Abstract: Disclosed is an attenuated, *Streptococcus pneumoniae* strain in which a pep27 is so mutated as not to be expressed even upon infection into human lung cells. A vaccine composition comprising the attenuated, live *Streptococcus pneumoniae* strain and a peptide vaccine composition comprising a pneumococcal pep27 peptide are provided for the prevention and treatment of pneumococcal infections. Also, a method for diagnosing pneumococcal infections and a diagnostic kit therefore are provided. The method features detecting a pneumococcal pep27 gene or pep27 peptide.

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Description

Title of Invention: VACCINE COMPOSITION FOR PREVENTION OR TREATMENT OF PNEUMOCOCCAL INFECTIONS COMPRISING VNCR/S OPERON-DISABLED STREPTOCOCCUS PNEUMONIA MUTANT STRAIN OR PEP27 PEPTIDE

Technical Field

[1] The present invention relates to an attenuated Streptococcus pneumonia strain with mutation in a vncR/S operon, which is overexpressed upon infection into human lung cells, and a vaccine composition for the prevention or treatment of pneumococcal infections comprising the same. Also, the present invention relates to a vaccine composition for the prevention or treatment of pneumococcal infections comprising a pneumococcal pep27 peptide. Further, the present invention is concerned with a method for diagnosing pneumococcal infections, featuring detection of a pneumococcal pep27 gene or peptide, and a diagnostic kit for pneumococcal infections.

Background Art

[2] Streptococcus pneumoniae is a naturally transformable, Gram-positive bacterial strain which causes various types of pneumococcal infection including bacterial pneumonia, otitis media, bacteremia, meningitis, etc. in both humans and animals [Willett, H. P. 1992. Streptococcus pneumoniae. In Zinsser Microbiology. Joklik, W.K., Willet, H.P., Amos, D.B. and Wilfert, CM., (eds). Prentice-Hall International, London, pp. 432-442]. The appearance of multi-drug resistant organisms makes it difficult to treat pneumococcal infection with antibiotics. 23-Valent polysaccharide vaccines [Pneumovax 23 (Merck) and Pnu-Imune 23 (Wyeth-Lederle)] currently commercially available for the prevention of pneumococcal infection comprise capsular polysaccharides (CPS) as effective antigens. However, they suffer from the disadvantages of lacking memory responses and not being effective when given to infants and young children due to the low antibody productivity thereof. In order to overcome the disadvantages of 23-valent vaccines, pneumococcal 7-valent conjugate vaccines [e.g., Prevnar (Wyeth-Lederle)], which usually comprise 7 types of CPS conjugated to a carrier protein, have been developed. In addition to being highly expensive, however, these vaccines find a narrow spectrum of applications for preventing pneumococcal infections because they offer protective effects on only 7 out of more than 95 types of pneumococci.

[3] Since almost all of the currently used pneumococcal vaccines are administered via
subcutaneous injection (IgG induction in sera), they do not provide effective early defense against *Streptococcus pneumoniae* which has the laryngeal mucous membranes as the source of infection.

Therefore, there have been attempts to develop a mucosal vaccine or a vaccine with a highly antigenic protein for use in preventing pneumococcal infections. Pneumolysin (Ply) toxoid is known as a virulence factor of the pneumococcus that binds to the cholesterol of a host cell to form pores in the cell, and thus attempts have been made to develop vaccines using an attenuated pneumolysin (PdB). However, pneumolysin shows very high toxicity in vitro and in vivo, and PdB alone shows no effects, but is found to elicit an increased survival rate against the pneumococcal infections only when given in combination with another virulence factor such as pneumococcal surface protein A (PspA), choline binding protein (CbpA), pneumococcal surface adhesion A (PsaA), Lyt A, etc. (Ogunniyi, A. D. et al., 2000, Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against the challenge of *Streptococcus pneumoniae*. Infect. Immun. 68:3028-3033). As such, candidate antigen proteins for conventional vaccines preventive of pneumococcal infections show low antigenicity or have no protective effects against all serotypes of the pneumococcus in addition to being administered via injection. In fact, vaccines which can act via the laryngeal mucosal membranes have not yet been developed to date. Thus, there remains a need to develop candidate antigen proteins or attenuated vaccines that can provide defense against early pneumococcal infections taking place at the laryngeal mucosal membranes.

Pneumococci are carried in the nasopharynx of healthy individuals which is a major reservoir for pneumococcal infections. In vivo pneumococci are placed under various types of environmental stress. A change of environmental niche in the host, such as penetration of pneumococci from the nasopharynx into the bloodstream, may trigger a dramatic change in morphology as well as gene expression. For instance, pneumococci in the nasopharynx are predominantly of a transparent colony phenotype and have been found to tend to express a small amount of capsules and a large amount of choline binding protein A (CbpA). On the contrary, pneumococci in the bloodstream are predominantly of the opaque colony morphology and have been found to tend to produce more capsule and less CbpA (Kim, J. O. et al., 1998, Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J. Infect. Dis. 177:368-377).

Recent studies with STM (signature-tagged mutagenesis), microarray and genome-based analysis of the virulence gene have showed that *Streptococcus pneumoniae* expresses different genes upon systemic infection by intraperitoneal injection or intravenous injection or upon nasal infection through mucosal membranes (Hava &
Camilli, 2002; Orihuela et al., 2003; Adamou et al., 2001; Paton et al., 2001; Polissi et al., 1998; Wizemann et al., 2001; Throup et al., 2000), and specific genes depending on the tissues (nasopharynx, lungs, blood, brain) through which the *Streptococcus pneumoniae* passes after infection (Orihuela et al., 2004; Hava & Camilli, 2002). Also, it was found that *Streptococcus pneumoniae* expresses in the blood different genes than it does when in the tissue (Oggioni et al., 2006) and that pneumococcal polysaccharide capsules disappear irrespective of serotypes upon the bacterial invasion into human lung cells (Hammerschmidt S et al. 2005, Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect. Immun. 73:4653-67). Interestingly, upon invasion of *Streptococcus pneumoniae* by contact with host epithelial cells, the amount of the capsule is substantially reduced while the expression of various factors is induced (Bergmann S, Hammerschmidt S. 2006, Versatility of pneumococcal surface proteins. Microbiology. 152(Pt 2):295-303).

**Disclosure of Invention**

**Technical Problem**

Starting from the idea of isolating and identifying the pneumococcal virulence factors induced upon invasion into human lung cells, the present inventors studied the possible use of virulence factors to develop diagnostic markers for pneumococcal diseases, therapeutic agents for inhibiting the expression thereof, and attenuated vaccines with mutant strains. Leading to the present invention, intensive and thorough research conducted by the present inventors resulted in the finding that the pneumococcal virulence factors which are induced upon invasion into human lung cells were excavated using microarray analysis. Subsequently, these inducible genes were confirmed with RT-PCR, after which mutant strains with mutations in these genes were established and characterized for growth, and deoxycholate- or antibiotic-induced lysis. The attenuation of the mutant strains was confirmed in vitro by quantitative analysis of the cytotoxicity and hemolytic activity on human lung cells and in vivo by the systemic and intranasal infection of mice. Also, immunization with the mutant strain was examined for protective effects on mice intranasally challenged with virulent pneumococcal strains.

**Solution to Problem**

[8] It is therefore an object of the present invention to provide an attenuated *Strep-tococcus pneumoniae* strain with a mutation in a vncR/S operon gene.

[9] It is another object of the present invention to provide a vaccine composition for the prevention or treatment of pneumococcal infections, comprising the attenuated, *Strep-tococcus pneumoniae* strain.

[10] It is a further aspect of the present invention to provide a peptide vaccine com-
position for the prevention or treatment of pneumococcal infections, comprising a pneumococcal pep27 peptide.

It is still a further aspect of the present invention to provide a diagnostic kit for pneumococcal infections, comprising a nucleotide sequence complementary to a pneumococcal pep27 gene or a fragment thereof, or an antibody to a pneumococcal pep27 peptide or a fragment thereof.

It is still another aspect of the present invention to provide a method for diagnosing pneumococcal infections, comprising detecting a pep27 gene or a fragment thereof, or a pep27 peptide or a fragment thereof in a subject.

**Advantageous Effects of Invention**

The vaccine composition comprising an attenuated strain of *Streptococcus pneumoniae* with a vncR/s operon mutant, more particularly a pep27 mutant in accordance with the present invention is useful in defending the invasion of *Streptococcus pneumoniae* through nasaopharynx mucosal membranes in its early stages. In addition, the *Streptococcus pneumoniae* mutant can be used as a model which is studied for pneumococcal infections. The peptide vaccine composition comprising a pep27 peptide as an active ingredient is also useful in the prevention and treatment of pneumococcal infections. A nucleotide sequence complementary to a pep27 gene or a fragment thereof or an antibody specific for a pep27 peptide or a fragment thereof can be used as an active ingredient in a diagnostic composition or kit used for pneumococcal infections as well as for the prevention and treatment of pneumococcal infections.

**Brief Description of Drawings**

FIG. 1 is a schematic view showing mutating by addition and deletion in accordance with the present invention.

FIG. 2 shows mRNA levels of the genes induced after pneumococcal infection into A549 cells.

FIG. 3 shows growth curves of D39 and a mutant thereof.

FIG. 4 is a graph showing penicillin-induced autolysis in mutants.

FIG. 5 is a graph showing cefotaxime-induced autolysis in mutants.

FIG. 6 is a graph showing DOC-induced autolysis in mutants.

FIG. 7 is a graph showing survival rates of mutants after treatment with DOC.

FIG. 8 is a graph showing hemolytic activity of mutants.

FIG. 9 is a graph showing cytotoxicity of mutants on A549 cells as measured by MTT assay.

FIG. 10 is a graph showing the attenuation of each mutant after intraperitoneal challenge.
FIG. 11 is a graph showing the attenuation of each mutant after intranasal challenge.
FIG. 12 is a graph showing the attenuation of each mutant after intranasal and intraperitoneal challenges.
FIG. 13 is a graph showing survival times of mice after intranasal challenge.
FIG. 14 is a graph showing cross-protective effects of pep27-deficient mutant vaccines.

Best Mode for Carrying out the Invention

In accordance with an aspect thereof, the present invention is directed to an attenuated strain of Streptococcus pneumoniae with a mutant gene in the vncR/S operon, preferably a mutant pep27 gene in the vncR/S operon.

Streptococcus pneumoniae, also named pneumococcus or Diplococcus pneumonia, is a Gram-positive bacterial strain and a member of the genus Streptococcus which undergoes cellular division along a single axis therein and thus grows in chains or pairs. Streptococcus pneumoniae is recognized as a major cause of pneumonia. Streptococcus pneumoniae infects the normal upper respiratory tract, causing pneumonia. In fact, S. pneumoniae is reported to be responsible for as much as about 70% of the bacterial pneumonia cases. Also, the organism causes many types of pneumococcal infection in various tissues other than pneumonia in the lung, including bacteremia in blood, osteomyelitis in the bone, otitis media in the ear, peritonitis in the stomach or the duodenum, pericarditis in the pericardium, and cellulitis in general wound sites. S. pneumoniae is known as the most common cause of pneumonia bacterial meningitis in infants and children. In addition, S. pneumoniae may be fatal to chronic patients with heart failure or diabetes and may propagate between individuals through the saliva or mucous discharges. The present invention provides a vaccine composition for the prevention and treatment of pneumococcal infections.

In the past, pneumococcal infections were treated with antibiotics of which penicillin is representative. Now, penicillin cannot perform its antibiotic function because overuse makes most pneumococcal strains resistant thereto. This has been true of the well-known antibiotics including cephalosporins, macrolides, tetracycline, quinolones, and clindamycin. So far, vancomycin, Levofloxacin and moxifloxacin have remained effective, but their use is subject to limitations. Further, antibiotics are applied only to infected individuals, and are not for the prevention of pneumococcal infections whereas vaccines can be used in that capacity. Several types of vaccines have been developed, but are required to be more effective. Therefore, there is a need for a vaccine that is highly effective in preventing pneumococcal infections.
Preferably, the strains of *Streptococcus pneumoniae* useful in the present invention include all the serotypes pathogenic to vertebrates including humans. As mentioned above, there are more than 90 serotypes of pneumococci, examples of strains which show various serotypes include D39, R6, G54, CGSP14, TIGR4, 70585, Hungary 19A-6 and Taiwan9F-14. Irrespective of the serotypes, any strain of *Streptococcus pneumoniae* may be used in the present invention as long as it causes pneumococcal infection in individual vertebrates. Recently, research found that strains of various serotypes show highly conserved sequences between corresponding genes, though their polysaccharides of capsule are different. This non-limitation can be understood from BLAST search results. As for the pep27 gene sequence useful in the present invention, for example, the D39 strain (type 2, sprO527) shares 100% homology with the R6 strain and 96% homology with Hungary19A-6 and TIGR4 (type 4), showing that pep27 genes are highly conserved across the serotypes of *Streptococcus pneumoniae*. From these results, it is understood that so long as it has high sequence homology between serotypes or strains of *Streptococcus pneumoniae*, any gene can be a target useful in constructing mutants of the present invention. Preferably, the present invention employs the D39 strain of *Streptococcus pneumoniae* to construct a mutant strain which is found to be a very effective vaccine when used on mice.

The vncR/S operon, one of those found in pneumococci, consists of vexl, vex 2, vex 3, pep27, vncR and vncS. Pep27, composed of 27 amino acid residues, is transported via a vex transporter system to induce growth inhibition and apoptosis. In detail, pep27 initiates the cell death program in *S. pneumoniae* through the signal transduction triggered via the two-component system (VncR/S) consisting of a membrane-bound histidine protein kinase (e.g., VncS) and a cytoplasmic effector termed a response regulator (e.g., VncR) (Novak et al., 1999). Existing as a component of the vancomycin-tolerant operon (vexl23-pep27-vncR/S), pep27 is involved in the induction of vancomycin tolerance in *Streptococcus pneumoniae*. As mentioned above, the expression of pep27 is regulated by the two-component system consisting of the membrane-bound histidine protein kinase (VncS) and the response regulator VncR. In addition, pep27 mRNA is transcribed together with a vex gene (transporter) gene which encodes a putative transmembrane protein, just downstream of which is located the VncR/S two-component system (Novak et al., 2000). Pep27 is known to play an important role in membrane permeabilization and shows anticancer properties (Lee et al., 2005), but has not yet been described as a specific factor which is induced upon the invasion of *S. pneumoniae* into lung cells.

Pep27 genes or their proteins, found to be involved in the cell cycle of *S.*
pneumoniae including growth and cell death, are differently named according to
serotypes of S. pneumoniae and may slightly differ in nucleotide or peptide sequence
from one type to another. However, so long as it actually functions as pep27, any
serotype can be used in preparing attenuated pneumococcal vaccines by being
damaged as described above. Preferably, all of the genes of S. pneumoniae which
perform exactly the function of the pep27 gene are included within the scope of
the present invention. For example, the pep27 gene is named SPCG_0563, sprO527 or
SP_0602 according to serotypes. Any of the genes which are functionally the same as
pep27 gene can be used as a target gene of the present invention. More preferably, the
pep27 peptide of the present invention may be damaged by mutating a gene repre-
ented by SEQ. ID. NO.: 1.

[38]
[39] In a preferred embodiment of the present invention, the attenuated vaccine can be
prepared by mutating pep27 alone or optionally in combination with one or more genes
of the vncR/S operon (GenBank accession number: NC_008533 (complete genome))
within which vncR, vncS, vexl, vex2 and vex3 as well as pep27 are present. Besides
pep27, at least one of the operon genes may be mutated.

[40]
[41] In another preferred embodiment, provided is a pneumococcal mutant in which at-
tenuation occurs at both pep27 and vex3 peptide or both pep27 and vncR peptides or
all of pep27, vncR and vex3 peptides. The attenuated strain can be constructed using a
method typical to the art, preferably through mutation, for example, gene deletion,
addition or substitution.

[42] vncR is differently named according to the serotypes of Streptococcus pneumoniae.
For example, it is named SPD_0524, sprO528, SPG_0547, SAK_0701, SAG0616,
SPCG_0564, SP_0603, SP70585_0665, SPP_0617, SPJ_0555, SPH_0697 or
SPT_0629. These genes or peptides and other serotype genes or peptides which are
actually identical to them are included within the scope of the vncR gene or peptide
useful in the present invention.

[43] As concerns vncS, it may be referred to by different names depending on the
serotypes of Streptococcus pneumoniae. For example, those referred to as SPD_0525,
sprO529, SPG_0548, SPCG_0565, SPJ_0556, SPH_0698, SP_0604, SPP_0618,
SP70585_0666, and SPT_0630 are given to vncS. Likewise, these genes or peptides
and other serotype genes or peptides which are substantially identical to or have the
same functional activity with them are included within the scope of the vncS gene or
peptide useful in the present invention.

[44] Vexl forms, along with vex2 and vex3, a transporter (ABC transporter) and is named
SPD_0521, sprO524, SP0599 or SAG0613 according to serotypes such as R6, D39 and TIGR4. So long as it is functionally identical to vexl, any gene may be used irrespective of serotypes in the present invention. Vex2 is responsible for the ATP-binding domain of the ABC transporter and is differently named SPD_0522, sprO525, SP0600 or SAG0614. Irrespective of these names, biologically identical or similar genes can be used in the present invention. Like Vexl, Vex3 is a transmembrane protein of the transporter and is differently named SPD_0523, sprO526, or SAG0615 according to the serotype. Without limitations as to the serotype, genes having actually the same function as in vex3 are used in the present invention. Thus, if necessary, not only the vex3 peptide, but also vexl or vex2 peptides adjacent thereto can be functionally damaged, which can be readily achieved using a well-known method.

As used herein, the term "attenuation" is intended to refer to modification of a virulent strain into a less virulent strain or a weaker pathogen. This attenuated strain refers to a strain which is significantly reduced in virulence related to clinical diseases while still replicating within a host. Preferably, the attenuated strain of the present invention is of such a low virulence or pathogenicity so as to allow itself to be administered as a vaccine. More preferably, the pneumococcal strains of the present invention are attenuated to the extent that they cannot cause clinical diseases while remaining replicable within hosts. The attenuated mutant can be obtained using a variety of different methods, such as point mutation, sequence change with related viruses, or nucleotide deletion.

The term "mutation", as used herein, is intended to mean all actions causing an alteration in the genetic function of a gene. In detail, "mutation" refers to the quantitative or qualitative change in a gene. In other words, a change at a molecular level in DNA, for example, substitution, deletion, addition, translocation or repetition at one or more nucleotides or a frame shift by the addition or deletion of one or two bases, induces a mutation which results in no production of an enzyme or a peptide encoded by the intact gene, or production of a functionally altered or disabled enzyme or peptide. The mutation can be achieved using typical methods without limitations. Preferred is a mutant which is functionally damaged by mutating a gene of interest, preferably by gene deletion. In more detail, the mutant of the present invention may be prepared by deleting a part of or a full length of the pep27 gene. In addition to the pep27 gene, genes around the pep27 gene, such as vncR, vncS, vexl, vex2 and vex3, may be damaged. Deletion methods and regions may be suitably chosen by those skilled in the art. Preferably, the regions given in Table 1 are deleted to prepare the mutants.

Table 1
In accordance with another aspect thereof, the present invention provides an attenuated vaccine composition for the prevention of pneumococcal infections, comprising a mutant strain of Streptococcus pneumoniae.

A vaccine is a biological preparation that improves immunity to a particular disease and typically contains an agent made of an attenuated microorganism. Thus, vaccination may mean the administration of an attenuated or dead virulent microorganism so as to impart immunogenicity to a particular infection. When given a vaccine, the

### TABLE 1

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Left arm (nt) 5'→3'</th>
<th>Right arm (nt) 5'→3'</th>
<th>Deleted region (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δvex2-3-&lt;br&gt;(MEO527R)&lt;br&gt;Spd0523-0524 included partially</td>
<td>Up: TTGCTTTCAGGCTTCTCTAG&lt;br&gt;DN: ACACACAAAAATTGGGCCG&lt;br&gt;NT site: 534172–534498</td>
<td>Up: ATTCCTATGATGCTGTG&lt;br&gt;CGCTAG&lt;br&gt;GACGATTTCTGCTGCT&lt;br&gt;NT site: 534499–534818</td>
<td></td>
</tr>
<tr>
<td>Δpem27&lt;br&gt;(EMpem27)&lt;br&gt;Spd (no number)</td>
<td>Up: GCCTTTCAGGCTTCTCTAG&lt;br&gt;DN: ACACACAAAAATTGGGCCG&lt;br&gt;NT site: 534453–534462</td>
<td>Up: ATTCCTATGATGCTGTG&lt;br&gt;CGCTAG&lt;br&gt;GACGATTTCTGCTGCT&lt;br&gt;NT site: 534663–534780</td>
<td></td>
</tr>
<tr>
<td>ΔvncR&lt;br&gt;(EMO524D)&lt;br&gt;Spd0524</td>
<td>Up: CCACTCTGCTCTCTG&lt;br&gt;DN: ACACACAAAAATTGGGCCG&lt;br&gt;NT site: 534940–535256</td>
<td>Up: ATTCCTATGATGCTGTG&lt;br&gt;CGCTAG&lt;br&gt;GACGATTTCTGCTGCT&lt;br&gt;NT site: 535257–535359</td>
<td></td>
</tr>
<tr>
<td>Δvnc8&lt;br&gt;(EMO525D)&lt;br&gt;Spd0525</td>
<td>Up: AGCCAGGCTATGGCTAGG&lt;br&gt;DN: ACACACAAAAATTGGGCCG&lt;br&gt;NT site: 535693–536011</td>
<td>Up: ATTCCTATGATGCTGTG&lt;br&gt;CGCTAG&lt;br&gt;GACGATTTCTGCTGCT&lt;br&gt;NT site: 536012–536389</td>
<td></td>
</tr>
<tr>
<td>Δvex1-3&lt;br&gt;(EMvex)&lt;br&gt;Spd0523</td>
<td>Up: CCACTCTGCTCTCTG&lt;br&gt;DN: ACACACAAAAATTGGGCCG&lt;br&gt;NT site: 531210–531533</td>
<td>Up: ATTCCTATGATGCTGTG&lt;br&gt;CGCTAG&lt;br&gt;GACGATTTCTGCTGCT&lt;br&gt;NT site: 531534–531777</td>
<td>53178–534469</td>
</tr>
</tbody>
</table>
agent stimulates the body’s immune system to recognize the agent as foreign, produce antibodies and remember it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

There are several types of vaccines currently in use. For example, vaccines are pathogens themselves or purified products derived from them such as DNA or antigenic peptides. Pathogens used in vaccines are themselves killed or left live, but attenuated. These represent different strategies used to try to reduce risk of illness, while retaining the ability to induce a beneficial immune response such as cellular and/or humoral immune response.

Preferable is an attenuated live vaccine containing a strain of *Streptococcus pneumoniae* in which the pep27 function is disabled by mutation.

The vaccine composition according to the present invention comprises a mutant *Streptococcus pneumoniae* strain with pep27 functionally disabled. Optionally, one or more peptides encoded by the vncR/S operon, selected from a group consisting of vncR, vncS, vex1, vex2, vex3 and combinations thereof, may be further functionally disabled in the *Streptococcus pneumoniae* mutant strain. More preferably, vncR, vncS and vex3 are functionally disabled in addition to pep27.

Even when administered through mucosal membranes, the vaccine composition comprising the mutant in accordance with the present invention can induce effective immunogenicity in the body. Over the conventional subcutaneous injections, the vaccine composition of the present invention is advantageous particularly for infants and children. In addition, the disabled pep27 cannot provide an invasion route to lung cells for the *Streptococcus pneumoniae* mutant strain of the vaccine composition and thus allows the mutant strain to act as an effective immunogen with weak virulence. Accordingly, the vaccine composition is effective in the prevention and treatment of pneumococcal infections. In addition, the disabled pep27 disrupts the mechanism through which *Streptococcus pneumoniae* of all serotypes invades lung cells, so that the vaccine composition of the present invention is prophylactic for a number of pneumococcal infections irrespective of the serotype of *Streptococcus pneumoniae*. In the following Examples, the mutant of the present invention was found to be 37% less hemolyzed than was the wild-type. An MTT assay showed that the virulence of the mutant is 13% less than that of the wild-type. When administered to mice, the vaccine composition comprising the pep27 peptide-disabled mutant was found to have a virulence 104- and 25-fold attenuated in systemic infected and nasopharynx infected models respectively, compared to the wild-type.

The vaccine composition comprising the mutant in accordance with the present invention can be applied in the prevention and treatment of all the diseases caused by
pneumococcal infection examples of which include pneumonia, otitis media, bacteremia, meningitis, osteomyelitis, peritonitis, pericarditis, and cellulitis, but are not limited thereto.

[60] As used herein, the term "administration" is intended to refer to the introduction of the composition of the present invention into a subject using a suitable method. So long as it delivers to the targeted tissue, any route can be used for administration of the composition of the present invention. For example, the administration may be carried out via oral, intra-abdominal, intravenous, intramuscular, subcutaneous, intradermal, intranasal, intrapulmonary, intrarectal, intravesicular, intraperineal, and transdermal routes, but is not limited thereto. Preferably, the vaccine composition of the present invention is administered through a intraperineal or mucosal route, with a greater preference for a nasaopharynx mucosal membrane route. Vaccination via nasaopharynx mucosal membranes may utilize an aerosol or drop administration system.

[62] No limitations are imparted to the subject which can be administered with the vaccine composition of the present invention. Vertebrates including humans, rats, mice, poultry, etc. are the subject.

[63] Also, the vaccine composition of the present invention must be administered in a pharmaceutically effective amount. The pharmaceutically effective amount varies depending on the gender and body surface area of the patient, the kind and severity of disease, the sensitivity to the drug, the route and frequency of administration, excretion rate, treatment duration, target cells, expression levels, and other factors well-known in the pharmaceutical art which can be easily determined by those skilled in the art.

[64] In a preferred embodiment, the vaccine composition of the present invention further comprises, in addition to the mutant, a pharmaceutically acceptable carrier or an adjuvant as an active ingredient.

[65] Examples of the carrier useful in the vaccine composition of the present invention include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia gum, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, amorphous cellulose, polyvinyl pyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate, and mineral oil.

[66] Any adjuvant typically used in the art can be used in the present invention without limitations. Examples of the adjuvant include cholera toxin binding unit, aluminum salts, lipid emulsion (MF-59), synthetic detergent (Tween), microspheres, liposomes, and mucoadhesive polymers, but are not limited thereto. New adjuvant forms, if developed, may also be used.

[67] The vaccine composition according to the present invention may be formulated into
dosage forms, for example, dosage forms such as powders, granules, tablets, capsules, suspensions, emulsions, syrups, etc., external applications, suppositories, sterile injections, etc.

For clinical practice, the composition of the present invention is usually formulated in combination with a diluent or excipient, such as a filler, a thickening agent, a binder, a wetting agent, a disintegrant, a surfactant, etc. Solid agents intended for oral administration of the composition of the present invention may be in the form of tablets, pills, powders, granules, capsules, and the like. These solid agents are formulated in combination with at least one excipient, such as starch, calcium carbonate, sucrose, lactose, or gelatine. Besides, a lubricant, such as magnesium stearate, talc and the like, may also be added. Liquid agents intended for oral administration include suspensions, internal use solutions, emulsion, syrups, and the like. In addition to a simple diluent such as water or liquid paraffin, various excipients, such as wetting agents, sweetening agents, aromatics, preservatives, and the like may be contained in the liquid agents for the oral administration of the composition of the present invention. Also, non-oral dosage forms of the composition of the present invention include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, freeze-dried agents, and suppositories. As concerns non-aqueous solutions and suspensions, they are made from propylene glycol, polyethylene glycol, vegetable oils such as olive oil, or injectable esters such as ethyl oleate. The basic materials of suppositories include witepsol, macrogol, Tween 61, cacao butter, laurin butter, and glycerogelatin.

In accordance with a further aspect thereof, the present invention provides a peptide vaccine composition for the prevention and treatment of pneumococcal infections, comprising a pep27 peptide of *Streptococcus pneumoniae*.

As described above, Pep27 is involved in the regulation of the cell cycle of *Streptococcus pneumoniae* by inducing apoptosis. When administered to a subject, an isolated pep27 peptide elicits immunogenicity in the subject to allow for effective defense against later pneumococcal infections.

Preferably, the peptide vaccine composition of the present invention comprises an immunogenic pep27 peptide or a fragment thereof as an active ingredient. The pep27 peptide may be encoded by a gene genetically modified from the intact gene. For use in the peptide vaccine of the present invention, the pep27 peptide may or may not be in the full-length form. So long as it substantially acts as an epitope, any fragment of pep27 or a peptide corresponding thereto can be used as an active ingredient of the peptide vaccine of the present invention. The administration of the peptide vaccine composition of the present invention can induce the production of antibodies specific for the mono- or multi-antigens of the pneumococcal pep27 peptide. All of the an-
tibodies need not be protective against pneumococcal infections. All or a part of the produced antibodies can provide an effective defense against the antigens which are encountered at a later date, thus protecting the subject from pneumococcal infections.

Preferably, the peptide vaccine composition of the present invention further comprises a vex3 peptide or a vncR peptide of *Streptococcus pneumoniae* and optionally a vex1 peptide or vex2 peptide. Further, the peptide vaccine composition of the present invention may contain all of the peptides encoded by the VncR/S operon.

So long as it delivers to the targeted tissue, any route can be used for administration of the peptide vaccine composition of the present invention. For example, the administration may be carried out via oral, intra-abdominal, intravenous, intramuscular, subcutaneous, intradermal, intranasal, intrapulmonary, intrarectal, intravesicular, intraperineal, and transdermal routes, but is not limited thereto. Preferably, the peptide vaccine composition of the present invention is administered through a intraperineal or mucosal route.

Also, no limitations are imparted to the subject which can be administered with the peptide vaccine composition of the present invention. The pharmaceutically effective amount of the peptide vaccine composition varies depending on the gender and body surface area of the patient, the kind and severity of disease, the sensitivity to the drug, the route and frequency of administration, excretion rate, treatment duration, target cells, expression levels, and other factors well-known in the pharmaceutical art which can be easily determined by those skilled in the art. The peptide vaccine may further comprise an additional active ingredient, a pharmaceutically acceptable carrier, or an adjuvant.

In accordance with still a further aspect thereof, the present invention provides a diagnostic kit for pneumococcal infection, comprising a nucleotide sequence complementary to a pep27 gene or a fragment thereof, or an antibody to a pep27 peptide or an immunogenic fragment thereof.

The nucleotide sequence useful in the present invention complementary to the pep27 gene or the fragment thereof may be in the form of a nucleotide primer/probe with about 80% or higher, preferably about 90% or higher or more preferably about 95% or higher homology with the antisense strand of the gene or the fragment. The nucleotide primer and/or probe useful in the diagnostic kit of the present invention is about 10 to 40 nt long. In a preferred embodiment, the nucleotide sequence is an oligonucleotide comprising at least 10 consecutive nucleotides of a DNA molecule coding for one of the polypeptides described in the present invention. Preferably, the oligonucleotide useful for use in the diagnostic kit of the present invention comprises 15 or more con-
secutive nucleotides of a DNA molecule coding for one of the polypeptides described in the present invention. Assay techniques with PCR or hybridization are well known in the art (Mullis et al., Cold Spring Harbor Symp. Quant. Biol. 51:263, 1987, and Erlich ed., PCR Technology, Stockton Press, NY, 1989). Accordingly, the primer or the probe can be used alone or in combination to detect a pep27 sequence presence in a sample.

As for the antibody to the pep27 peptide or a fragment thereof, it may be a whole form having the full length of two heavy chains and two light chains or a functional fragment thereof which specifically binds to the pep27 peptide. In this context, the term "functional fragment of antibody" means a fragment retaining an antigen-binding function as exemplified by Fab, F(ab'), F(ab')2, Fv, diabody and triabody.

The antibody or the functional fragment thereof can be used to determine the presence or absence of the pep27 in a subject. So long as it is typically used in the art to detect a peptide or an antibody, any method such as Western blotting, can be used to detect a peptide bound to the antibody without limitations.

In accordance with still another aspect thereof, the present invention provides a method for diagnosing pneumococcal infection in a subject by determining the presence of a pep27 gene or a fragment thereof or a pep27 peptide or a fragment thereof in the subject. Preferably, the method may be carried out using the diagnostic kit of the present invention.

The detection may be carried out on a sample from a subject. The sample may be selected from tissues, cells, blood, serum, plasma, ascites, hydrothorax, cerebrospinal fluid, saliva, urine, sperm, and feces.

The presence of the pep27 gene in a subject can be determined by hybridizing the pep27 gene with a complementary nucleotide sequence such as a primer or a probe. If necessary, the corresponding gene can be amplified using a suitable method such as PCR.

The isolation of a peptide of interest can be achieved using a well-known method. Peptides can be quantitatively measured using a variety of methods.

Quantitative methods of assaying peptides include Western blotting, immunoprecipitation assay, complement fixation assay, fluorescence activated cell sorter (FACS) and protein chips, but are not limited thereto. Through these assay methods, antigen-antibody complexes can be quantitatively compared between a normal control and that
of the patient, providing information about the pneumococcal infection and/or prophylactic or therapeutic effects related thereto.

**Mode for the Invention**

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as limiting the present invention. Also, the reference documents described herein are incorporated by reference in their entirety.

The statistical analysis of data was performed using student's t-test (paired or unpaired T test). When given, the data are represented by the average+standard deviation of two or four measurements. The difference of the average survival time between groups was analyzed with Mann-Whitney U test (2-tailed) while a Fisher Exact test was used to analyze the difference of overall survival rates between groups (P < 0.05;*, P < 0.01; **, P < 0.001; ***)..

**EXAMPLES**

**EXAMPLE 1: Search for Inducible Pneumococcal Genes for Infection**

**i) Bacteria, Cell Line, Experimental Animals and Transformation**

The pneumococcal strains used in the example are given in Table 2, below. The pneumococcal strain D39 (Type 2) and mutants derived therefrom were cultured in Todd Hewitt (THY) broths.

For the transformation of the D39 strains, they were cultured to a middle point of exponential phase in a Casitone-Tryptone (CAT)-based medium: one liter of CAT based medium contained 10 g of enzymatic casein hydrolysate (Difco Laboratories, USA), 5 g of tryptophan (Difco Laboratories), 1 g of yeast extract (Difco Laboratories), 5 g of NaCl, 5 mg of choline (Sigma, USA), 0.2 % glucose (Sigma, USA), and 16.6 mM dipotassium phosphate(Sigma, USA). A complete transformation medium was prepare from CAT broth by addition of (per liter) 147 mg of CaCl₂ and 2 g of fetal bovine serum (fraction V; Sigma). Competence was controlled by appropriate addition of the competence-specific peptide and quantified as erythromycin-resistant transformants obtained after exposure of cells to DNA in culture medium, as described previously[Havarstein, L. S.et al., 1995, An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U.S.A. 92:11140-11144]. Encapsulated strain D39 (type 2) was grown in brain heart infusion broth (Difco Laboratories, USA) or Todd-Hewitt broth (Difco Laboratories, USA) and transformed as described previously [Bricker, A. L. et al.,
Transformation of a type 4 encapsulated strain of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. 172:131-135. For selection of pneumococcal transformants, erythromycin was added to the growth medium at a concentration of 2.5 µg/ml.

ii) **Human Lung Cell Culture**

The A549 human lung carcinoma (ATCC CCL-185) strain (ATCC TIB-71) was purchased from the American Type Culture Collection and cultured in a DMEM medium (Gibco BRL, Gaithersburg, Md) supplemented with 4.5% glucose, 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, Md.), 100 U/ml penicillin G and 100 µg/ml streptomycin at 37°C under a 5% CO₂ atmosphere condition. 4-week-old male ICR mice (Orient, Korea) were used for infection experiments.

iii) **Virulence Assay**

Intraperitoneal or intranasal challenge with a highly virulent capsular type 2 strain (D39) and its isogenic mutants was performed to evaluate the effect, if any, of mutant target genes on the virulence of *S. pneumoniae*. Bacteria were cultured at 37°C overnight on 10% [vol/vol] horse serum-added brain heart infusion agar (Difco Laboratories, USA) or Todd-Hewitt agar (Difco Laboratories, USA) (supplemented with erythromycin as required) and then grown in serum broth (10% [vol/vol] horse serum in brain heart infusion broth (Difco Laboratories, USA) or Todd-Hewitt broth (Difco Laboratories, USA)) for 3 h at 37°C to give ca. 108 CFU/ml [Ogunniyi, A. D.et al., 2000]. Each bacterial culture was then diluted in serum broth to suitable concentrations, and groups of 10 CDI mice were infected intraperitoneally with 0.1 mL of either D39 or mutants or intranasally with 10 µL of D39 or mutants. The survival of the challenged mice was monitored four times daily for the first 5 days, twice daily for the following 5 days, and once daily until 21 days post challenge.

Table 2
iv) RNA Isolation and Microarray Assay

Bacterial RNA was isolated from the A549 cells infected with *S. pneumoniae* using the method of Orihuela et al. (2004). In this regard, *S. pneumoniae* grown in DMEM was inoculated at a density of 4 x 10^8 CFU/mL into the human lung cell line A549 (4 x 10^6, MOI 100:1) which was then incubated at 37°C for 10 min or 2 h. Thereafter, the bacteria which remained unattached was removed by washing and the cells were harvested in the presence of RNAprotect™ (Qiagen, Valencia, Calif.) for protecting bacterial RNA. Ultrasonication (Sonorex ultrasonic cleaners Model RK52H, Bandelin, Germany) for 5 min disrupted the cells but not the bacteria. The cell lysate thus obtained was centrifuged at 800 g for 5 min to remove cell debris, followed by additional centrifugation at 4,600 g for 10 min to obtain the bacteria. RNA was isolated from the pellet (bacterial/eukaryotic pellet) using RNeasy minikitTM (Qiagen). Bacterial RNA was enriched by removing eukaryotic RNA from the bacterial/eukaryotic pellet with MICROBEnrichTM (Ambion, Austin, Tex.). For quantitative analysis, the bacterial RNA thus isolated was measured for OD_{260} while electrophoresis on agarose gel gave qualitative information on RNA.

Also, the RNA was analyzed using a microarray method. After infection of a human lung cell line A547 with the virulent pneumococcal strain D39 for 10 min and 2 hrs, transcripts of the bacteria were monitored for expression change during the infection.
on a gene chip (Genomic Tree, Korea) while the pneumococcal strain which was not used for the infection served as a control.

**EXAMPLE 2: Construction of Mutant Pneumococcal Strain**

Pneumococcal chromosomal DNA was isolated from D39 and hrcA strains using the method of Rubin et al (2004). A mutagenic DNA fragment in which ermB (erythromycin-resistant gene cassette) is partially substituted for a target gene of *Streptococcus pneumoniae* was constructed by PCR. Transformation with the DNA fragment resulting in a mutant. In detail, a mutagenic DNA fragment was made by inserting ermB in substitution for a mid-portion of a pneumococcal gene by the Tripartite PCR method established by the lab of the inventors [Kwon et al, 2003; Lau et al, 2002]. In this context, selected regions (left arm and right arm, except for a middle region) of a target gene were amplified from pneumococcal chromosomal DNA. Separately, chromosomal DNA from the hrcA mutant strain which expresses ermB was used as a template for amplifying an ermB gene (860 bp) (Table 3). A 3’ primer for the left arm DNA fragment of the target gene was designed to contain a 5’ terminal primer sequence of ermB while a 5’ primer for the right arm DNA fragment was designed to contain a 3’ terminal primer sequence of ermB. The DNA fragments resulting from primary PCR showed sequence structures in which overlaps were found between a 3’ terminus of the left arm DNA fragment and a 5’ terminus of ermB and between a 3’ terminus of ermB and a 5’ terminus of the right arm DNA fragment. Accordingly, PCR was performed on the three DNA fragments, that is, the left arm, ermB and the right arm, in one tube in the presence of the 5’ primer used for the left arm and the 3’ primer used for the left arm to afford a DNA construct in which the three DNA fragments were linked to one (FIG. 1, Table 4).

The mutant DNA template thus constructed was transformed into *Streptococcus pneumoniae* by the method of Havarstein et al (1995) using competence stimulating peptide-1, followed by selection of erythromycin-resistant colonies on CAT agar containing 1 μg/ml of erythromycin. Colony PCR was performed to examine whether the selected erythromycin-resistant mutants contained the exact deletion region. The mutant was deposited with KCTC (Korean Collection for Type Cultures; Korean Research Institute of Bioscience and Biotechnology (KRIBB), Korea), and assigned accession number KCTC 11375BP.
[Table 3]

**TABLE 3**

Sequence of ermB Cassette Amplicon

<table>
<thead>
<tr>
<th>ermB orientation</th>
<th>Direction: 5' → 3'</th>
<th>SEQ ID NO. or Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse orientation F</td>
<td>ACGCACGACACCTGTOGGATCAA</td>
<td>Kim et al., 2001 (SEQ. ID. NO.: 7)</td>
</tr>
<tr>
<td>Reverse orientation R</td>
<td>CTCAGACTTTCAGGAGTTGGTC</td>
<td>Kim et al., 2001 (SEQ. ID. NO.: 8)</td>
</tr>
<tr>
<td>Same orientation F</td>
<td>AGTCCGCGCCGCCTCTAGGAT</td>
<td>9</td>
</tr>
<tr>
<td>Same orientation R</td>
<td>CGGGCCCCC1TTTGTGGTGA</td>
<td>10</td>
</tr>
</tbody>
</table>

※ F, Forward primer; R, Reverse primer

[Table 4]

**TABLE 4**

Primer Sequences for Mutant Construction

<table>
<thead>
<tr>
<th>Primer sequence (5' → 3')/ MK0527R</th>
<th>SEQ ID NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L up tctctatcag cctcaagcag</td>
<td>11</td>
</tr>
<tr>
<td>L dn atcaaaaaa ttttggggccg gttgaagca agcgcataaa cg</td>
<td>12</td>
</tr>
<tr>
<td>R up attctatgag tcgcgtccga ctgacggatt gttgctatga</td>
<td>13</td>
</tr>
<tr>
<td>R dn gttatctgtag agcgccttg</td>
<td>14</td>
</tr>
</tbody>
</table>

※ the underlined part is a nucleotide sequence complementary to ErmB cassette. L, left arm primer; R, right arm primer

[121] Table 4

[122] EXAMPLE 3: Assay for Mutant Properties

[123] 1) Assay Methods of Mutant Properties

[124] [125] Hemolytic Activity [Lock RA et al., 1996]
Using the method of Lock et al. (1996), hemolytic activity was assessed to compare virulence between the mutants and the wild-type. The mutants were grown to an OD550 of 0.3 in THY broth, followed by centrifugation. The cell pellets thus obtained were suspended in lysis buffer (50 mM Tris-Cl, 1 mM DTT, 0.1% Triton X-100) and incubated at 37°C for 5 min in the presence of 1 mM PMSF and 0.1% deoxychloric acid (DOC). Sonication disrupted the cells. After centrifugation, the supernatant was removed and diluted in series. Dilutions were incubated together with one volume of washed 1.5% sheep or horse erythrocytes in 96-well microtiter plates to determine hemolytic activity. The hemolytic titer was defined as the reciprocal of the highest dilution that can lyse 50% of the erythrocytes, which is determined by the absorbance at 540 nm of released hemoglobin [Lock, R. A. et al., 1996, Sequence variation in the *Streptococcus pneumoniae* pneumolysin gene affecting hemolytic activity and electrophoretic mobility of the toxin. Microb. Pathog. 21:71-83].

Cytotoxicity by MTT Assay [Smirnov, 1999]

The cytotoxicity of the mutants were assessed using an MTT assay in which MTT is reduced into formazan by the mitochondrial dehydrogenase of living cells, giving a purple color [Smirnov, 1999]. A549 cells (human lung cell line) were seeded at a density of 1x104 cells/well on 96-well plates and incubated to form a monolayer of cells, after which the medium was changed with antibiotic-free DMEM. The cells were infected with 1x106 CFU of the pneumococcal strains (MOI 100: 1) for 4 hrs and DMEM containing antibiotics was replaced as the medium, followed by measuring absorbance at 540 nm on an ELISA reader.

Deoxycholate (DOC)- or Antibiotic-Induced Autolysis

When a mutant culture with an absorbance of 0.3 ~ 0.4 at 550 nm was incubated in the presence of 0.05 % deoxycholic acid or antibiotic, it was measured for a change in absorbance at 550 nm or for the number of living cells.

ELISA Assay

Enzyme-linked immunosorbent assay (ELISA) assay was performed to measure antibody titers. Sera or saliva from mice immunized with mutants were pooled by group and plated on ELISA plates coated with whole cell lysates of D39 or the mutants. In detail, cell lysates of the mutants were diluted to a concentration of 500 ng/ml in TPBS, placed on 96-well plates, incubated at 37°C for 1 hr with a 1% BSA blocking solution, and washed. Again, they were incubated at 37°C for 1 hr with multi-diluted primary antibody and washed. Incubation with the secondary antibody IgG-HRP was followed by color development with TMB. Absorbance at 540 nm was
measured using an ELISA reader (Softmax, USA).

2) Assay Results

i) Growth Properties of Mutant Pneumococcal Strains with Infection-Inducible Genes Disabled

Selected were the genes which were found to increase three or more times in expression level upon the infection of the human lung cell line A549 with the mutants, compared to the non-infected control wild-type D39, as measured by microarray analysis with RNA isolated 10 min and 2 hrs after the infection (Table 5). Of them, some was confirmed with respect to gene expression by RT-PCR (FIG. 2).

Table 5

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Infection for 10 min</th>
<th>Infection for 2 hrs</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>pep27</td>
<td>9.97</td>
<td>97.08</td>
<td>Secreted protein recognized by VncR/S</td>
</tr>
<tr>
<td>vncR</td>
<td>18.40</td>
<td>18.07</td>
<td>a cytoplasmic effector, termed response regulator VncR</td>
</tr>
</tbody>
</table>

When drawing respective growth curves of the mutants, MK0527R was observed to have stationary phase prolonged for 2 hrs, compared to the wild-type, and thus showed delayed autolysis even in the stationary phase (FIG. 3).

ii) Antibiotic-Induced Lysis

In order to confirm the observation that some mutants underwent delayed cell death even when they reached the stationary phase, antibiotic-induced autolysis was analyzed. Because *Streptococcus pneumoniae* was induced to undergo autolysis by penicillin G, growth curves were drawn in the presence of penicillin G (FIG. 4). Again, the D39 wild-type strain was measured for lysis rate in the presence of cefotaxime, a cephalosporin-derived antibiotic. After addition of cefotaxime to the cultures, it took 90 min for D39 to decrease in absorbance by half, but 110 min was measured with
MK0527R because of autolysis resistance (FIG. 5).

iii) Deoxycholate (DOC)-Induced Autolysis

*Streptococcus pneumoniae* is sensitively induced to undergo lysis by DOC. In order to examine resistance to antibiotic-induced autolysis, bacteria lysis rates were measured in the presence of 0.05% DOC. The time taken for absorbance to decrease by half was measured to be 240 sec for the wild-type and 270 sec for MK0527R (FIG. 6).

As for live cells treated with DOC, they were counted as 10 cells for the wild-type D39 5 min post-treatment and 1x10^3 cells for MK0527R even 40 min post-treatment, demonstrating the DOC resistance of the mutant (FIG. 7).

iv) Hemolytic Activity

Hemolytic activity was measured to examine whether the mutants were attenuated in virulence. Because it induces serious damage upon infection into cells, the wild-type caused a lot of hemoglobin to be released and increased absorbance to an OD$_{550}$ of 0.29. As for MK0527R, its hemolytic activity was found to significantly decrease to 63% of the level of the wild-type (FIG. 8).

DOC induces the expression of the autolytic enzyme LytA in *Streptococcus pneumoniae* [Chastanet A et al., 2001] which releases pneumolysin in a LytA-dependent manner [Mitchell TJ et al., 1997]. The slower lysis in the mutant MK0527R (vex3-pep27-vncR deleted) strain than in the wild-type indicated that autolysis is inhibited by mutating the genes. Particularly, the wild-type were perfectly lzed after in vitro treatment with DOC for 5 whereas as many as 10^3 CFU of the MK0527R strain survived after DOC treatment for 40 min, demonstrating that the mutation significantly inhibits the autolysis.

When undergoing lysis, *Streptococcus pneumoniae* releases various intracellular factors including pneumolysin (a main pneumococcal toxin) which can disrupt host cells, and lipoteichoic acid, a cell wall component, which causes inflammation [Jedrzejas MJ, 2001]. Accordingly, the lysis inhibition in the mutant strains was coincident with a significant reduction of virulence in infected mice models.

v) Measurement of Cytotoxicity by MTT Assay

The mutant strains were quantitatively measured for cytotoxicity on human lung cell line A549 using an MTT assay in which MTT is reduced into formazan by the mitochondrial dehydrogenase of living cells, giving a purple color (FIG. 9). D39 was more virulent than was the mutant strain as demonstrated by an OD of 0.477, which was decreased by 13% compared to that of the mutant MK0527R.
vi) **LD$_{50}$ of Mutant Strains**

In order to ensure the safety of the mutant strains when used as live vaccines, the lethal dose thereof was measured using mice as targets. Table 6 lists the median lethal doses LD$_{50}$ of the mutant strains (the doses required to kill half the members of systemically (intraperitoneally) and intranasally administered mouse models). Upon intranasal challenge, the MK0527R strain was 25-fold attenuated in virulence, compared to the wild-type D39. Upon systemic challenge, the virulence of the mutant was $10^4$-fold reduced compared to that of the wild-type. The MK0527R strain was too much attenuated to determine its accurate median lethal dose because it must be peritoneally challenged at a density of $1 \times 10^9$ or greater CFU.

Table 6

<table>
<thead>
<tr>
<th>Mutant Name</th>
<th>Injection type</th>
<th>LD$_{50}$</th>
<th>Fold attenuation$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK0527R (pep27, vex3, VncR gene deleted)</td>
<td>Intranasal</td>
<td>$&gt;5 \times 10^8$</td>
<td>$&gt;25$</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>$&gt;1 \times 10^8$</td>
<td>$&gt;1 \times 10^4$</td>
</tr>
<tr>
<td>D39</td>
<td>Intranasal</td>
<td>$2 \times 10^7$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>$10^4$</td>
<td>1</td>
</tr>
</tbody>
</table>

$^*$Fold attenuation: LD$_{50}$ of Mutant/LD$_{50}$ of Wild-type

The MK0527R strain was too attenuated in virulence to measure LD$_{50}$ as demonstrated by the survival of all mice models which were intraperitoneally injected at a dose of $1 \times 10^8$ cells with the strain. As a live, attenuated vaccine, this mutant strain was intraperitoneally injected three times to produce IgG antibodies which were, however, found to have weak defense potentials. There were no differences in mean survival time period between mice challenged with the MK0527R strain and the control. In contrast, the MK0527R mutant was found to act as an effective vaccine upon intranasal challenge, suggesting that the IgG antibodies produced upon intraperitoneal challenge (systemic infection) did not contain a defensive component against D39 whereas the IgG antibodies produced upon intranasal challenge contained a defensive component against D39. This result may be elucidated by the following hypothesis: D39 is predominantly of opaque morphology in THY broth, and when in-
traperitoneally injected the pneumococcal strain is immediately introduced into the bloodstream and thus remains in an opaque form without the induction of phase transition. In contrast, upon intranasal infection, the D39 strain is predominantly of transparent morphology and then is introduced into the bloodstream, undergoing a phase transition into opaque morphology. Thus, it is likely that intranasal challenge induces the expression of phase transition-related proteins whereas systemic (intraperitoneal) challenge does not induce phase transition. Hence, it is inferred that the expression of phase transition-related proteins be induced upon intranasal infection, but not upon systemic (intraperitoneal) infection. In this study, the genes induced upon infection into lung cells are probably involved in phase transition. As these genes are disabled by mutation, the mutant strains do not undergo phase transition and grow with opaque (or transparent) morphology. Thus, while being resistant to lysis, the mutant strains cannot invade the cells. In addition, all the antibodies produced against the mutant strains are, in our opinion, directed toward opaque (or transparent) types, so that they can effectively defend against opaque (or transparent) pneumococcal strains introduced into the bloodstream upon intranasal infection, but cannot directly fight against transparent (or opaque) strains.

In consequence, the deletion of 0526, 0527 and 0528 genes, or the pep27 gene induces positive regulation in the expression of the autolysin in pneumococcal strains, so that the mutant strains limitedly undergo lysis, showing reduced cytotoxicity.

EXAMPLE 4: Effects of the Mutant-Containing Vaccine

1) Measurement of Effects of the Vaccine

i) Measurement of Vaccine Effects using a Systemic Infection Model

Mice were intraperitoneally immunized as described previously [Ogunniyi, A. D. et al, 2000]. Attenuated, mutant strains were intraperitoneally injected at LD$_{10}$ (corresponding to a dose of ca. 1x10$^6$ ~ 1x10$^7$ cells) three times at regular intervals of two weeks. Two weeks after the third immunization, serum samples were obtained by retroocular bleeding. On week 2 after the final immunization, the mice were intraperitoneally challenged with the highly virulent, encapsulated type 2 strain (D39). Prior to the challenge, the bacteria were incubated at 37°C overnight on blood agar and then grown in meat extract broth ((brain heart infusion broth, Difco Laboratories, USA) or (Todd-Hewitt broth, Difco Laboratories, USA)) supplemented with 10% (v/v) sheep serum. Stationary incubation at 37°C for 3 hrs gave 1x10$^8$ CFU/ml of the bacteria which was then diluted to 1x10$^4$ CFU per ml of inoculate. The presence of serotype-specific capsules was confirmed by the Quellung reaction with antisera purchased from Statens
Seraminstitut (Copenhagen, Denmark). The survival of the challenged mice was monitored every four hours for the first 7 days, and once daily until 21 days post challenge.

ii) Measurement of Vaccine Effects using a intranasal Infection Model

Mice were immunized three times at a frequency of once every two weeks with LD$_{50}$ (ca. 1x10$^7$ ~ 1x10$^8$ cells) of the attenuated, mutant strains through the intranasal mucosal membranes. Two weeks after the final immunization, virulent D39 strain was infected at a dose of 3x10$^7$ CFU through the nasoopharynx. Survival was monitored as described above.

2) Results

Measurement of in vivo Attenuation of Each Mutant

In order to re-examine if each mutant strain was attenuated, the survival time of the mice infected therewith was assessed. In this context, D39 and the mutants each was intraperitoneally injected at a dose of 1x10$^4$ CFU before monitoring the time periods of survival. No mice were dead when immunized with the MK0527R mutant, demonstrating that virulence at 1x10$^4$ CFU was significantly attenuated (P<0.0001) (FIG. 10).

Because intraperitoneal injection is a systemic infection model, but is not a normal infection route of Streptococcus pneumoniae [Salyers AA and Whitt DD, 1994], the bacteria were infected at a dose of 2x10$^7$ CFU through an intranasal route, followed by monitoring the survival of the mice. Mice infected with D39 survived for 111 hrs. On the other hand, none of the mice infected with MK0527R were dead within two weeks post-infection, showing a significant attenuation in the virulence of the mutant (P<0.001) (FIG. 11). MK0527 is a pneumococcal mutant strain in which vex3-pep27-vncR genes are deleted. A pep27-deficient mutant was also found to show the same phenotype and attenuation as in MK0527R (FIG. 12). Mutants in which vex1, vex2, vex3, vncR and vncS were respectively deleted were found to be attenuated in virulence, but with no significance (FIG. 12).

EXAMPLE 5: Measurement of Antibody Titer after Immunization with Vaccines Containing Mutants

i) Antibody Titer after Systemic (Intraperitoneal) Immunization

The MK0527R strain, which was attenuated as demonstrated in vivo and in vitro in the above experiments, was intraperitoneally challenged at a dose of 1x10$^6$ ~ 1x10$^7$ CFU three times at intervals of two weeks into groups of 10 mice, and then IgG
antibody titer was measured to be 6400.

ii) Antibody Titer and Defense after Intranasal Immunization

In order to examine the effects of the vaccine against intranasal infection, the attenuated MK0527R strain was intranasally infected at a dose of $1 \times 10^7$ ~ $1 \times 10^8$ CFU three times at intervals of two weeks and 10 days after the final infection, the wild-type D39 strain was subjected to intranasal challenge at a dose of $3 \times 10^7$ CFU. A significant increase in mean survival rate was found in the MK0527R-immunized mice (P<0.05) (FIG. 13).

EXAMPLE 6: Cross-Protective Effect of Vaccine Containing pep27-Deficient Mutant

The pep27 mutant strain was infected at a dose of $1 \times 10^7$ CFU three times at intervals of a fortnight into groups of 15 mice through an intranasal route, two weeks after which the mice were challenged with $3.5 \times 10^8$ CFU of virulent D39 type 2 or $4.1 \times 10^8$ CFU of virulent type 4.

When infected with the hetero-serotype type 4, the mice immunized with the type 2 pep27 mutant were found to show a significant defense effect as shown in FIG. 14 (**, P<0.01). The vaccine was found to significantly defend the pathogens. Each point represents one mouse and each horizontal line represents the mean time period of survival in each group.

EXAMPLE 7: Measurement of the Effect of the Peptide Vaccine

A pep27 peptide serving as an immunogen was measured for vaccine effect as follows.

10 id of a mixture containing 10 µg of a pep27 peptide and a suitable amount of a cholera toxin binding unit was infected three times at 14 day intervals into rats through the nasaopharynx. The rat nasaopharynx was cleansed with PBS, followed by measuring IgA antibody titers. A virulent D39 strain ($1 \times 10^7$ CFU/10 id) was dropped into the nasaopharynx, followed by analyzing the protective effects of the peptide vaccine.

Industrial Applicability

As described hitherto, the vaccine composition comprising an attenuated *Strep. toccoccus pneumoniae* strain with the mutation of a vncR/S operon gene, particularly, a pep27 gene in accordance with the present invention guarantees excellent protective
effects against a variety of serotypes of pneumococcal strains and is suitable for use in intranasal administration without any adjuvants, enjoying the advantage of avoiding the inconvenience of an injection. In addition, nucleotide sequences complementary to the pep27 gene or fragments thereof or antibodies specific for pep27 peptides or fragments thereof can be used as active ingredients for diagnosst kits or reagents of pneumococcal infections, finding applications in the diagnosis, prevention, and treatment of pneumococcal diseases.

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.
RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

Issued pursuant to Rule 7.1

TO: RHEE, Dong-Kwon
    Natural Sciences Campus, Sungkyunkwan University
    300 Choamhun-dong, Jangan-gu, Suwon-si, Gyeonggi-do 440-746
    Republic of Korea

1. IDENTIFICATION OF THE MICROORGANISM

   Identification reference given by the DEPOSITOR:
   Streptococcus pneumoniae
   D39 Δpep27

   Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
   KCTC 11375BP

2. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

   The microorganism identified under 1 above was accompanied by:
   [ ] a scientific description
   [ ] a proposed taxonomic designation
   (Mark with a cross where applicable)

3. RECEIPT AND ACCEPTANCE

   This International Depositary Authority accepts the microorganism identified under 1 above,
   which was received by it on August 14, 2008.

4. RECEIPT OF REQUEST FOR CONVERSION

   The microorganism identified under 1 above was received by this International Depositary Authority on
   and a request to convert the original deposit to a deposit
   under the Budapest Treaty was received by it on

5. INTERNATIONAL DEPOSITARY AUTHORITY

   Name: Korean Collection for Type Cultures
   Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB)
            111 Gwahangno, Yuseong-gu,
            Daejeon 305-086
            Republic of Korea
   Signature(s) of person(s) having the power to represent the International Depositary Authority or authorized official(s):
   OH, Hee-Mook, Director
   Date: September 5, 2008
Claims


[Claim 2] The attenuated *Streptococcus pneumoniae* strain as set forth in claim 1, wherein the vncR/S operon gene is a pep27 gene.

[Claim 3] The attenuated *Streptococcus pneumoniae* strain as set forth in claim 2, wherein the vncR/S operon gene further comprises at least one selected from among vex1, vex2, vex3, vncR and vncS in addition to the pep27 gene.

[Claim 4] The attenuated *Streptococcus pneumoniae* strain as set forth in claim 2, wherein the pep27 gene is represented by SEQ. ID. NO. 1.

[Claim 5] A vaccine composition for prevention or treatment of pneumococcal infections, comprising as an active ingredient the attenuated, *Streptococcus pneumoniae* strain of one of claims 1 to 3.

[Claim 6] The vaccine composition as set forth in claim 5, being suitable for administration through an intramuscular or intranasal route.

[Claim 7] The vaccine composition as set forth in claim 6, wherein the intranasal route uses a intranasal mucosal membrane.

[Claim 8] A peptide vaccine composition for prevention or treatment of pneumococcal infections, comprising a pneumococcal pep27 peptide as an active ingredient.

[Claim 9] The peptide vaccine composition as set forth in claim 8, further comprising a peptide selected from a group consisting of vex1, vex2, vex3, vncR, vncS and combinations thereof.

[Claim 10] The peptide vaccine composition as set forth in claim 8, further comprising a peptide selected from a group consisting of vex1, vex2, vex3, vncR, vncS and combinations thereof.

[Claim 11] A diagnostic kit for pneumococcal infections, comprising, as an active ingredient, a nucleotide sequence complementary to a pep27 gene or a fragment thereof, or an antibody against a pep27 peptide or a fragment thereof.

[Claim 12] A method of diagnosing pneumococcal infection, comprising detecting a pep27 gene or a fragment thereof, or a pep27 peptide or a fragment thereof in a subject of interest.
[Fig. 1]

Recombination

[Fig. 2]

RT-PCR result:

control  10min infection  2hour infection

16s rRNA

spr0528