composition, including the use of any other agents according to the intended use, the patient's age, weight, general health, gender, and diet, the time of administration, the route of administration, and rate of the excretion of the composition; the duration of the treatment; other drugs used in combination or coincidentally with the specific composition; and like factors well known in the medical arts. Typically, the composition may be administered at a daily dose of from $10^4$ to $10^8$ CFU once or in a divided dosage manner.

**[0049]** Hereinafter, the present invention will be described in more retail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1

Screening of Target Genes to be Inactivated through Comparison of *Salmonella Gallinarum* Virulence Genes

**[0050]** The first step of preparing avirulent avian *Salmonella* strains was the screening of target virulence genes to be inactivated. *Salmonella* Pathogenicity Island-1 (SPI-1), and *Salmonella* Pathogenicity Island-2 (SPI-2), both of which are type three secretion system gene clusters essential for the delivery of the pathogenicity of *Salmonella*, and spvRABCD and faeHI, both of which are genes on virulence plasmids, were determined as target genes, and the data base of the NCBI was searched for the nucleotide sequences of the target genes (*Salmonella enterica* subsp. *enterica* serovar *Gallinarum* str. 287/91, NC 011274). Because the nucleotide sequence of spvRABCD of *Salmonella Gallinarum* had not yet been disclosed, primers were synthesized with reference to the nucleotide sequence of the same name gene of *Salmonella typhimurium* (*Salmonella typhimurium* LT2 plasmid pSLT, NC 003277), which has high nucleotide sequence homology with *Salmonella Gallinarum*. As for the faeHI operon, the information of its nucleotide sequence was obtained from *Salmonella Gallinarum* virulence plasmid minor fimbrial subunit genes (AF005899).

Example 2

Preparation of Avirulent Variants by Inactivation of Virulence Genes of *Salmonella Gallinarum* and by Integration of the Inactivated Sites

**[0051]** 2-1. Inactivation of Virulence Genes of *Salmonella Gallinarum*

**[0052]** To delete TTSS-related virulence genes of the wild-type *Salmonella Gallinarum* (SGSC No. 2293) as determined in Example 1, the one-step deletion method using lambda Red recombinase, developed by Datsenko K A et al., (Datsenko K A et al, PNAS, (2000); 97(12):6640-6645), was employed.

**[0053]** A chloramphenicol resistant gene of pKD3 was used as an antibiotic marker for identifying insertion into a target site of chromosome. Using a pair of the primers SPI-1-P1 (SEQ ID NO: 5) and SPI-1-P2 (SEQ ID NO: 6) of Table 1, which correspond to 50 bp of 5' flanking region of the avrA and 50 bp of 3' flanking region of the invH gene, wherein SPI-1 comprising from avrA to invH is target for deletion, and a part of the chloramphenicol resistant gene of pKD3, respectively, a polymerase chain reaction (hereinafter referred to as "PCR") was performed [Sambrook et al, Molecular Cloning, a Laboratory Manual (1989), Cold Spring Harbor Laboratories], with pKD3 as a template. The obtained PCR product was gene fragment about 1100 bp long.

**[0054]** In this regard, a PCR EL premix kit (BIONEER) was used and 30 cycles of denaturation at 94° C. for 30 sec, annealing at 55 ° C. for 30 sec and elongation at 72° C. for 1 min was conducted. The PCR product was separated in 0.8% agarose gel by electrophoresis and eluted at a desired band size.

**[0055]** According to the method of Datsenko K A et al., the 1100 bp-long gene fragment was introduced into pKD46-transformed, competent wild-type *Salmonella Gallinarum*, which was then spread over LB plates containing chloramphenicol (30 mg/L), As for the resulting transformant, its gene was examined by PCR using a pair of the primers SPI-1-P3 (SEQ ID NO: 7) and SPI-1-P4 (SEQ ID NO: 8), which correspond to regions about 1 kb distant from both ends of the deletion target gene, respectively. The PCR product thus obtained was 3100 bp long, indicating that the SPI-1 gene cluster was inactivated.

**[0056]** The resulting strain was cultured at 37° C., a condition of removing the pKD46 vector, to select a strain that could not grow on an LB plate containing ampicillin (100 mg/L).

**[0057]** Subsequently, the antibiotic marker inserted into the inactivated gene cluster was removed by transformation with pCP20. The removal of the antibiotic marker was identified by PCR using the primers SPI-1-P3 & SPI-1-P4. The resulting PCR product was 2000 bp long, also indicating the inactivation.

**[0058]** Afterwards, the strain which was now free of the antibiotic marker was cultured at 42° C. (a condition of removing pCP20) to select a strain that could not grow on an LB plate containing ampicillin. The SPI-1 gene cluster-inactivated strain thus obtained was named SG3-d1 (*Salmonella Gallinarum* SG2293::ΔSPI-1).

**[0059]** SPI-2, spv, and fae gene clusters were also inactivated in the same manner as in the SPI-1 gene cluster. The resulting gene cluster-inactivated strains were named SG3-d2 (*Salmonella Gallinarum* SG2293::ΔSPI-2, Accession No. KCCM 11009P), SG3-ds (*Salmonella Gallinarum* SG2293::Δspv), and SG3-df (*Salmonella Gallinarum* SG2293::Δfae), respectively. Primers used for deleting genes and for identifying gene deletion are summarized in Table 2, below.

TABLE 2

| | |
|---|---|
| Primers for deletion of SPI-1 gene from chromosome | |
| SPI-1-P1 (SEQ ID NO: 5) | TTATGGCGCTGGAAGGATTTCCTCTGGCAGGCAACCT TATAATTTCATTAGTGTAGGCTGGAGCTGCTTC |
| SPI-1-P2 (SEQ ID NO: 6) | ATGCAAAATATGGTCTTAATTATATCATGATGAGTTC AGCCAACGGTGATCATATGAATATCCTCCTTAG |
| Primers for Deletion of SPI-2 Gene from Chromosome | |
| SPI-2-P1 (SEQ ID NO: 9) | ACCCTCTTAACCTTCGCAGTGGCCTGAAGAAGCATAC CAAAAGCATTTATGTGTAGGCTGGAGCTGCTTC |
| SPI-2-P2 (SEQ ID NO: 10) | ACTGCGTGGCGTAAGGCTCATCAAAATATGACCAATG CTTAATACCATCGCATATGAATATCCTCCTTAG |
| Primers for Deletion of spvRABCD gene from virulence plasmid | |
| spv-P1 (SEQ ID NO: 13) | GTGCAAAAACAGGTCACCGCCATCCTGTTTTTGCACA TCAAA ACATTTTTGTGTAGGCTGGAGCTGCTTC |
| spv-P2 (SEQ ID NO: 14) | TTACCCCAACAGCTTGCCGTGTTTGCGCTTGAACATA GGGAT GCGGGCTTCATATGAATATCCTCCTTAG |
| Primers for Deletion of faeHI gene from virulence plasmid | |
| fae-P1 (SEQ ID NO: 17) | TTACCGATATTCAATGCTCACCGCCAGGGAGGTATGC CAGCG GGACGGTAGTGTAGGCTGGAGCTGCTT C |
| fae-P2 (SEQ ID NO: 18) | ATGAAAATAACGCATCATTATAAATCTATTATTTCCG CC CTGGCCGCGCTCATATGAATATCCTCCTTAG |

TABLE 2-continued

| | |
|---|---|
| Primers for identificaion of SPI-1 gene deletion from chromosome | |
| SPI-1-P3 (SEQ ID NO: 7) | ATGTTCTTAACAACGTTACTG |
| SPI-1-P4 (SEQ ID NO: 8) | AGGTAGTACGTTACTGACCAC |
| Primers for identification of SPI-2 gene deletion from chromosome | |
| SPI-2-P3 (SEQ ID NO: 11) | TGTTCGTACTGCCGATGTCGC |
| SPI-2-P4 (SEQ ID NO: 12) | AGTACGACGACTGACGCCAAT |
| Primers for spvRABCD gene deletion from virulence plasmid | |
| spv-P3 (SEQ ID NO: 15) | GACCATATCTGCCTGCCTCAG |
| spv-P4 (SEQ ID NO: 16) | CAGAGCCCGTTCTCTACCGAC |
| Primers for faeHI gene deletion from virulence plasmid | |
| fae-P3 (SEQ ID NO: 19) | CAGGCTCCCCTGCCACCGGCT |
| fae-P4 (SEQ ID NO: 20) | CAGGCCAACTATCTTTCCCTA |

**[0060]** 2-2. Integration of Type III Secretion System-Related Virulence Genes Inactivation

**[0061]** To integrally inactivate the gene clusters in one strain, the SG3d1 strain was sequentially subjected to the inactivation of SPI-2, spvRABCD, and faeHI gene clusters, using a method similar to that of Example 2-1.

**[0062]** To begin with, PCR was performed using the primers SPI-2-P1 (SEQ ID NO: 9) and SPI-2-P2 (SEQ ID NO.: 10) for the purpose of inactivating the SPI-2 cluster gene, with pKD4 serving as a template, resulting a 1600 bp gene fragment. This PCR product was introduced into the SG3-d1 strain in which pKD46 vector retrained (Example 1-2), followed by spreading the bacteria over an LB plate containing kanamycin (50 mg/L). As for the resulting transformant, its gene was examined by PCR using a pair of the primers SPI-2-P3 (SEQ ID NO: 11) and SPI-2-P4 (SEQ ID NO: 12), which correspond to both flanking regions of the deletion target gene. The PCR product thus obtained was 3600 bp long, indicating that the SPI-2 gene cluster was inactivated.

**[0063]** The resulting strain was cultured at 37° C., a condition of removing the pKD46 vector, to select a strain that could not grow on an LB plate containing ampicillin (100 mg/L).

**[0064]** Subsequently, the antibiotic marker inserted into the inactivated gene cluster was removed by transformation with pCP20. The removal of the antibiotic marker was identified by PCR using the primers SPI-1-P3 & SPI-1-P4 in case of SPI-1 and the primers SPI-2-P3 & SPI-2-P4 in case of SPI-2. The resulting PCR product was 2000 bp long, also indicating that the inactivation had taken place.

**[0065]** Afterwards, the strain free of the antibiotic marker was cultured at 42° C. (a condition of removing pCP20) to select a strain that could not grow on an LB plate containing ampicillin. The SPI-1 and SPI-2 gene cluster-inactivated strain thus obtained was named SG3-d1d2 (*Salmonella Gallinarum* SG2293::ΔSPI-1ΔSPI-2, Accession No. KCCM 11010P).

**[0066]** In SG-d1d2 strain, spvRABCD and faeHI gene clusters were further inactivated. To this end, the spvRABCD gene cluster (the kanamycin-resistant gene of pKD4 was used as an antibiotic marker) was inactivated in the same manner as in the inactivation of SPI-1 in Example 1-2, while the inactivation of the faeHI gene cluster (the chloramphenicol-resistant gene of pKD3 was used as an antibiotic marker) was conducted in the same manner as in the inactivation of SPI-2 in the SPI-1-inactivated strain. As for the resulting transformants, their genes were examined by PCR using the primer set spv-P3 (SEQ ID NO: 15) and spv-P4 (SEQ ID NO: 16) for spvRABCD deletion, and the primer set fae-P3 (SEQ ID NO: 19) and fae-P4 (SEQ ID NO: 20) for faeHI deletion, which correspond to regions about 1 kb distant from both ends of the respective deletion target genes. The PCR products thus obtained were 3600 bp, 3100 bp long respectively, indicating that the spvRABCD and faeHI gene clusters were inactivated. The resulting strain was cultured at 37° C., a condition of removing the pKD46 vector, to select a strain that could not grow on an LB plate containing ampicillin (100 mg/L). The *Salmonella Gallinarum* strain in which all of the four gene clusters SPI-1, SPI-2, spvRABCD and faeHI were integrally inactivated was named SG3-d4 (*Salmonella Gallinarum* SG2293:: ΔSPI-1ΔSPI-2ΔspvRABCDΔfaeHI) and deposited under accession No. KCCM 11011P.

**[0067]** 2-3. Sequencing of *Salmonella Gallinarum* spvRABCD Operon

**[0068]** Nowhere has the genetic information on spvRABCD of *Salmonella Gallinarum* (SGSC No. 2293) been disclosed yet. Its nucleotide sequence was analyzed in the present invention. For this, primers were synthesized as summarized in Table 3, below.

TABLE 3

| | |
|---|---|
| spv-S1 (SEQ ID NO: 21) | GGTCAATTAAATCCACTCAGAA |
| spv-S2 (SEQ ID NO: 22) | ACGGGAGACACCAGATTATC |
| spv-S3 (SEQ ID NO: 23) | TTCAGTAAAGTGGCGTGAGC |
| spv-S4 (SEQ ID NO: 24) | CCAGGTGGAGTTATCTCTGC |
| spv-S5 (SEQ ID NO: 25) | ACTGTCGGGCAAAGGTATTC |
| spv-S6 (SEQ ID NO: 26) | TTTCTGGTTACTGCATGACAG |
| spv-S7 (SEQ ID NO: 27) | TCCAGAGGTACAGATCGGC |
| spv-S8 (SEQ ID NO: 28) | GAAGGAATACACTACTATAGG |
| spv-S9 (SEQ ID NO: 29) | GTGTCAGCAGTTGCATCATC |

TABLE 3-continued

| | |
|---|---|
| spv-S10 (SEQ ID NO: 30) | AGTGACCGATATGGAGAAGG |
| spv-S11 (SEQ ID NO: 31) | AAGCCTGTCTCTGCATTTCG |
| spv-S12 (SEQ ID NO: 32) | AACCGTTATGACATTAAGAGG |
| spv-S13 (SEQ ID NO: 33) | TAAGGCTCTCTATTAACTTAC |
| spv-S14 (SEQ ID NO: 34) | AACCGCTTCTGGCTGTAGC |
| spv-S15 (SEQ ID NO: 35) | CCGTAACAATGACATTATCCTC |

**[0069]** The analysis result is given in SEQ ID NO: 3.

Example 3

Assay of Virulence Gene-Inactivated *Salmonella Gallinarum* SG2-d4 for Avirulence by Measurement of Invasion Efficiency into Avian Epithelial Cell

**[0070]** *Salmonella Gallinarum* and *Salmonella pullorum*, which are unique *Salmonella* species due to the lack of a motile flagella, are specifically infected to avian cells and can invade other animal cells but at very low efficiency. In this example, an in vitro cell invasion assay was conducted (Henderson S C et al, Infect Immun, (1999); 67(7):3580-3586) on the avian epithelial cell line BAT (Budgerigar Abdominal Tumor), provided from M D. Lee, Georgia University. The avirulent *Salmonella Gallinarum* variants SG3-d1d2 and SG3-d4, developed by the above-described gene deletion method, were expected to invade the host cell with very low efficiency by reduced level of TTSS-related protein. A recent research review on the infection mechanisms of pathogenic microorganisms has it that even when only a specific gene of SPI-1 is deleted, the *Salmonella* strain shows a decrease in invasion efficiency into epithelial cells (Lostroh C P et al. Microbes Infect, (2001); 3(14-15):1281-1291).

**[0071]** In the present invention, TTSS-related gene deletion was proven to lead to a decrease in virulence by measuring the efficiency of the invasion of the avian *Salmonella* variants into the avian epithelial cell line BAT.

**[0072]** Invasion efficiency into avian epithelial cells was measured on 24-well plates in triplicate, and mean values of three measurements were given. The BAT cell line was cultured at 37° C. in DMEM supplemented with 10% fetal bovine serum, 1 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin under the condition of 5% $CO_2$. The BAT cell line was seeded at a density of $2.5\times10^5$ cells/well into 24-well plates and incubated at 37° C. for 1~2 days in a 5% $CO_2$ incubator to form monolayers of cells. After distribution of the cell and incubation for one day, the culture medium was changed out with antibiotic-free DMEM. For comparison of invasion efficiency, wild-type *Salmonella Gallinarum* SG3 (SGSC: 2293), the virulence gene-inactivated *Salmonella Gallinarum* variants SG3-d1, SG3-d2, SG3-d1d2 and SG3-d4, and SG9R, which is a commercially available live vaccine, were employed, with the non-pathogenic *E. coli* MG1655 serving as a control.

**[0073]** After being primarily seed cultured, all of test bacteria were vigorously incubated for 4~5 hours in a main LB medium, and the cultures were diluted to $OD_{600}$=1.0. To 200 μL of the animal cells incubated in the antibiotic-free medium, 200 μL of each of the culture dilutions was added so that the bacteria were aliquoted at a concentration of $2.0\times10^8$ cfu/ml per well. The plates were incubated at 37° C. for one hour in a 5% $CO_2$ atmosphere to allow the bacteria to penetrate into the epithelial cells. Thereafter, the medium was aspirated off and the plates were washed with 1×PBS to remove remaining microorganisms. Then, the epithelial cells were incubated at 37 ° C. for 2 hours in the presence of 50 μg/ml gentamycin in a 5% $CO_2$ incubator to clear the microorganisms remaining outside the cells. The antibiotic was removed by washing with 1×PBS. To examine the microorganisms which succeeded in penetrating into the epithelial cells, the animal cells were lyzed for 15~30 min in 500 μl of 0.1% Triton X-100. The cell lysates were spread over LB plates and incubated overnight at 37° C. so that the microorganisms that had grown could be counted. To calculate the invasion efficiency, 200 μL of the microorganism culture with $OD_{600}$=1.0 was also incubated.

Invasion Efficiency(%)=Count of Microorganisms invaded to Cell/Count of Microorganisms within Culture Medium ($OD_{600}$=1.0)×100

**[0074]** The BAT cell invasion efficiencies of the four transformed *Salmonella Gallinarum* variants prepared by the inactivation of virulence gene clusters were calculated.

**[0075]** Of them, the variant in which only the SPI-1 gene, cluster, responsible for cell invasion mechanism, was inactivated, was decreased in invasion efficiency by 84% compared to the wild-type. The SG3-d1d2 variant with the deletion of both SPI-1 and SPI-2 and the SG3-d4 variant with the deletion of all the four gene clusters were found to decrease in cell invasion efficiency by approximately 89% and 91%, respectively, compared to the wild-type *Salmonella Gallinarum* (SG3). The variants of the present invention were also remarkably reduced in invasion ability, in comparison to that of the commercially available live vaccine Nobilis SG9R. These data demonstrated that the inactivation of TTSS-related gene clusters decreases the virulence of *Salmonella Gallinarum* (see Table 4 and FIG. **2**).

TABLE 4

| | Strain | Property | Genotype | Index of Internalization (%) |
|---|---|---|---|---|
| Control Group | MG1655 | Avirulent *E. coli* | Wild type | 2% |
| | SG3 | Virulent *Salmonella Gallinarum* (Wild-type, SGSC No. 2293) | Wild type | 100% |
| | Nobilis SG9R | *Salmonella Gallinarum* Live vaccine (commercially available) | SG:: ΔrecA | 67% |
| Test Group (avirulent *Salmonella Gallinarum*) | SG3-d1 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-1 | 16% |
| | SG3-d2 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-2 | 34% |
| | SG3-d1d2 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-1/ ΔSPI-2 | 11% |

TABLE 4-continued

| Strain | Property | Genotype | Index of Internalization (%) |
|---|---|---|---|
| SG3-d4 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-1/ ΔSPI-2/ Δspv/Δfae | 9% |

(SG3 100% = 0.36% invasion efficiency in practice)

**[0076]** The avirulence of *Salmonella Gallinarum* variant SG3-d4 was confirmed in vitro test which shows extremely low in invasion efficiency into avian epithelial cells, as was reconfirmed in animal tests and the results are given in Example 4.

## Example 4

### Assay of *Salmonella Gallinarum* SG3-d4 for Avirulence by Measuring Mortality of Chickens

**[0077]** The Research Institute of veterinary Science, Seoul National University, was entrusted with this assay. One-week-old brown egg layers (Hy-Line chicken) were employed in this assay, and they were divided into many groups of 10 which were separated in respective chicken houses before infection with pathogens. No vaccine programs were used on the experimental animals after they hatched.

**[0078]** Five avian *Salmonella* strains including the wild-type *Salmonella Gallinarum* SG3 (SGSC: 2293), the virulent gene cluster-inactivated *Salmonella Gallinarum* SG3-d2 and SG3-d4 (identified to decrease in virulence by in vitro invasion assay), the commercially available live vaccine Nobilis SG9R, and the non-pathogenic E, coli MG1655 were employed in the in vivo assay.

**[0079]** After being primarily seed cultured, the five strains were vigorously incubated for 4-5 hours to $OD_{600}$=1.0 in a main LB medium, and the concentration of each of the cell cultures was adjusted to $1.0\times10^8$ cfu/ml. The bacteria was subcutaneously injected at an adjusted dose into the chickens which were the monitored for two weeks for mortality. Subsequently, the chickens which were alive were autopsied to examine lesions and to isolate bacteria.

**[0080]** For the two weeks after artificial, infection of the pathogens ($1.0\times10^8$ cfu/mL), the chickens infected with *Salmonella*. Gallinarum (SG3) were observed and showed typical external syndromes such as low motility, blue diarrhea and low uptake of feedstuff, and looked to be dying. The mortality was not high, but an autopsy disclosed lesions in almost all the chickens.

**[0081]** In contrast, the chicken group infected with the *Salmonella Gallinarum* variant (SG3-d4) the avirulence of which was proven by in vitro invasion assay were observed to actively move and not die although some of them had diarrhea during the two weeks. Also, they were found to have almost no lesions in the autopsy. Therefore, the *Salmonella Gallinarum* variant of the present invention was again proven to have greatly decreased virulence. The chicken groups infected with the SG3-d2 variant in which the gene responsible for primary invasion into host cells remains intact while the SPI-2 gene involved in systemic infection and survival over phagocytosis is inactivated, or with the SG3-ds variant in which the spv gene known to participate in pathogenicity is inactivated, were observed to have low or no mortality (%). Thus, even the inactivation of single gene clusters had a great influence on the reduction of pathogenicity (see Table 5).

TABLE 5

| | Strain | Property | Geno-type | Mortal-ity (%) | Frequency of lesions in live birds (%) |
|---|---|---|---|---|---|
| Control Group | MG1655 | Avirulent *E. coli* | Wild-type | 0% | 20% (2/10) |
| | SG3 | Virulent *Salmonella Gallinarum* (Wild-type, SGSC No. 2293) | Wild-type | 20% | 88% (7/8) |
| | Nobilis SG9R | *Salmonella Gallinarum* Live vaccine (commercially available) | SG:: ΔrecA | 0% | 40% (4/10) |
| Test Group (avirulent *Salmonella Gallinarum*) | SG3-d1 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-1 | 40% | 17% (1/6) |
| | SG3-d2 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-2 | 10% | 0% (0/9) |
| | SG3-ds | Virulence gene-deleted *Salmonella Gallinarum* | SG:: Δspv | 0% | 20% (2/10) |
| | SG3-d4 | Virulence gene-deleted *Salmonella Gallinarum* | SG:: ΔSPI-1/ΔSPI-2/ Δspv/Δfae | 0% | 10% (1/10) |

**[0082]** According to autopsy findings, the liver and spleen were swollen and weakened, with the significant frequency of greenish brown or bluish green liver lesions, in the chicken group infected with the wild-type *Salmonella Gallinarum* (SG3). Like the commercially available live vaccine Nobilis SG9R or the non-pathogenic *E. coli* MG1655, however, the virulent gene cluster-inactivated variants of the present invention (SG3-d1d2 and SG3-d4) were found to produce almost no lesions, and were demonstrated to be harmless to chickens.

## Example 5

### Comparison of the Productivity of ΦCJ1 Bacteriophage Specific to *Salmonella Gallinarum* Variants

**[0083]** Ultimately, the development of avirulent *Salmonella* stains is to apply to the production of *Salmonella*-specific lytic bacteriophages. The *Salmonella* variants prepared in Example 2 were proven to have greatly attenuated virulence in Examples 3 and 4. Finally, ΦCJ1 (Korean Patent Application No. 10-2008-121500/US20100135962), which specifically infects avian *Salmonella*, was used to examine a difference in bacteriophage productivity between the wild-type and the avirulent *Salmonella Gallinarum* variants.

**[0084]** The avian-specific bacteriophage ΦCJ1 was cultured on a mass scale, with the wild-type *Salmonella Gallinarum* strain (SG3) or the variant serving as a host cell. For this, each bacterial strain was cultured to an $OD_{600}$ of 0.5 ($2.5\times10^{10}$ colony forming units (cfu)) in 50 ml of LB broth in a flask with agitation. ΦCJ1 was inoculated at $1.25\times10^{9}$ pfu (plaque forming unit) to form an MOI (multiplicity of infection) of 0.05, and allowed to stand for 20 min at 37° C., followed by additional incubation at 37° C. for 4 hours. Chloroform was added in an amount of 2% of the final volume and shakes for 20 min. After passage of the supernatant through a 0.2 μm filter, the titer of ΦCJ1 was counted.

**[0085]** ΦCJ1 was produced at a titer of $6\times10^{11}$ pfu/ml from the wild-type strain (SG3) and at a titer of $8\times10^{10}$ pfu/ml from the avirulent *Salmonella Gallinarum* variant (SG3-d4). These data demonstrated that the avirulent variants prepared by inactivating virulence gene clusters have no problems with infection with bacteriophages and can be used as host cells for producing bacteriophages (see Table 6). In addition, ΦCJ2 (US 20100158870) and ΦCJ3 (US 20100166709), which were both developed by the same applicant, were produced using the variant as a host cell. The host cell was found to allow the production of ΦCJ2 at a titer of approximately $2\times10^{10}$ pfu/ml and ΦCJ3 at a titer of approximately $5\times10^{9}$ pfu/ml. Like ΦCJ1, ΦCJ2 and ΦCJ3 were produced from the variant of the present invention, without significant difference from the wild-type.

TABLE 6

| | Strain | Property | Genotype | Production Titer of ΦCJ1 (pfu/ml) |
|---|---|---|---|---|
| Control Group | SG3 | Virulent *Salmonella Gallinarum* (Wild-type, SGSC No. 2293) | Wild type | $6\times10^{11}$ |
| Test Group (avirulent *Salmonella Gallinarum*) | SG3-d4 | Virulence Gene-Deleted *Salmonella Gallinarum* | SG3:: ΔSPI-1/ΔSPI-2/ Δspv/Δfae | $8\times10^{10}$ |

**[0086]** As described hitherto, the avirulent *Salmonella Gallinarum* variants, prepared by inactivating virulence genes, according to the present invention are useful as host cells for effectively producing *Salmonella*-specific lytic bacteriophages on an industrial scale with the advantage of cost saving. The avirulent *Salmonella Gallinarum* variants simplify the purification process taken to remove toxicity after bacteriophage production, thus greatly reducing the production cost and solving the safety problem of the products. In addition, the variants can be used as live vaccines that guarantee higher immunological effects and safety than do conventional vaccines.

**[0087]** Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

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<400> SEQUENCE: 1

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tttgtttact atactgcttc ccgccgccgg attggcctcc acatattcat ttaatcgttg 180

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gagaaaaggt tcttcaccac acagacgttc acaaatatta tcttcatgaa tttttactaa 300

gttcataaat tcaagctgaa gttttttggc gagcgccagg ctaaaaatac cgcattcaga 360

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ggtctccttt atagaggaaa caagctcatg gactgacata acaaatttaa gatttaactc 660

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tccccaaacg atacataacc ttcttacgat aaatactgac ggtttttgtt cccagaccaa 1260

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tcgcctgcgt gacaccggga ggtagattcc accaggcgta ttgattgata ttaaacaata 1380

cctcttccgg cgtctcgccg gcattaaaag catacgtagc agccacggtt ttcttttgtg 1440

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<400> SEQUENCE: 3

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<220> FEATURE:
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 7

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<210> SEQ ID NO 12
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<210> SEQ ID NO 14
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: spv-P2 primer

<400> SEQUENCE: 14

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<210> SEQ ID NO 15
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gaccatatct gcctgcctca g 21

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acgggagaca ccagattatc 20

<210> SEQ ID NO 23
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<400> SEQUENCE: 23

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actgtcgggc aaaggtattc 20

<210> SEQ ID NO 26
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tccagaggta cagatcggc 19

<210> SEQ ID NO 28
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gtgtcagcag ttgcatcatc 20

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aaccgttatg acattaagag g 21

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taaggctctc tattaactta c 21

<210> SEQ ID NO 34
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aaccgcttct ggctgtagc 19

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**1-14**. (canceled)

**15**. A method for treating *Salmonella Gallinarum*-infectious fowl typhoid in animals by administering *Salmonella Gallinarum* variants as an effective ingredient, wherein pathogenecity of the *Salmonella Gallinarum* variants is reduced by inactivating at least one gene selected from the group consisting of the *Salmonella* Pathogenicity Island-1, *Salmonella* Pathogenicity Island-2, spvRABCD and faeHI.

**16**. The method of claim **15**, wherein the inactivation of the gene is carried out using a method selected from the group consisting of modification of single or plural nucleotides in the gene, deletion of single or plural genes, insertion of an exogenous gene into the genes, deletion of all of the gene clusters, and a combination thereof.

**17**. The method of claim **15**, wherein the pathogenecity of the *Salmonella Gallinarum* variants is reduced by deleting an entire gene cluster of *Salmonella* pathogenicity island-2 of SEQ ID NO:2.

**18**. The method of claim **17**, wherein the *Salmonella Gallinarum* variant is deposited under accession No. KCCM 11009P.

**19**. The method of claim **15**, wherein the pathogenecity of the *Salmonella Gallinarum* variants is reduced by deleting all gene clusters of *Salmonella* Pathogenicity Island-1 of SEQ ID NO: 1, *Salmonella* Pathogenicity Island-2 of SEQ ID NO: 2, spvRABCD of SEQ ID NO: 3, and faeHI of SEQ ID NO: 4.

**20**. The method of claim **19**, wherein the *Salmonella Gallinarum* variant is deposited under accession No. KCCM 11011P.

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