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(57) Abstract: The present invention relates to compositions and their use for stimulating an immune response to *Streptococcus suis* in animals, preferably swine, or humans. Preferred compositions of the invention are able to illicit immune responses to two or more serotypes of *S. suis*. Methods of making and using compositions of the invention are provided.



WO 2013/033092 A2

STREPTOCOCCUS SUIIS PILUS ANTIGENS

SEQUENCE LISTING

[0001] This application contains a sequence listing in accordance with 37 C.F.R. 1.821 –
1.825. The sequence listing accompanying this application is hereby incorporated by reference
5 in its entirety.

BACKGROUND OF THE INVENTION

A. Field of the Invention

[0002] The present invention relates to compositions and associated methods of stimulating
an immune response, preferably a protective one, to *Streptococcus suis* in animals and humans.

10 B. Description of the Related Art

[0003] *Streptococcus suis* is a gram-positive bacterium that infects pigs and causes a wide
range of serious diseases. It is widely recognised as causing rapidly progressive and fatal sepsis
in infant pigs, and is associated with meningitis, polyarthritis, pneumonia, septicemia,
endocarditis, encephalitis, polyserositis, and abscesses in swine of all ages. For unknown
15 reasons, adult pigs do not succumb to this infection but demonstrate asymptomatic
nasopharyngeal carriage. A more fulminant bacteraemic infection occurs in neonatal pigs, and
infant piglets can be infected after early contact with colonised adult females. The bacterium is
endemic in nearly all countries with an extensive pig industry and is capable of being transmitted
from pigs to humans. Humans may be infected when they handle infected pig carcasses or meat,
20 and infection can be life-threatening. Large outbreaks in China have raised public health
concerns worldwide. In humans, meningitis is the most common presentation of *S. suis*.
Pneumonia, arthritis, septicaemia, endocarditis, and deafness are other known outcomes of
infection. Nevertheless, the mechanisms of *S. suis* pathogenesis in humans and pigs remain
poorly understood.

25 [0004] Currently, eradication of *S. suis* from pig populations is not considered feasible.
Antibiotic treatment in pigs is rarely successful, possibly because of poor antibiotic penetration
of the porcine tonsillar tissues, which act as a source of infection. Resistance to macrolides,
lincosamides, and tetracycline is common, limiting efforts to clear tonsillar carriage.

Development of a vaccine targeted against the most virulent or prevalent strains of *S. suis* might prevent colonisation of female pigs and protect those working with pigs. But to date, only inconsistent results have resulted with vaccination attempts. These inconsistent results may have occurred, at least in part, due to a lack of cross-reactivity of the vaccines to different serotypes of *S. suis*. For example, based on the capsular polysaccharide, there are at least 35 different serotypes of *S. suis*. Theoretically, one or more surface proteins of *S. suis* could act as an antigen for multiple serotypes of *S. suis* and be the basis of a vaccine against *S. suis*. However, identifying appropriate *S. suis* surface proteins has proven elusive.

[0005] Pilus protein structures have been proposed to exist in *S. suis*, see Jacques et al. 1990, and were recently discovered on the surfaces of Streptococcal species that cause invasive disease in humans. Pilus protein structures have an important role in adhesion and attachment to host cells in gram-negative bacteria. See Telford et al. (2006). It has been proposed that pilus structures serve a similar role in Streptococcal bacteria. Recently, putative pilus gene clusters were discovered in *S. suis*. Holden et al. (2009) described three putative pilus pathogenicity islands, and Takamatsu et al. (2009) described four of them (i.e. srtBCD, srtE, srtF and srtG). Takamatsu et al. (2009) also described the occurrence of these different pilus clusters in serotype 2, the primary serotype found in human infections, and other serotypes. Most of the examined isolates originated from Japan and Thailand. Available sequence analyses indicated that genes encoding for pilus proteins are clustered at the same genetic locus and are part of the same operon (pilus islands). It is not clear whether these putative pilus islands actually encode for pilus components. Study of the srtF cluster has suggested that pili at least from this cluster may be dispensable for critical steps of *S. suis* pathogenesis of infection. See Fittipaldi et al. (2010). In contrast, a surface antigen of *S. suis* serotype 2 with features of a pilus ancillary protein has been identified and shown to have immunoprotective effects in mice against *S. suis* infection. See Garibaldi et al. (2010).

[0006] The present invention provides novel immunogenic compositions based on putative *S. suis* pilus proteins or subunits isolated from swine that are useful for treating, reducing, or even preventing infection from one or more serotypes of *S. suis* in swine. These compositions may also be useful in preventing or prophylactically treating humans at risk of *S. suis* infection.

SUMMARY OF THE INVENTION

[0007] The invention provides immunogenic compositions that comprise at least one *Streptococcus suis* peptide that is putatively a pilus peptide. Preferably, these immunogenic compositions also include a physiologically-acceptable vehicle. Immunogenic compositions of the invention are useful for the prophylaxis, or even prevention, of *S. suis* infections, preferably of infection by multiple serotypes of *S. suis*.

[0008] *Streptococcus suis* pilus peptides are found in one or more *S. suis* serotypes and are encoded by one or more *S. suis* polynucleotides or contiguous fragments thereof that are described herein. Preferably, the *S. suis* serotypes include, or consist of, at least two serotypes selected from serotypes 1, 2, 7, and 9. For example, the *S. suis* serotypes may include, or consist of, a combination of serotypes 1 and 2; a combination of serotypes 1 and 7; a combination of serotypes 1 and 9; a combination of serotypes 2 and 7; a combination of serotypes 2 and 9; or a combination of serotypes 7 and 9. More preferably the combination includes, or consists of, a combination of serotypes 1, 2, and 7; a combination of serotypes 1, 2, and 9; a combination of serotypes 1, 7, and 9; or a combination of serotypes 2, 7, and 9. Most preferably, the *S. suis* serotypes include, or consist of, a combination of serotypes 1, 2, 7, and 9.

[0009] *Streptococcus suis* pilus peptides of the invention include peptides that are at least 98%, 95%, 90%, 85%, or even 80% homologous to and/or identical with any peptide having the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or a contiguous fragment thereof, and that is immunoreactive to *S. suis*, preferably such immunoreactivity yields a prophylactic or protective effect against *S. suis*. Exemplary *S. suis* pilus peptides of the invention comprise any one of the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or a contiguous fragment(s) thereof, that is(are) immunoreactive to *S. suis*. Preferably, *S. suis* pilus peptides of the invention include the amino acid motif LPXTG (SEQ ID NO: 28). Herein, a contiguous fragment comprises at least 5, preferably at least 8, more preferably at least 10, and even more preferably at least 15 contiguous amino acids or more of one of the aforesaid sequences.

[0010] In another aspect, the invention provides nucleic acid sequences that encode one or more *S. suis* pilus peptides, antibody constructs, or antibody conjugates. The gene sequences

coding for the *S. suis* pilus peptides comprise a nucleic acid sequence that is at least 98%, 95%, 90%, 85%, or even 80% homologous to and/or identical with the sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, or a contiguous fragment(s) thereof, coding for a peptide that is immunoreactive to *S. suis*, preferably such immunoreactivity yields a prophylactic or protective effect against *S. suis*. Exemplary nucleic acid sequences of the invention include any one of the sequences of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, or a contiguous fragment thereof that encodes a peptide of at least 5, preferably at least 8, more preferably at least 10, or even more preferably of at least 15 amino acids that is immunoreactive to *S. suis*. Preferably, *S. suis* pilus genes are those coding for a peptide that comprises the amino acid motif LPXTG (SEQ ID NO: 28).

[0011] A *S. suis* pilus peptide of the invention includes but is not limited to a peptide that comprises:

- i) a peptide comprising SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7;
- ii) a peptide that is at least 80% homologous to and/or identical with a peptide of i),
- iii) a contiguous fragment of a peptide of i) or ii);
- iv) a peptide of i), ii), or iii) further comprising SEQ ID NO: 28;
- v) a fragment of iii) or iv) comprising at least 5, preferably at least 8, more preferably at least 10, even more preferably at least 15 contiguous amino acids that are included within SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7;
- vi) a peptide that is encoded by a polynucleotide comprising the sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13;
- vii) a peptide that is encoded by a polynucleotide that is at least 80% homologous to or identical with a polynucleotide of vi); or
- viii) a peptide that is encoded by a polynucleotide that comprises at least 15, preferably at least 24, more preferably at least 30, even more preferably at least 45 contiguous

nucleotides of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO:11, SEQ ID NO: 12, or SEQ ID NO: 13,

wherein the *S. suis* pilus peptide is immunoreactive to *S. suis* such that administering the *S. suis* pilus peptide to a subject yields a prophylactic or protective effect against *S. suis* or a disease or adverse health condition that is associated with *S. suis* as compared to an untreated subject. Preferably, a therapeutically effective amount of the *S. suis* pilus peptide is administered to the subject such that one or more clinical symptoms of an *S. suis* infection is lessened as compared to an untreated subject.

[0012] Optionally, immunogenic compositions of the invention may include a carrier molecule. Where a carrier molecule is present, one or more *S. suis* pilus peptides may be conjugated to the carrier molecule. Carrier molecules that may be included in the invention comprise, but are not limited to, diphtheria toxoid, tetanus toxoid, keyhole limpet hemocyanin, bovine serum albumin, or combinations thereof. Carrier molecules included in immunogenic compositions of the invention may, themselves, be immunogenic. Preferably, the carrier molecules are non-toxic. Carrier molecules and *S. suis* pilus peptides may be conjugated to one another.

[0013] A physiologically-acceptable vehicle that is included in an immunogenic composition of the invention may be a pharmaceutically or veterinarily acceptable carrier, adjuvant, or combination thereof.

[0014] Any of the *S. suis* pilus peptides provided herewith or any immunogenic compositions comprising one or more of the *S. suis* pilus peptides provided can be used as a medicament, preferably as vaccine or immunogenic composition, most preferably for the prophylaxis or treatment of a subject against a *S. suis* infection.

[0015] Methods of the invention include, but are not limited to, a method of provoking an immune response against a *S. suis* infection in a subject comprising the administering of an immunogenic composition that comprises one or more *S. suis* pilus peptides as defined herein to the subject. Preferably, the immune response is provoked against more than one serotype of *S. suis*. Compositions of the invention may be used to treat or prevent a *S. suis* infection or a disease or adverse health condition that is associated with an *S. suis* infection. Preferably, the immune response that is provoked reduces the incidence of or severity of one or more clinical

signs associated with or caused by infection with one or more *S. suis* serotypes or a disease or adverse health condition that is associated with *S. suis*.

[0016] Herein, suitable subjects and subjects in need to which compositions of the invention may be administered include animals and humans. Preferably, animals are non-human mammals selected from the group consisting of a porcine, a murid, an equid, a lagomorph, and a bovid. Most preferably, the subject is a porcine (i.e. swine or pig).

[0017] The invention also provides a method of reducing the incidence or severity of one or more clinical signs associated with or caused by *S. suis* infection that comprises administering an immunogenic composition of the invention that comprises one or more *S. suis* pilus peptides as provided herewith such that the incidence of or the severity of a clinical sign of the *S. suis* infection is reduced by at least 10%, preferably by at least 20% or 30%, more preferably by at least 40% or 50%, even more preferably by at least 60% or even 70%, and most preferably by at least 80%, 90%, 95%, or even 100% relative to a subject that has not received the immunogenic composition. Clinical signs include lameness, meningitis, sepsis, septicemia, pneumonia, endocarditis, arthritis, endophthalmitis, deafness, and death. Any of these clinical signs may result from an infection with a *S. suis* having the serotype of strains P1/7 or 891591 or any of *S. suis* serotypes 1, 2, 7, or 9. Other clinical signs that are considered by clinicians in the field as being indicative of *S. suis* infection or a disease or adverse health condition that is associated with *S. suis* may also be used.

[0018] According to a further aspect, the present invention also relates to a method for the prophylaxis of a *S. suis* infection, wherein the *S. suis* infection may be caused by serotype 2, any other serotype of *S. suis*, or a combination of multiple *S. suis* serotypes, and the method comprises the administration of an immunogenic composition of the invention that comprises one or more *S. suis* pilus peptides as provided herewith.

[0019] Additionally, a method of preparing any of the immunogenic compositions of the invention is provided herewith. This method comprises mixing at least one *S. suis* pilus peptide of the invention with a physiologically-acceptable vehicle. One or more diluents or adjuvants may be admixed with the *S. suis* pilus peptide and the physiologically-acceptable vehicle. Optionally, this method comprises mixing one or more *S. suis* pilus peptides of the invention with a carrier molecule such that the one or more *S. suis* pilus peptides and carrier molecule are

covalently coupled or conjugated to one another. Such conjugates may be multivalent or univalent. Multivalent compositions or vaccines include an immuno-conjugation of multiple *S. suis* peptides with a carrier molecule.

[0020] In a further aspect, the invention provides a method of producing one or more *S. suis* pilus peptides that comprises transforming a host cell, preferably a prokaryotic cell such as *E. coli*, with a nucleic acid molecule that encodes for any of the *S. suis* pilus peptides as provided herewith. Alternatively, the host cell may be a eukaryotic cell such as an animal cell, protist cell, plant cell, or fungal cell. Preferably the eukaryotic cell is a mammalian cell such as CHO, BHK or COS, or a fungal cell such as *Saccharomyces cerevisiae*, or an insect cell such as Sf9.

[0021] Another aspect of the invention provides a method of producing one or more *S. suis* pilus peptides that induce an immune response against at least one serotype of *S. suis*, and preferably two or more serotypes of *S. suis*. This method comprises culturing an expression vector that has been transformed with a nucleic acid coding for and expressing one or more of the *S. suis* pilus peptides disclosed herein. The expressed proteins are either retained by the expression organism or secreted into the culture medium. Expression is conducted under conditions sufficient to produce a *S. suis* pilus peptide capable of inducing an immune response to *S. suis*. The one or more *S. suis* serotypes to which the *S. suis* pilus peptides induce an immune response preferably include serotypes 1, 2, 7, and 9.

[0022] Methods of making compositions of the invention may further comprise admixing the conjugate of one or more *S. suis* pilus peptides and a carrier molecule with a physiologically-acceptable vehicle such as a pharmaceutically- or veterinary-acceptable carrier, adjuvant, or combination thereof. Those of skill in the art will recognize that the choice of vehicle, adjuvant, or combination thereof will be influenced by the delivery route, personal preference, and animal species among other factors.

[0023] In another aspect, the invention provides a method of diagnosing a *S. suis* infection in a subject. This method comprises providing one or more *S. suis* pilus peptides; contacting the one or more *S. suis* pilus peptides with a sample obtained from the subject; and identifying the subject as either having a *S. suis* infection, previously being infected with *S. suis*, or having been vaccinated against *S. suis* if an antibody capable of specifically binding the one or more *S. suis* pilus peptides is detected in the sample. Preferably, a marker is included in an immunogenic

composition used to vaccinate against *S. suis* such that vaccinated subjects may be distinguished from non-vaccinated subjects.

[0024] In another respect, the invention provides a method of ascertaining that a subject has been previously exposed to a *S. suis* infection and is able to express an immune response to *S. suis*. This method comprises providing one or more *S. suis* pilus peptides; contacting the one or more *S. suis* pilus peptides with a sample obtained from the subject; and identifying the subject as having a *S. suis* infection if an antibody capable of specifically binding the one or more *S. suis* pilus peptides is detected in the sample. Preferably, the method includes the ability to detect and distinguish any marker that is indicative of a vaccinated subject so that a false positive for infection can be eliminated.

[0025] The invention also provides kits that comprise an immunogenic composition that comprises one or more *S. suis* pilus peptides; a container for packaging the immunogenic composition; a set of printed instructions; and a dispenser capable of administering the immunogenic composition to an animal. Optionally, the one or more *S. suis* pilus peptides may be packaged together or separately. If a carrier is included, the one or more *S. suis* pilus peptides may be conjugated to the carrier, or if the carrier is supplied in a separate container, a means of conjugating the one or more *S. suis* pilus peptides and carrier, as well as appropriate printed instructions, may be supplied.

[0026] The invention also provides kits for vaccinating an animal comprising a set of printed instructions; a dispenser capable of administering the immunogenic composition provided herewith comprising one or more *S. suis* pilus peptides to an animal; and wherein at least one of *S. suis* pilus peptides effectively immunizes the animal against at least one disease or adverse health condition that is associated with *S. suis* infection. Preferably, the one or more *S. suis* pilus peptides are selected from those provided herewith. Kits of the invention may further comprise a veterinary acceptable carrier, adjuvant, or combination thereof.

[0027] The dispenser in a kit of the invention is capable of dispensing its contents as droplets; and the immunogenic composition comprises the *S. suis* pilus peptides as provided herewith included in the kit is capable of reducing the severity of at least one clinical sign of a *S. suis* infection when administered intranasally, orally, intradermally, or intramuscularly to an animal. Preferably, the severity of a clinical sign is reduced by at least 10% preferably by at

least 20%, more preferably by at least 30%, even more preferably by at least 50% or by at least 60% or 70%, and most preferably by at least 80%, 90%, or even 100% as compared to an untreated, infected animal.

[0028] Methods for the treatment or prophylaxis of infections caused by *S. suis* are also disclosed. The method comprises administering an effective amount of the immunogenic composition of the present invention to a subject, wherein the treatment or prophylaxis is selected from the group consisting of reducing signs of *S. suis* infection, reducing the severity of or incidence of clinical signs of *S. suis* infection, reducing the mortality of subjects from *S. suis* infection, and combinations thereof.

[0029] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs at the time of filing. All patents and publications referred to herein are incorporated by reference herein.

[0030] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0032] FIG. 1. Schematic presentations of the sortase (srt) genes and the flanking regions are identified as follows: (FIG. 1A) SrtA genes of *S. suis* serotype 2 isolates P1/7 and 891591 and flanking genes; (FIG. 1B) SrtB, C, and D genes and flanking genes of P1/7. Homologous genes in *Streptococcus pneumoniae* are included for comparison; (FIG. 1C) SrtE genes and flanking genes of P1/7 and 891591; and (FIG. 1D) additional srt and flanking genes of 891591.

Homologous genes in other Streptococcal spp. included for comparison. In all schematics, the higher numbers indicate the SSU numbers as indicated in the P1/7 genome; and the lower numbers indicate the target numbers as used in the described studies and throughout this specification.

5 [0033] FIG. 2. Recombinant proteins expression in plasmid pET46 after induction by IPTG and as detected on a Coomassie stained gel is shown. Lane numbers correspond to clone (i.e. gene) numbers. Lane 1: “—” is non-induced *E. coli* cells containing plasmid pET46. Expected sizes of the expressed fusion proteins are indicated at the bottom of the lanes. Lane M is a molecular weight marker.

10 [0034] FIG. 3. An SDS-PAGE (Coomassie stained gel) analysis of fractions collected during purification of fragment 35.2. Lanes: L = lysate of induced *E. coli* (pET-46/35.2) cells; F = flow-through fraction; W = wash fraction; E = elution fraction; M = molecular weight marker.

[0035] FIG. 4. Affinity purified pilus proteins as analyzed with SDS-PAGE (Coomassie stained gel) is shown. Lane numbers correspond to protein clone numbers. M is a molecular weight marker.

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[0036] FIG. 5A. SDS-PAGE (Coomassie stained gel) analysis of affinity purified pilus proteins is shown. FIG. 5B. Western blot analysis on purified pilus proteins using rabbit antibodies directed against the purified pilus proteins is shown. Lane numbers correspond to clone numbers.

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[0037] FIG. 6. Western blot analysis of cell wall proteins extracted from various *S. suis* isolates using rabbit antibodies directed against the various purified pilus proteins is shown. Lane numbers correspond to the various *S. suis* strain isolates used for preparation of the cell wall extracts. Bottom rows show pre-immune sera, and top rows show final bleed sera. FIG. 6A shows antiserum generated against protein 3. FIG. 6B shows antiserum generated against protein 11. FIG. 6C shows antiserum generated against protein 12. FIG. 6D shows antiserum generated against protein 16. FIG. 6E shows antiserum generated against protein 25. FIG. 6F shows antiserum generated against protein 35-2. FIG. 6G shows antiserum generated against

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protein 35-1. FIG. 6H shows monoclonal antibodies directed against MRP and antiserum generated against protein 35-1.

[0038] FIG. 7. Electron micrograph (EM) examinations of various *S. suis* isolates for pilus expression are shown. Pili are expressed in *S. pneumoniae* isolates (FIG. 7A, from Barocchi et al., 2006, Proc. Nat. Acad. Sci. 103: 2875) and in *S. suis* serotype 2 isolate 040910-1 (FIG. 7B). FIGs. 7C-E shows pili expressed in *S. suis* serotype 2 isolates P1/7 (FIG. 7C), 17/3 (FIG. 7D) and 89/1591 (FIG. 7E). Arrows indicate some of the gold-labelled particles associated with the pili and randomly distributed among the grid.

[0039] FIG. 8. Schematic presentation of the procedure used to inactivate genes 3 (FIG. 8A), 12 (FIG. 8B) and 35 (FIG. 8C). The inactivated genes as well as the flanking genes are indicated in white and gray. Cloned regions are indicated in black. Primers are indicated by an arrow and numbered. Primer are described in Table 4.

[0040] FIG. 9A-9C. Schematic presentation of the procedure used to inactivate expression of putative pilus genes. The gene of interest is depicted by a white arrow ($\Rightarrow$); flanking regions are light gray; the spectinomycin resistance gene is a dark gray arrow ($\Rightarrow$); vectors are depicted with solid lines; and chromosomal DNA is depicted by dashed lines.

[0041] FIG. 10A-10C. Genotype of mutant isolates verified by polymerase chain reaction (PCR) using primers 5 and 6 (complete) and 7 and 8 (out) for pilus gene 3 (FIG. 10A); primers 15 and 16 (complete) and 17 and 18 (out) for pilus gene 12 (FIG. 10B) and primers 25 and 26 (complete) and 27 and 28 (out) for pilus gene 35 (FIG. 10C).

[0042] FIG. 11. SDS-PAGE (Coomassie stained gel) analysis of affinity purified pilus proteins. Lane numbers correspond to clone numbers.

[0043] FIG. 12. Western blot analyses of putative pilus proteins (3, 11, 12, 16, 25, 35.1 and 35.2) and purified cell wall proteins (1, 5, 8, 9, 10, 15, 20, 23 and 29) against the convalescent sera directed the various serotype 1 isolates. FIGs. 12A and 12B are analyses of serotype 1, strain 6112. FIGs. 12C and 12D are analyses of serotype 1, strain 6388. Lane numbers correspond to the protein numbers. Expected sizes of the purified proteins are indicated at the bottom of the lanes. Molecular weight sizes based on a protein marker are indicated at the left. A solid rectangle () marks the position of a positive reactivity of the serum at the

expected molecular size; a dotted rectangle () marks the position of a negative reactivity of the serum at the expected molecular size.

[0044] FIG. 13. Western blot analysis of putative pilus proteins (3, 11, 12, 16, 25, 35.1 and 35.2) and purified cell wall proteins (1, 5, 8, 9, 10, 15, 20, 23 and 29) against the convalescent sera directed the various serotype 2 isolates. FIGs. 13A-13C show serotype 2, strain 3; FIGs. 13D-13F show serotype 2, strain 10; and FIGs. 13G-13I show serotype 2, strain P1/7. Lane numbers correspond to the protein numbers. Expected sizes of the purified proteins are indicated at the bottom of the lanes. A solid rectangle () marks the position of a positive reactivity of the serum at the expected molecular size; a dotted rectangle () marks the position of a negative reactivity of the serum at the expected molecular size.

[0045] FIG. 14. Western blot analysis of putative pilus proteins (3, 11, 12, 16, 25, 35.1 and 35.2) and purified cell wall proteins (1, 5, 8, 9, 10, 15, 20, 23 and 29) against the convalescent sera directed the various serotype 7 isolates. FIGs 14A and 14B are serotype 7, strain 7711; FIGs. 14C and 14D are serotype 7, strain 7917; and FIGs. 14E and 14F are serotype 7, strain 8039. Lane numbers correspond to the protein numbers. Expected sizes of the purified proteins are indicated at the bottom of the lanes. A solid rectangle () marks the position of a positive reactivity of the serum at the expected molecular size; a dotted rectangle () marks the position of a negative reactivity of the serum at the expected molecular size.

[0046] FIG. 15. Western blot analysis of putative pilus proteins (3, 11, 12, 16, 25, 35.1 and 35.2) and purified cell wall proteins (1, 5, 8, 9, 10, 15, 20, 23 and 29) against the convalescent sera directed the various serotype 9 isolates. FIGs. 15A and 15B are serotype 9, strain 8067; and FIGs. 15C and 15D are serotype 9, strain 8017. Lane numbers correspond to the protein numbers. Expected sizes of the purified proteins are indicated at the bottom of the lanes. A solid rectangle () marks the position of a positive reactivity of the serum at the expected molecular size; a dotted rectangle () marks the position of a negative reactivity of the serum at the expected molecular size.

[0047] FIG. 16A-16D. Western blot analysis of cell wall proteins extracted from various *S. suis* isolates using rabbit antibodies directed against the various purified pilus proteins. Lane numbers correspond to the various isolates used for preparation of the cell wall extracts: (Top row, FIGs. 16A and 16B) antiserum generated against protein 3 purified using a native

purification procedure; (Bottom row, FIGs. 16C and 16D) antiserum generated against protein 3 purified using a denatured purification procedure.

[0048] FIG. 17A-17D. Western blot analysis of cell wall proteins extracted from various *S. suis* isolates using rabbit antibodies directed against the various purified pilus proteins. Lane numbers correspond to the various isolates used for preparation of the cell wall extracts: (Top row, FIGs. 17A and 17B) antiserum generated against protein 12 purified using a native purification procedure; (Bottom row, FIGs. 17C and 17D) antiserum generated against protein 12 purified using a denatured purification procedure.

[0049] FIG. 18A-18D. Western blot analysis of cell wall proteins extracted from various *S. suis* isolates using rabbit antibodies directed against the various purified pilus proteins. Lane numbers correspond to the various isolates used for preparation of the cell wall extracts: (Top row, FIGs. 18A and 18B) antiserum generated against protein 35-1 purified using a native purification procedure; (Bottom row, FIGs. 18C and 18D) antiserum generated against protein 35-1 purified using a denatured purification procedure.

DETAILED DESCRIPTION

[0050] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, protein chemistry and immunology, which are within the skill of the art. Such techniques are explained in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Vols. I, II and III, Second Edition (1989); *DNA Cloning: A Practical Approach*, Vols. I-IV, Second Edition (D. N. Glover and B. D. Hames eds., 1996); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization: A Practical Approach* (B. D. Hames & S. J. Higgins eds. 1984); *Animal Cell Culture*, Third Edition (J. R. W. Masters ed. 2000); *Immobilized Cells and Enzymes* (J. Woodward, ed., IRL press, 1986); Perbal, B., *A Practical Guide to Molecular Cloning*, Second Edition (B. Perbal, 1988); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Protein purification methods – a practical approach* (E.L.V. Harris and S. Angal, eds., IRL Press at Oxford University Press, 1990); and *Handbook of Experimental Immunology*, Vols. I-IV, Fifth Edition (D. M. Weir and C. C. Blackwell eds., 1997, Blackwell Scientific Publications).

[0051] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular nucleic acid molecules, polypeptide sequences or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

A. Definitions

[0052] As used in this specification and the claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more antigens, reference to "an excipient" includes mixtures of two or more excipients, and the like.

[0053] The terms "*S. suis* peptide", "*S. suis* pilus peptide" and variants thereof refer to the putative *S. suis* pilus peptides and proteins that are described herein unless otherwise specifically stated.

[0054] An "immunogenic or immunological composition" refers to a composition of matter that comprises at least one *S. suis* pilus peptide as provided herewith that elicits in the host a cellular or antibody-mediated immune response to *S. suis*. In a preferred embodiment of the present invention, the immunogenic composition comprises one or more of the *S. suis* pilus peptides disclosed herein and induces an immune response and, more preferably, confers protective immunity against one or more of the clinical signs of a *S. suis* infection or a disease or condition associated with a *S. suis* infection.

[0055] The term "fragment" refers to a contiguous polypeptide or truncated and/or substituted form of a *S. suis* pilus peptide or a gene coding for such a *S. suis* pilus peptide that includes one or more epitopes and thus elicits the immunological response against *S. suis*.

Preferably, such fragment is a contiguous fragment or truncated and/or substituted form of any of the *S. suis* pilus peptides or any of the *S. suis* pilus genes provided herewith. In general, such truncated and/or substituted forms, or fragments will comprise at least six contiguous amino acids from the full-length *S. suis* pilus peptide. More preferably, the truncated or substituted forms, or fragments will have at least 10, more preferably at least 15, and still more preferably at least 19 contiguous amino acids from the full-length *S. suis* pilus peptide. Such fragments can be

identified using any number of epitope mapping techniques, well known in the art. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by concurrently synthesizing large numbers of peptides on solid supports, the peptides
5 corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known and described in the art, see e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) Proc. Natl. Acad. Sci. USA 81:3998-4002; and Geysen et al. (1986) Molec. Immunol. 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino
10 acids such as by, e.g., x-ray crystallography and two-dimensional nuclear magnetic resonance. See Epitope Mapping Protocols, supra. Synthetic antigens are also included within the definition, for example, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens. See, e.g., Bergmann et al. (1993) Eur. J. Immunol. 23:2777-2781; Bergmann et al. (1996), J. Immunol. 157:3242-3249; Suhrbier, A. (1997), Immunol. and Cell Biol. 75:402-
15 408; and Gardner et al., (1998) 12th World AIDS Conference, Geneva, Switzerland, June 28-July 3, 1998. (The teachings and content of each of the aforementioned references are all incorporated by reference herein.)

[0056] The term “immunoreactive to *S. suis*” as used herein means that the peptide or fragment elicits an immunological response against *S. suis*.

20 [0057] An “immune response” or “immunological response” means, but is not limited to, the development of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, an immune or immunological response includes, but is not limited to, one or more of the following effects: the production or activation of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells, directed specifically to an antigen or
25 antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or a protective immunological (memory) response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction in number of symptoms, severity of symptoms, or the lack of one or more of the symptoms associated with infection by the pathogen, a delay in the of

onset of viremia, reduced viral persistence, a reduction in the overall viral load and/or a reduction of viral excretion.

[0058] “Protection against *S. suis*”, “protective immunity” , and similar phrases, mean an immune response against *S. suis*, respectively, generated by an immunization schedule that results in fewer deleterious effects than would be expected in a non-immunized subject that has not been previously exposed to *S. suis*. That is, the severity of the deleterious effects of the infection are lessened in an immunized subject because the subject’s immune system is resistant to *S. suis*. Infection may be reduced, slowed, or possibly fully prevented, in an immunized animal, preferably a pig or human. Herein, where complete prevention of infection is meant, it is specifically stated. If complete prevention is not stated then the term includes partial prevention.

[0059] Herein, “reduction of the incidence and/or severity of clinical signs” or “reduction of clinical symptoms” means, but is not limited to, reducing the number of infected subjects in a group, reducing or eliminating the number of subjects exhibiting clinical signs of infection, or reducing the severity of any clinical signs that are present in the subjects, in comparison to wild-type infection. For example, it should refer to any reduction of pathogen load, pathogen shedding, reduction in pathogen transmission, or reduction of any clinical sign symptomatic of *S. suis* infection, respectively, such as arthritis or meningitis, or any other recognized clinical sign. Preferably these clinical signs are reduced in subjects receiving the composition of the present invention by at least 10% in comparison to subjects not receiving the composition and become infected. More preferably clinical signs are reduced in subjects receiving the composition of the present invention by at least 20%, more preferably by at least 30%, even more preferably by at least 40%, and most preferably by at least 50% or more.

[0060] As used herein, “a pharmaceutical- or veterinary-acceptable carrier” includes solvents, dispersion media, coatings, adjuvants, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. In some preferred embodiments, and especially those that include lyophilized immunogenic compositions, stabilizing agents for use in the present invention include stabilizers for lyophilization or freeze-drying.

[0061] “Adjuvants” as used herein, can include aluminum hydroxide and aluminum phosphate, saponins e.g., Quil A, QS-21 (Cambridge Biotech Inc., Cambridge MA), GPI-0100

(Galenica Pharmaceuticals, Inc., Birmingham, AL), water-in-oil emulsion, oil-in-water emulsion, water-in-oil-in-water emulsion. The emulsion can be based in particular on light liquid paraffin oil (European Pharmacopea type); isoprenoid oil such as squalane or squalene; oil resulting from the oligomerization of alkenes, in particular of isobutene or decene; esters of acids or of alcohols containing a linear alkyl group, more particularly plant oils, ethyl oleate, propylene glycol di-
5 (caprylate/caprate), glyceryl tri-(caprylate/caprate) or propylene glycol dioleate; esters of branched fatty acids or alcohols, in particular isostearic acid esters. The oil is used in combination with emulsifiers to form the emulsion. The emulsifiers are preferably nonionic surfactants, in particular esters of sorbitan, mannide (e.g. anhydromannitol oleate), glycol,
10 polyglycerol, propylene glycol, and of oleic, isostearic, ricinoleic or hydroxystearic acid, which are optionally ethoxylated, and polyoxypropylene-polyoxyethylene copolymer blocks, in particular the Pluronic products, especially L121. See Hunter et al., The Theory and Practical Application of Adjuvants (Ed. Stewart-Tull, D. E. S.). John Wiley and Sons, NY, pp51-94 (1995) and Todd et al., Vaccine 15:564-570 (1997). Exemplary adjuvants are the SPT emulsion
15 described on page 147 of "Vaccine Design, The Subunit and Adjuvant Approach" edited by M. Powell and M. Newman, Plenum Press, 1995, and the emulsion MF59 described on page 183 of this same book, incorporated herein by reference.

[0062] A further instance of an adjuvant is a compound chosen from the polymers of acrylic or methacrylic acid and the copolymers of maleic anhydride and alkenyl derivative.
20 Advantageous adjuvant compounds are the polymers of acrylic or methacrylic acid which are cross-linked, especially with polyalkenyl ethers of sugars or polyalcohols. These compounds are known by the term carbomer (Phameuropa Vol. 8, No. 2, June 1996). Persons skilled in the art can also refer to U.S. Patent No. 2,909,462 which describes such acrylic polymers cross-linked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably not more than
25 8, the hydrogen atoms of at least three hydroxyls being replaced by unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2 to 4 carbon atoms, e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may themselves contain other substituents, such as methyl. The products sold under the name Carbopol®; (BF Goodrich, Ohio, USA) (i.e. polymers of acrylic acid cross-linked with
30 polyalkenyl ethers or divinyl glycol) are particularly appropriate. They are cross-linked with an allyl sucrose or with allyl pentaerythritol. Among then, there may be mentioned Carbopol®

974P, 934P and 971P. Most preferred is the use of Carbopol® 971P. Among the copolymers of maleic anhydride and alkenyl derivative, are the copolymers EMA (Monsanto), which are copolymers of maleic anhydride and ethylene. The dissolution of these polymers in water leads to an acid solution that will be neutralized, preferably to physiological pH, in order to give the adjuvant solution into which the immunogenic, immunological or vaccine composition itself will be incorporated.

[0063] Further suitable adjuvants include, but are not limited to, the RIBI adjuvant system (Ribi Inc.), Block co-polymer (CytRx, Atlanta GA), SAF-M (Chiron, Emeryville CA), monophosphoryl lipid A, Avridine lipid-amine adjuvant, heat-labile enterotoxin from *E. coli* (recombinant or otherwise), cholera toxin, IMS 1314 or muramyl dipeptide, or naturally occurring or recombinant cytokines or analogs thereof or stimulants of endogenous cytokine release, among many others.

[0064] “Diluents” can include water, saline, dextrose, ethanol, glycerol, and the like. “Isotonic agents” can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others.

[0065] “Stabilizers” can include albumin and alkali salts of ethylenediaminetetracetic acid, among others.

[0066] Herein, “effective dose” means, but is not limited to, an amount of antigen that elicits, or is able to elicit, an immune response that yields a reduction of clinical symptoms in a subject to which the antigen is administered.

[0067] “Isolated” means altered “by the hand of man” from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not “isolated,” but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is “isolated”, as the term is employed herein.

[0068] The terms “vaccination” or “vaccinating” or variants thereof, as used herein means, but is not limited to, a process which includes the administration of a *S. suis* pilus antigen that, when administered to a subject, elicits, or is able to elicit directly or indirectly, an immune response in the subject against *S. suis*.

[0069] “Mortality” in the context of the present invention refers to death caused by *S. suis* infection, and includes the situation where the infection is so severe that an animal is euthanized to prevent suffering and provide a humane ending to its life.

[0070] An “effective amount” for purposes of the present invention, means an amount of an immunogenic composition capable of inducing an immune response that reduces the incidence of or lessens the severity of *S. suis* infection in a subject. Particularly, an effective amount refers to colony forming units (CFU) per dose.

[0071] “Long-lasting protection” shall refer to “improved efficacy” that persists for at least 3 weeks, but more preferably at least 3 months, still more preferably at least 6 months in an animal, preferably a pig. It is most preferred that the long lasting protection shall persist until the average age at which porcine animals are marketed for meat.

[0072] “Sequence homology”, as used herein, refers to a method of determining the relatedness of two sequences. To determine sequence homology, two or more sequences are optimally aligned, and gaps are introduced if necessary. However, in contrast to “sequence identity”, conservative amino acid substitutions are counted as a match when determining sequence homology. In other words, to obtain a polypeptide or polynucleotide having sequence homology with a reference sequence, 85%, preferably 90%, even more preferably 95% of the amino acid residues or nucleotides in the reference sequence must match or comprise a conservative substitution with another amino acid or nucleotide, or a number of amino acids or nucleotides up to 15%, preferably up to 10%, even more preferably up to 5% of the total amino acid residues or nucleotides, not including conservative substitutions, in the reference sequence may be inserted into the reference sequence. Preferably the homologous nucleotide sequence comprises at least a stretch of 50 nucleotides, more preferably 100 nucleotides, even more preferably 250 nucleotides, and most preferably 500 or more nucleotides. Preferably, the homologous amino acid sequence comprises at least a stretch of 10 amino acids, more preferably 20 amino acids, even more preferably 35 amino acids, and most preferably 50 or more amino acids.

[0073] A “conservative substitution” refers to the substitution of an amino acid residue or nucleotide with another amino acid residue or nucleotide having similar characteristics or

properties including size, hydrophobicity, etc., such that the overall functionality does not change significantly.

[0074] “Sequence Identity” as it is known in the art refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, namely a reference sequence and a given sequence to be compared with the reference sequence. Sequence identity is determined by comparing the given sequence to the reference sequence after the sequences have been optimally aligned to produce the highest degree of sequence similarity, as determined by the match between strings of such sequences. Upon such alignment, sequence identity is ascertained on a position-by-position basis, e.g., the sequences are “identical” at a particular position if at that position, the nucleotides or amino acid residues are identical. The total number of such position identities is then divided by the total number of nucleotides or residues in the reference sequence to give % sequence identity. Sequence identity can be readily calculated by known methods, including but not limited to, those described in Computational Molecular Biology, Lesk, A. N., ed., Oxford University Press, New York (1988), Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H. G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinge, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988), the teachings of which are incorporated herein by reference. Preferred methods to determine the sequence identity are designed to give the largest match between the sequences tested.

[0075] Methods to determine sequence identity are codified in publicly available computer programs which determine sequence identity between given sequences. Examples of such programs include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research, 12(1):387 (1984)), BLASTP, BLASTN and FASTA (Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al., NCVI NLM NIH Bethesda, MD 20894, Altschul, S. F. et al., J. Molec. Biol., 215:403-410 (1990), the teachings of which are incorporated herein by reference). These programs optimally align sequences using default gap

weights in order to produce the highest level of sequence identity between the given and reference sequences. As an illustration, a polynucleotide having a nucleotide sequence having at least 85%, preferably 90%, even more preferably 95% "sequence identity" to a reference nucleotide sequence, it is intended that the nucleotide sequence of the given polynucleotide is identical to the reference sequence except that the given polynucleotide sequence may include up to 15, preferably up to 10, even more preferably up to 5 point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, a polynucleotide having at least 85%, preferably 90%, even more preferably 95% identity relative to the reference nucleotide sequence has up to 15%, preferably 10%, even more preferably 5% of the nucleotides in the reference sequence deleted or substituted with another nucleotide, or a number of nucleotides up to 15%, preferably 10%, even more preferably 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, a polypeptide having a given amino acid sequence having at least, for example, 85%, preferably 90%, even more preferably 95% sequence identity to a reference amino acid sequence, it is intended that the given amino acid sequence of the polypeptide is identical to the reference sequence except that the given polypeptide sequence may include up to 15, preferably up to 10, even more preferably up to 5 amino acid alterations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a given polypeptide sequence having at least 85%, preferably 90%, even more preferably 95% sequence identity with a reference amino acid sequence, up to 15%, preferably up to 10%, even more preferably up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 15%, preferably up to 10%, even more preferably up to 5% of the total number of amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or the carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in the one or more contiguous groups within the reference sequence. Preferably, residue positions which are not identical differ by

conservative amino acid substitutions. However, conservative substitutions are not included as a match when determining sequence identity.

[0076] The invention provides immunogenic compositions, as well as uses and methods of making the compositions, that are useful for the treatment, reduction, or even the prevention of infection from one or more serotypes of *S. suis*.

B. *S. suis* Pilus Proteins

[0077] Herein, are disclosed putative genes that encode *S. suis* pilus proteins or protein fragments from multiple serotypes. The encoded *S. suis* pilus proteins and fragments (collectively “*S. suis* pilus polypeptides” herein) also are disclosed. The disclosed putative pilus genes are referenced herein as genes 3, 11, 12, 16, 25 and 35—SEQ ID NOs: 8-13, respectively. Gene 35 is disrupted and encodes two protein fragments referred to herein as either 35.1 and 35.2 or as 35-1 and 35-2. Proteins 3, 11, 12, 16, and 25 are respective SEQ ID NOs: 1-5 herein; protein fragments 35.1 and 35.2 are respective SEQ ID NOs: 6 and 7 herein.

[0078] These *S. suis* pilus polypeptides form the bases of compositions that are immunogenic, preferably in swine, humans, or both. Multiple *S. suis* pilus polypeptides may be combined with each other to yield immunogenic compositions that result in a protective immunity, either partial or complete, against multiple *S. suis* serotypes. Individual *S. suis* pilus polypeptides may be immunogenic against multiple *S. suis* serotypes. It is proposed that these immunogenic compositions, based on one or more *S. suis* pilus polypeptides, will overcome the deficiencies of prior vaccines and provide protection, either partial or complete, against multiple serotypes, preferably against *S. suis* serotypes 1, 2, 7, 9, or combinations thereof.

[0079] Generally, Streptococcal spp. pilus genes are located near sortase (srt) genes in a Streptococcal genome (Telford et al., 2006, incorporated herein by reference). In addition, pilus genes usually contain the amino acid motif LPXTG (SEQ ID NO: 28) (Telford et al., 2006). The *S. suis* pilus genes 3, 11, 12, 16, 25 and 35 of the present invention are located near putative sortase genes in the *S. suis* genomes from which they originate. See FIG. 1 and Example 1. Further, each of the putative *S. suis* pilus polypeptides 3, 11, 12, 16, 25, 35-1, and 35-2 of the invention include the amino acid motif LPXTG (SEQ ID NO: 28).

[0080] Those of skill in the art will recognize that the *S. suis* pilus genes disclosed herein may be mutated, either by deletion(s), insertion(s), substitution(s), or combinations thereof, such that they either yield the same *S. suis* pilus polypeptides disclosed herein (i.e. the mutation(s) are silent) or yield *S. suis* pilus proteins or protein fragments that are immunogenically indistinguishable from or very similar to the *S. suis* pilus polypeptides of SEQ ID NOs: 1-7. Preferably, a mutated *S. suis* pilus gene shares at least 85% homology to and/or identity with one of SEQ ID NOs: 8-13.

[0081] Similarly, those of skill in the art will recognize that the *S. suis* pilus polypeptides may also be mutated, either by deletion(s), insertion(s), substitution(s), or combinations thereof, such that they either yield *S. suis* pilus proteins or protein fragments that are immunogenically indistinguishable from or very similar to the *S. suis* pilus polypeptides of SEQ ID NOs: 1-7. Preferably, a mutated *S. suis* pilus protein or protein fragment shares at least 85% homology to and/or identity with one of SEQ ID NOs: 1-7.

C. Expression Systems

[0082] Expression systems that can be used for the production the *S. suis* pilus peptides provided herein, are well known in the art and include, but not limited to, bacterial expression systems, yeast expression systems, insect cell or mammalian expression systems. Vectors and methods for making and/or using vectors (or recombinants) for expression of the *S. suis* pilus peptides provided herewith can be by or analogous to the methods disclosed in: U.S. Patent Nos. 4,603,112, 4,769,330, 5,174,993, 5,505,941, 5,338,683, 5,494,807, 4,722,848, 5,942,235, 5,364,773, 5,762,938, 5,770,212, 5,942,235, 382,425, PCT publications WO 94/16716, WO 96/39491, WO 95/30018, Paoletti, "Applications of pox virus vectors to vaccination: An update," PNAS USA 93: 11349-11353, October 1996, Moss, "Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety," PNAS USA 93: 11341-11348, October 1996, Smith et al., U. S. Patent No. 4,745,051, (recombinant baculovirus), Richardson, C.D. (Editor), Methods in Molecular Biology 39, "Baculovirus Expression Protocols" (1995 Humana Press Inc.), Smith et al., "Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector", Molecular and Cellular Biology, Dec., 1983, Vol. 3, No. 12, p. 2156-2165; Pennock et al., "Strong and Regulated Expression of Escherichia coli B-Galactosidase in Infect Cells with a Baculovirus vector," Molecular and Cellular Biology Mar.

1984, Vol. 4, No. 3, p. 399-406; EPA0 370 573, U. S. application No. 920,197, filed October 16, 1986, EP Patent publication No. 265785, U. S. Patent No. 4,769,331 (recombinant herpesvirus), Roizman, "The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors," PNAS USA 93:11307-11312, October 1996, Andreansky et al.,
 5 "The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors," PNAS USA 93: 11313-11318, October 1996, Robertson et al. "Epstein-Barr virus vectors for gene delivery to B lymphocytes", PNAS USA 93: 11334-11340, October 1996, Frolov et al., "Alphavirus-based expression vectors: Strategies and applications," PNAS USA 93: 11371-11377, October 1996, Kitson et al., J. Virol. 65,3068-3075,1991; U. S.
 10 Patent Nos. 5,591,439, 5,552,143, WO 98/00166, allowed U. S. applications Serial Nos. 08/675,556, and 08/675,566 both filed July 3,1996 (recombinant adenovirus), Grunhaus et al., 1992,"Adenovirus as cloning vectors," Seminars in Virology (Vol. 3) p. 237-52, 1993, Ballay et al. EMBO Journal, vol. 4, p. 3861-65,Graham, Tibtech 8,85-87, April, 1990, Prevec et al., J. Gen Virol. 70,42434, PCT WO 91/11525, Felgner et al. (1994), J. Biol. Chem. 269,2550-2561,
 15 Science, 259: 1745-49,1993 and McClements et al., "Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease", PNAS USA 93: 11414-11420, October 1996, and U. S. Patent Nos. 5,591,639, 5,589,466, and 5,580,859, as well as WO 90/11092, WO93/19183, WO94/21797, WO95/11307, WO95/20660, Tang et al., Nature and
 20 Furth et al. Analytical Biochemistry, relating to DNA expression vectors, inter alia. See also WO 98/33510; Ju et al., Diabetologia, 41: 736-739,1998 (lentiviral expression system); Sanford et al., U. S. Patent No. 4,945,050; Fischbach et al. (Intracel), WO 90/01543; Robinson et al., seminars in Immunology vol. 9, pp. 271-283 (1997), (DNA vector systems); Szoka et al., U. S. Patent No. (method of inserting DNA into living cells); McCormick et al., U. S. Patent No. 5,677,178 (use
 25 of cytopathic viruses); and U. S. Patent No. 5,928,913 (vectors for gene delivery), as well as other documents cited herein.

[0083] A preferred cloning strategy is described in the Examples below. Briefly, selected pilus genes of *S. suis* serotype 2 isolates P1/7 or 89/1591 (herein also referred to as 891591) were cloned and expressed in *E. coli* Novablue; or alternatively, transformants were screened with
 30 polymerase chain reaction (PCR), plasmid constructs having the expected genetic sequence were

transformed into *E. coli* BL21 (DE3), expression with IPTG was induced, and the resulting proteins were sequenced.

[0084] Regardless of the cloning strategy used, the selected transformants were cloned and expressed in *E. coli* using the pET46 Ek/LIC vector. Those of skill in the art will be familiar with the uses of this vector as it is commercially available and instructions are provided by the manufacturer. Vector pET46 Ek/LIC has an N-terminal his fusion and a tightly controlled T7 promoter. It also yields high expression of genetic inserts. The his fusion can be removed using an enterokinase using techniques known in the art.

[0085] Expression of cloned inserts as examined with Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Coomassie Brilliant Blue (CBB) staining indicated that all proteins, except protein 4, were expressed at their predicted molecular size. Very little, if any, protein 4 were detected in pET-46 clones, and no protein 4 was detected in *E. coli* BL21 clones.

D. Carriers Molecules

[0086] While not preferred, in certain aspects, the *S. suis* pilus peptides of the invention may be conjugated or covalently linked to carrier molecules. Preferred carriers for animal use are bovine serum albumin and Keyhole Limpet Hemocyanin. Protein carriers suitable for human use include tetanus toxoid, diphtheria toxoid, acellular pertussis vaccine (LPF toxoid), cross-reacting materials (CRM's) which are antigenically similar to bacterial toxins but are non-toxic by means of mutation. For example, CRM 197 obtained according to Pappenheimer, et al, Immunochemistry, 9, 891-906 (1972), and other bacterial protein carriers, for example meningococcal outer membrane protein may be used. Preferably, the carrier protein itself is an immunogen.

[0087] The *S. suis* pilus peptides of the invention may be covalently coupled to the carrier by any convenient method known to the art. While use of a symmetric linker such as adipic acid dihydrazide, as described by Schneerson et al, J. Experimental Medicine, 152, 361-376 (1980), or a heterobifunctional linker such as N-succinimidyl 3-(2-pyridyldithio) propionate as described by Fattom et al, Infection and Immunity, 56, 2292-2298 (1988) are within the scope of the invention, it is preferred to avoid the use of any linker but instead couple a *S. suis* peptide of the

invention directly to the carrier molecule. Such coupling may be achieved by means of reductive amination as described by Landi et al J. Immunology, 127, 1011-1019 (1981).

[0088] The size of the immunogenic composition, as defined by average molecular weight, is variable and dependent upon the chosen *S. suis* pilus peptide(s) and the method of coupling of the *S. suis* pilus peptide(s) to the carrier. Therefore, it can be as small as 1,000 daltons (10^3) or greater than 10^6 daltons. With the reductive amination coupling method, the molecular weight of the *S. suis* pilus peptide(s) is usually within the range of 5,000 to 500,000, for example 300,000 to 500,000, or for example 5,000 to 50,000 daltons.

[0089] Carrier molecules, i.e. peptides, derivatives and analogs thereof, and peptide mimetics that specifically bind a *S. suis* pilus peptide of the invention can be produced by various methods known in the art, including, but not limited to solid-phase synthesis or by solution (Nakanishi et al., 1993, Gene 137:51-56; Merrifield, 1963, J. Am. Chem. Soc. 15:2149-2154; Neurath, H. et al., Eds., The Proteins, Vol II, 3d Ed., p. 105-237, Academic Press, New York, N.Y. (1976), incorporated herein in their entirety by reference).

[0090] The *S. suis* pilus peptides of the invention or the antibodies or binding portions thereof of the present invention may be administered in injectable dosages by solution or suspension of in a diluent with a pharmaceutical or veterinary carrier.

[0091] Toxicity and therapeutic efficacy of such molecules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population).

[0092] The vaccines of the invention may be multivalent or univalent. Multivalent vaccines are made from immuno-conjugation of multiple *S. suis* pilus peptides with a carrier molecule.

[0093] In one aspect, the *S. suis* pilus peptide compositions comprise an effective immunizing amount of the immunogenic conjugate, preferably in combination with an immunostimulant; and a physiologically acceptable vehicle. As used in the present context, "immunostimulant" is intended to encompass any compound or composition which has the ability to enhance the activity of the immune system, whether it be a specific potentiating effect in combination with a specific antigen, or simply an independent effect upon the activity of one

or more elements of the immune response. Immunostimulant compounds include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP. Methods of utilizing these materials are known in the art, and it is well within the ability of the skilled artisan to
5 determine an optimum amount of stimulant for a given vaccine. More than one immunostimulant may be used in a given formulation. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation.

[0094] The compositions may, if desired, be presented in a pack or dispenser device which
10 may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration preferably for administration to a mammal, especially a pig. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological
15 products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

E. Adjuvants

[0095] In order to further increase the immunogenicity of the immunogenic compositions provided herewith, and which contain one or more *S. suis* pilus peptides may also comprise one
20 or more adjuvants.

[0096] The adjuvant may be purified by any of the techniques described previously or known in the art. The preferred purification technique is silica gel chromatography, in particular the "flash" (rapid) chromatographic technique, as described by W. Clark Still et al, J. Organic Chemistry, 43, 2923-2925 (1978). However, other chromatographic methods, including HPLC,
25 may be used for purification of the adjuvant. Crystallization may also be used to purify the adjuvant. In some cases, no purification is required as a product of analytical purity is obtained directly from the synthesis.

[0097] The vaccine compositions of the invention are prepared by physically mixing the adjuvant with the *S. suis* pilus peptide(s) under appropriate sterile conditions in accordance with

known techniques to produce the adjuvanted composition. Complexation of the *S. suis* peptide(s) and the adjuvant is facilitated by the existence of a net negative charge on the conjugate which is electrostatically attracted to the positive charge present on the long chain alkyl compound adjuvant.

5 [0098] It is expected that an adjuvant can be added in an amount of about 100 μ g to about 10 mg per dose, preferably in an amount of about 100 μ g to about 10 mg per dose, more preferably in an amount of about 500 μ g to about 5 mg per dose, even more preferably in an amount of about 750 μ g to about 2.5 mg per dose, and most preferably in an amount of about 1 mg per dose. Alternatively, the adjuvant may be at a concentration of about 0.01% to 75%,
10 preferably at a concentration of about 2% to 30%, more preferably at a concentration of about 5% to 25%, still more preferably at a concentration of about 7% to 22%, and most preferably at a concentration of 10% to 20% by volume of the final product.

F. Physiologically-Acceptable Vehicles

[0099] The vaccine compositions of this invention may be formulated using techniques
15 similar to those used for other pharmaceutical polypeptide compositions. Thus, the adjuvant and *S. suis* pilus peptide(s), preferably conjugated to carrier molecule and/or admixed with an adjuvant may be stored in lyophilized form and reconstituted in a physiologically acceptable vehicle to form a suspension prior to administration. Alternatively, the adjuvant and conjugate may be stored in the vehicle. Preferred vehicles are sterile solutions, in particular, sterile buffer
20 solutions, such as phosphate buffered saline. Any method of combining the adjuvant and the conjugate in the vehicle such that improved immunological effectiveness of the immunogenic composition is appropriate.

[0100] The volume of a single dose of the vaccine of this invention may vary but will be generally within the ranges commonly employed in conventional vaccines. The volume of a
25 single dose is preferably between about 0.1 ml and about 3 ml, preferably between about 0.2 ml and about 1.5 ml, more preferably between about 0.2 ml and about 0.5 ml at the concentrations of conjugate and adjuvant noted above.

G. Formulation

[0101] Immunogenic compositions and vaccines of the invention comprise at least one *S. suis* pilus peptide. Preferably, the *S. suis* pilus peptide(s) is in a physiologically-acceptable vehicle. An adjuvant and/or diluent may also be included in immunogenic compositions and
5 vaccines of the invention. Optionally, a carrier may be conjugated to a *S. suis* pilus peptide prior to admixing with a physiologically-acceptable vehicle.

[0102] The formulations of the invention comprise an effective immunizing amount of one or more immunogenic compositions or antibodies thereto and a physiologically acceptable vehicle. Vaccines comprise a therapeutically effective immunizing amount of one or more
10 immunogenic compositions and a physiologically acceptable vehicle. The formulation should suit the mode of administration.

[0103] Antibodies generated against immunogenic conjugates of the present invention by immunization with an immunogenic conjugate can be used in passive immunotherapy and generation of antiidiotypic antibodies for treating or preventing infections of *S. suis*.

[0104] The immunogenic composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The immunogenic composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium
15 carbonate, etc.
20

H. Effective Dose

[0105] The compounds described herein can be administered to a subject at therapeutically effective doses to treat *S. suis*-associated diseases. The dosage will depend upon the host receiving the vaccine as well as factors such as the size, weight, and age of the host.

[0106] The precise amount of immunogenic conjugate or antibody of the invention to be employed in a formulation will depend on the route of administration and the nature of the subject (e.g., species, age, size, stage/level of disease), and should be decided according to the judgment of the practitioner and each subject's circumstances according to standard clinical techniques. An effective immunizing amount is that amount sufficient to treat or prevent an *S. suis* infectious disease in a subject such that at least one clinical sign of *S. suis* is reduced by at
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least 10%—preferably by 20-40%, more preferably by 50-70%, even more preferably by 80-95%, and most preferably by 96-100%—as compared to an untreated subject infected with *S. suis*. Effective doses may also be extrapolated from dose-response curves derived from animal model test systems and can vary from 0.001 mg/kg to 100 mg/kg of the active ingredient(s) in the immunogenic composition or vaccine.

[0107] Toxicity and therapeutic efficacy of compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduces side effects.

[0108] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in animals, especially pigs, or humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in subjects. Levels in plasma can be measured, for example, by high performance liquid chromatography.

[0109] The immunogenicity of a composition can be determined by monitoring the immune response of test animals following immunization with the composition by use of any immunoassay known in the art. Generation of a humoral (antibody) response and/or cell-mediated immunity, may be taken as an indication of an immune response. Test animals may include pigs, mice, hamsters, dogs, cats, rabbits, cows, horses, sheep, etc., or human subjects.

[0110] The immune response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to the immunogenic conjugate, as assayed by known techniques, e.g., enzyme linked immunosorbent assay (ELISA), immunoblots, immunoprecipitations, etc.; or, by protection of immunized hosts from infection by the pathogen and/or attenuation of symptoms due to infection by the pathogen in immunized hosts as determined by any method known in the art, for assaying the levels of an infectious disease agent, e.g., the bacterial levels (for example, by culturing of a sample from the subject), etc. The levels of the infectious disease agent may also be determined by measuring the levels of the antigen against which the immunoglobulin was directed. A decrease in the levels of the infectious disease agent or an amelioration of the symptoms of the infectious disease indicates that the composition is effective.

[0111] The therapeutics of the invention can be tested *in vitro*, or *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in pigs or humans. For example, *in vitro* assays that can be used to determine whether administration of a specific therapeutic compound is indicated include *in vitro* cell culture assays in which appropriate cells from a cell line or cells cultured from a subject having a particular disease or disorder are exposed to, or otherwise administered a therapeutic, and the effect of the therapeutic on the cells is observed.

[0112] Alternatively, the therapeutic may be assayed by contacting the therapeutic composition to cells (either cultured from a subject or from a cultured cell line) that are susceptible to infection by the infectious disease agent but that are not infected with the infectious disease agent, exposing the cells to the infectious disease agent, and then determining whether the infection rate of cells contacted with the therapeutic composition was lower than the infection rate of cells not contacted with the therapeutic composition. Infection of cells with an infectious disease agent may be assayed by any method known in the art.

[0113] In addition, the therapeutic composition can be assessed by measuring the level of the molecule against which the antibody is directed in the animal model or human subject at suitable time intervals before, during, or after therapy. Any change or absence of change in the amount of the molecule can be identified and correlated with the effect of the treatment on the subject. The level of the molecule can be determined by any method known in the art.

[0114] After vaccination of a subject to a *S. suis* serotype(s) using the methods and compositions of the present invention, any binding assay known in the art can be used to assess

the binding between the resulting antibody and the particular molecule. These assays may also be performed to select antibodies that exhibit a higher affinity or specificity for a particular antigen.

I. Detection and Diagnostic Methods

5 [0115] Antibodies, or binding portions thereof, resulting from the use of *S. suis* pilus peptides of the present invention are useful for detecting in a sample the presence of *S. suis* bacteria. This detection method comprises the steps of providing an isolated antibody or binding portion thereof raised against an *S. suis* pilus peptide of the invention, adding to the isolated antibody or binding portion thereof a sample suspected of containing a quantity of *S. suis*, and
10 detecting the presence of a complex comprising the isolated antibody or binding portion thereof bound to *S. suis*.

[0116] The antibodies or binding portions thereof of the present invention are also useful for detecting in a sample the presence of a *S. suis* pilus peptide. This detection method comprises the steps of providing an isolated antibody or binding portion thereof raised against a
15 *S. suis* pilus peptide, adding to the isolated antibody or binding portion thereof a sample suspected of containing a quantity of the *S. suis* peptide, and detecting the presence of a complex comprising the isolated antibody or binding portion thereof bound to the *S. suis* peptide.

[0117] Immunoglobulins, particularly antibodies, (and functionally active fragments thereof) that bind a specific molecule that is a member of a binding pair may be used as
20 diagnostics and prognostics, as described herein. In various embodiments, the present invention provides the measurement of a member of the binding pair, and the uses of such measurements in clinical applications. The immunoglobulins in the present invention may be used, for example, in the detection of an antigen in a biological sample whereby subjects may be tested for aberrant levels of the molecule to which the immunoglobulin binds, and/or for the presence of
25 abnormal forms of such molecules. By "aberrant levels" is meant increases or decreases relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disease. The antibodies of this invention may also be included as a reagent in a kit for use in a diagnostic or prognostic technique.

[0118] In one aspect, an antibody of the invention that immunospecifically binds to a *S. suis* pilus peptide may be used to diagnose, prognose, or screen for a *S. suis* infection.
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[0119] In another aspect, the invention provides a method of diagnosing or screening for the presence of a *S. suis* infection or immunity thereto, comprising measuring in a subject the level of immunospecific binding of an antibody to a sample derived from the subject, in which the antibody immunospecifically binds a *S. suis* pilus peptide in which an increase in the level of the immunospecific binding, relative to the level of the immunospecific binding in an analogous sample from a subject not having the infectious disease agent, indicates the presence of *S. suis*.

[0120] Examples of suitable assays to detect the presence of *S. suis* peptides or antagonists thereof include but are not limited to ELISA, radioimmunoassay, gel-diffusion precipitation reaction assay, immunodiffusion assay, agglutination assay, fluorescent immunoassay, protein A immunoassay, or immunoelectrophoresis assay.

[0121] Immunoassays for the particular molecule will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cultured cells, in the presence of a detectably labeled antibody and detecting the bound antibody by any of a number of techniques well-known in the art.

[0122] The binding activity of a given antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

[0123] An additional aspect of the present invention relates to diagnostic kits for the detection or measurement of *S. suis*. Kits for diagnostic use are provided that comprise in one or more containers an anti-*S. suis* peptide antibody, and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-*S. suis* peptide antibody can be labeled (with a detectable marker, e.g., a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). Accordingly, the present invention provides a diagnostic kit comprising, an anti-*S. suis* peptide antibody and a control immunoglobulin. In a specific embodiment, one of the foregoing compounds of the container can be detectably labeled. A kit can optionally further comprise in a container a predetermined amount of an *S. suis* pilus peptide recognized by the antibody of the kit, for use as a standard or control.

J. Administration to a Subject

[0124] Routes of administration include but are not limited to intravenous, intranasal, oral, intradermal, and intramuscular. Other routes include subcutaneous, intracutaneous,

intravascular, intraarterial, intraperitoneal, intrathecal, intratracheal, intracutaneous, intracardial, intralobal, intramedullary, intrapulmonary, intravaginal, and via drinking water.

[0125] Depending on the desired duration and effectiveness of the treatment, the compositions according to the invention may be administered once or several times, also
5 intermittently, for instance on a daily basis for several days, weeks or months and in different dosages. Preferably, compositions of the invention are administered in one, two, or three doses, more preferably in two doses, and most preferably in a single dose.

EXAMPLES

[0126] The following examples are included to demonstrate preferred embodiments of the
10 invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain
15 a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: Identification of Pilus Genes of *S. suis*

[0127] Six putative pilus genes of *S. suis* were identified by comparative genetic analysis with other Streptococcal species. See FIG. 1 and SEQ ID Nos: 8-13. The putative pilus genes were cloned and expressed in *E. coli* as described herein.

A. Materials and Methods

[0128] Bacterial strains and growth conditions. The *E. coli* isolates Nova Blue and BL21(DE3) (Novagen) were used. *E. coli* strains were grown in Luria broth and plated on Luria broth containing 1.5% (w/v) agar. If required, media or plates contained 100 µg/ml of ampicillin (Boehringer, Mannheim, Germany).

25 [0129] Cloning. For cloning the *E. coli* expression vector pET-46 Ek/LIC (Novagen) was used. Primers used for cloning are listed below in Table 1. Primers were designed as recommended by the manufacturer (Novagen). The 5'ends of the primers incorporating the LIC sequences are underlined. Stop codons introduced into the reverse primers are shown in bold. For cloning, the regions encoding the signal peptide, as well as, the C-terminal hydrophobic
30 anchor regions were excluded. The selected gene fragments were amplified by polymerase chain

reaction (PCR), and amplified fragments were annealed into the vector after being treated with T4 DNA polymerase as recommended by the manufacturer (Novagen; user protocol TB163Rev. 10307). The generated plasmids were transformed into *E. coli* strain NovaBlue (Novagen). The insert size of the resulting clones was verified by PCR.

5 Table 1. Polynucleotide primers used for cloning.

Gene #	Primers used for cloning 5' to 3'	DNA source	Product size (bp)
3	<u>GACGACGACAAGATTGGAGCAGGCACTATTACTGTC</u> (SEQ ID NO: 14)	P1/7	1233
	<u>GAGGAGAAGCCCGGTTATGCTTGTGCTGTGTTATTCTC</u> (SEQ ID NO: 15)		
11	<u>GACGACGACAAGATCGAAACGAATCGGTCAGATGCA</u> (SEQ ID NO: 16)	891591	2670
	<u>GAGGAGAAGCCCGGTTACACAACGCGATATTGTTCACC</u> (SEQ ID NO: 17)		
12	<u>GACGACGACAAGATTGAGGGAAATCCTACAGTTACC</u> (SEQ ID NO: 18)	891591	1428
	<u>GAGGAGAAGCCCGGTTACACAACGCGATATTGTTCACC</u> (SEQ ID NO: 19)		
16	<u>GACGACGACAAGATTGGGATTGAAGTGCAAGAAACACC</u> (SEQ ID NO: 20)	P1/7	4738
	<u>GAGGAGAAGCCCGGTTAGCCCTGCTCATTGGCCACTTG</u> (SEQ ID NO: 21)		
25	<u>GACGACGACAAGATAGATGCCAGTCAAGTTTCGACG</u> (SEQ ID NO: 22)	P1/7	654
	<u>GAGGAGAAGCCCGGTTAAATTCCAGGAGGTGTAACAGG</u> (SEQ ID NO: 23)		
35-1	<u>GACGACGACAAGATTGCCGAAGATACGGCTAGCACA</u> (SEQ ID NO: 24)	P1/7	790
	<u>GAGGAGAAGCCCGGTTAACCATGAATCCACGTACAGTT</u> (SEQ ID NO: 25)		
35-2	<u>GACGACGACAAGATAGCAAGAGAAGGTGAAGTGAAC</u> (SEQ ID NO: 26)	P1/7	958
	<u>GAGGAGAAGCCCGGTTAGAAGTTATCTGCTGTCAATGC</u> (SEQ ID NO: 27)		

[0130] Expression. Plasmid DNA isolated from positive clones was used to transform *E. coli* strain BL21(DE3). For protein expression, cells (10 ml) were grown to the exponential growth phase. IPTG (1 mM) was added to the cells, and they were allowed to grow another 3-4 hours at 37°C. Subsequently, cells were harvested and suspended in water (super quality) to an OD₆₀₀ nm of 20.

[0131] Detection of expressed proteins. Proteins were denatured as described by the manufacturer (Invitrogen) and subsequently separated on a 4-12% Bis-Tris gel using MOPS buffer (NUPAGE, Invitrogen). Proteins in gels were visualized using a Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO) staining.

[0132] Sequencing. Sequencing was performed by BaseClear (Quick shot HT sequencing; long run). The sequence data were analysed using the program DNASTar (Lasergene Seqman).

[0133] Protein Purification. Proteins were affinity purified from solubilised cell pellets using Ni-nitrilotriacetic acid (Ni²⁺ NTA) column chromatography as described by the manufacturer (Qiagen). Briefly, cells were grown exponentially; 1 mM IPTG was added; and the cells were allowed to grow another 4 hr at 37°C. Subsequently, cells were harvested and lysed. The cleared supernatants were loaded onto Ni²⁺ NTA agarose columns. The columns were washed as described by the manufacturer (Qiagen). Subsequently, to remove LPS present in *E. coli* lysates more extensively, columns were washed with five column volumes of 10 mM Tris-HCl, pH 8.0 and 10 column volumes of 10 mM Tris-HCl pH 8.0 containing 60% of isopropanol. This procedure was repeated twice. Columns were washed one additional time with five column volumes of 10 mM Tris-HCl, pH 8.0, and proteins were eluted as described by the manufacturer (Qiagen). Purified proteins were further concentrated using Amicon Ultra-4 5000 MWCO filters (Millipore). Imidazole was removed by washing the filters with 20 volumes of PBS + 150 mM NaCl+5% glycerol three times.

[0134] Protein amount was determined after SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were visualized using SYPRO-orange (Molecular Probes, Sunnyvale, Calif.) staining according to the manufacturer's recommendations. Signals were detected on a multi-imager (Typhoon; Molecular Dynamics). A known bovine serum albumin (BSA) concentration range was used as a standard, to calculate the amounts of protein present in the gel. The Image Quant 5.2 program of Molecular Dynamics was used for the calculations.

B. Results

[0135] Identification of putative pilus genes by comparative genome analysis.

Bioinformatic tools were used to screen the genomes of *S. suis* strains P1/7 and 891591. Protein sequences genomically linked to putative sortase genes and containing the characteristic amino acid motif LPXTG (SEQ ID NO: 28) were identified. BLAST analyses were used to analyze the level of % identity of the identified proteins to proteins present in data libraries. Five putative sortase genes (srtA-srtE) were identified in the genome of P1/7 (see FIG. 1).

[0136] Results show that the srtA gene of P1/7 is preceded by two genes, identified herein as target genes 3 and 16, encoding proteins containing amino acid motif LPXTG (SEQ ID NO: 28). See FIG. 1A. Similarity was observed between the protein encoded by gene 3 and the Spb1 of *S. agalactiae* ($E = 1.9e-05$), whereas the protein encoded by gene 16 showed similarity ($E = 4e-09$) to IntA (Internalin) of *Listeria monocytogenes*. Target gene 3 is also found in 891591. The protein encoded by 891591 differs from the protein encoded by P1/7 at four amino acid positions. In strain 891591 the 5'-end of the IntA encoding gene was flanked by a gene encoding a IS4 transposase. The 3'-end of the IntA encoding gene, as well as, the gene encoding the Spb1 seemed to be located at a different position on the chromosome, but were also flanked to genes encoding IS4 transposases.

[0137] As shown in FIG. 1B, the srtB, srtC, and srtD genes of P1/7 are linked on the chromosome and are preceded by two genes, herein target genes 25 and 35, that contain an amino acid domain LPXTG (SEQ ID NO: 28). The protein encoded by gene 25 showed similarity to cell wall surface anchor family proteins of *S. pyogenes* ($E = 4e-32$), whereas the protein encoded by gene 35 showed similarity to cell wall surface anchor family proteins of *S. pneumoniae* ($E = 1e-49$). Until now genes homologous to the srtB, C, and D genes of P1/7 were not identified in 891591. For comparison FIG. 1B also shows the srtB, C, and D genes of *S. pneumoniae*, as well as, the genes linked to these sortase genes. The rggA, B and C genes of *S. pneumoniae* have been described as being involved in pilus formation; the rrgB gene being the gene encoding the main pilus subunit (Barocchi et al., 2006).

[0138] The genes flanking the srtE gene of P1/7 are shown in FIG. 1C. None of these genes contain the amino acid motif LPXTG (SEQ ID NO: 28). In 891581, identical genes were observed flanking the srtE gene. A srt gene present in 891591 is shown in FIG. 1D. This srt gene is flanked by two genes containing LPXTG domains (SEQ ID NO: 28). See FIG. 1D. Genes homologous to this sortase gene, as well as, the flanking genes are not present in P1/7.

However, the genes showed a high level of identity to genes present in *Streptococcus pyogenes* (GAS), *S. agalactiae* (GBS) and *S. pneumoniae* and were shown to be involved in pilus formation (Telford et al., 2006). The T6, san1518 and rrgB genes were shown to encode the main pilus subunits (Telford et al., 2006; Barocchi et al., 2006).

5 [0139] In conclusion, by comparative genome analysis four putative pilus genes (genes 3, 16, 25 and 35—SEQ ID NOs: 8, 11, 12 and 13, respectively) were identified in P1/7 and two putative pilus genes (genes 11 and 12—SEQ ID NOs: 9 and 10, respectively) were identified in 891591. Target gene 3 was found in P1/7 and 891591. In 891591, the 5'-end and the 3'-end of target gene 16 are located at different positions of the chromosome.

10 [0140] Cloning of putative *S. suis* pilus genes. Genes 3, 16, 25, and 35 were amplified using chromosomal DNA of strain P1/7. Genes 11 and 12 were amplified using chromosomal DNA of strain 891591. In P1/7, genes 16 and 35 were shown to be nonfunctional. Gene 16 contains a nonsense mutation after codon 49. Therefore, a 4738 bp fragment (about a 190 kDa product) located 3' of the mutation was amplified. Gene 35 (about 1954 bp) contains a
15 frameshift mutation after codon 261 (i.e. after 860 bp), so gene 35 was cloned into two fragments. A 790 bp fragment (designated as 35-1 or 35.1) located 5' of the frameshift, and a 958 bp fragment (designated as 35-2 or 35.2) located 3' of the mutation were amplified. The expected products of the respective fragments are about 32 kDa and 38 kDa. Amplified PCR products were cloned into pET-46 Ek/LIC. The insert size of the resulting clones was verified
20 by PCR. Actual sizes matched well with the calculated sizes.

[0141] Sequencing. Sequence analysis of the inserts cloned into pET-46 Ek/LIC revealed that the DNA sequences of all target genes, except for target gene 11, were identical to the sequences reported for P1/7 and 891591 (see SEQ ID NOs: 1, and 3-7, respectively). Sequence of target gene 11 cloned into pET-46 Ek/LIC showed a (T→G) mutation at position 1185 of the
25 nucleotide sequence (see SEQ ID NO: 9), which yielded a mutation of aspartic acid to glutamic acid (D→E) (see SEQ ID NO: 2).

[0142] Expression of recombinant proteins. The expression of the recombinant proteins was analysed after induction of the recombinant proteins by IPTG (FIG. 3). Non-induced *E. coli* cells did not express a fusion protein (lane 1, “—” of FIG. 2). In contrast, induced *E. coli* cells
30 expressed the various fusion polypeptides (lanes 2-8 of FIG. 2). The level of induction differed between the various fusion polypeptides. High levels of expression were observed for the fusion

polypeptides 11, 12, 16, 25, 35.1 (or 35-1 herein) and 35.2 (or 35-2 herein); whereas protein 3 seemed to be expressed at a lower level. In general, the size of the expressed fusion proteins matched well with the theoretical molecular sizes. However, the size of fusion protein 16 differed significantly from the calculated molecular size (calculated size = 190 kD; expressed size = 40kD, respectively).

[0143] Protein purification. All pET-46 clones seemed to over express the proteins in a soluble form, and proteins were purified from these clones. One liter (1 L) cultures were used for purification. Protein expression was induced with IPTG, and cells were lysed. All proteins were purified using native purification conditions and a Ni-NTA column. After purification, all collected fractions were analyzed on SDS-PAGE. As a representative, FIG. 3 shows the data as obtained for fragment 35.2. Flow-through fractions containing the purified protein were collected and concentrated in eight fractions. Fractions were analyzed using NU-PAGE. The fractions were pooled, and their protein content was determined. As shown in FIG. 4, considerable amounts of all of the putative pilus proteins were purified. Except for protein 16, all proteins were expressed at their expected molecular sizes. The expected size for protein 16 is 190 kDa; however, the expressed protein was only about 40 kDa. Depending on the target protein, the amount of purified protein obtained varied between 1.9 and 16 mg (see Table 2). The purified proteins can be used for the generation of antibodies.

Table 2. Amount of proteins purified using native purification conditions.

Pili (gene)	Total amount of protein (mg)
3	1.9
11	3.9
12	3.3
16	4.2
25	16.0
35.1	1.9
35.2	11.1

[0144] Summary. By comparative genome analysis four (4) putative pilus genes (genes 3, 16, 25, and 35, cloned in two fragments 35-1 and 35-2) were identified in P1/7 and two putative pilus genes (genes 11 and 12) were identified in 891591. Target gene 3 was found in P1/7, as well as, in 891591. In 891591, the 5'-end and the 3'-end of target gene 16 are located at different positions of the chromosome. All target genes were successfully cloned using pET-46 Ek/LIC,

and fusion proteins were expressed in *E. coli*. The level of expression observed in *E. coli* differed between the various fusion proteins. High levels of expression were observed for the fusion proteins 11, 12, 16, 25, and fragments 35.1, and 35.2; whereas; fusion protein 3 seemed to be expressed at a lower level. Except for the fusion protein 16, the size of the expressed fusion proteins matched well with the theoretical sizes. Proteins were successfully purified from *E. coli* (pET-46) clones. Depending on the target protein, the amount of purified protein obtained varied between 1.9 and 16 mg.

EXAMPLE 2: Assessment of Pilus Formation in *S. suis* Serotype 2 Isolates

[0145] Electron microscopy was used to examine the surface of various *S. suis* serotype 2 isolates for the presence of pilus structures. In addition, immuno-blot detection of cell wall extracts from individual serotype 2 isolates with antiserum specific for the pilus proteins 3 and 11 revealed a high-molecular weight ladder pattern similar to those observed for pili identified in various Streptococcal spp.

A. Materials and Methods

[0146] Bacterial isolates and growth conditions. The *S. suis* isolates used in this study are shown in Table 3. *S. suis* cells were grown in Todd-Hewitt broth (TH) (Code CM189, Oxoid) and incubated overnight under aerobiosis at 37°C. Overnight cultures were diluted 10-fold in TH broth and grown to an optical density of 0.5 at 600 nm.

Table 3. *S. suis* strains used.

Strain	Serotype	Presence of ^a		Clinical source ^b	Virulence ^c	Reference
		MRP ^f	EF ^g			
6388	1	s	+	organs	HV	Laboratory collection
6112	1	s	+	organs	HV	Laboratory collection
4501	1	s	+	organs	HV	Laboratory collection
(6290)						
4005 (3)	2	+	+	organs	HV	Laboratory collection
3881 (10)	2	+	+	tonsil	HV	Laboratory collection
P1/7	2	+	+	--	V	J. Arends, Amsterdam, the Netherlands
10M7E27	2	-	-	tonsil	HV	MRP, EF negative mutant obtained from 3881
89/1591	2	-	-	organs	WV/AV ^d	M. Gottschalk, Quebec, Canada
6254	2	unknown		--	ND	BIV, USA

Strain	Serotype	Presence of ^a		Clinical source ^b	Virulence ^c	Reference
		MRP ^f	EF ^g			
040910-1	2	unknown		heart	ND	BIV, Japan
040910-2	2	unknown		heart	ND	BIV, Japan
235/02	2	unknown		--	ND	M. Rodriguez Ortega, Spain
17/03	2	unknown		--	ND	M. Rodriguez Ortega, Spain
7711	7	unknown		CNS	ND	Laboratory collection
7917	7	unknown		CNS	ND	Laboratory collection
8039	7	unknown		CNS	ND	Laboratory collection
7997	9	*	-	organs	V ^e	Laboratory collection
8067	9	-	-	CNS	V	Laboratory collection
8017	9	-	-	CNS	V	Laboratory collection

^a - = absent ; + = present; s = protein with a lower molecular mass is present; * = variant of proteins with a higher molecular mass is present

^b typical *S. suis* organs: liver, kidney, spleen. CNS = central nervous system

5 ^c HV = highly virulent; V = virulent; WV = weakly virulent; AV = avirulent; ND = virulence not determined (see Stockhofe-Zurwieden et al., 1996; Vecht et al., 1992, Vecht et al., 1991)

^d depending on the animal model used (see Berthelot-Herault et al., 2005. Can J. Vet. Res. 69:236-240)

10 ^e compared to serotype 2 strains a higher dose of infection is required to obtain clinical signs of disease

^f MRP = muramidase-released protein

^g EF =extracellular factor

[0147] Generation of rabbit-anti sera. Rabbit antisera against the purified putative pilus proteins were generated at BioGenes GmbH (Berlin, Germany) using a 28 day schedule and following a typical protocol. The generation and collection of rabbit antisera is well-known in the art, and any of several standard protocols may be used. Pre-immune serum (20 ml) and antiserum (60 ml) were collected.

[0148] Preparation of cell wall extracts from *S. suis* cells. *Streptococcus suis* cultures were grown in TH broth to OD600 nm of 0.5. Cells (10 ml) were collected by centrifugation and resuspended in 1 ml of 30 mM Tris-HCl pH 8.0; 3 mM MgCl₂ and 25% of sucrose. Lysozyme (Sigma, M9901) and/or mutanolysin (Sigma, L6876) were added to a concentration of 1.0 mg/ml and 125 units/ml, respectively. After incubation for 3 hours (h) at 37°C protoplasts were collected by centrifugation. Supernatants containing the cell wall proteins were collected and frozen at -20°C until use.

[0149] Electron microscopical examination. *Streptococcus suis* cells were grown in TH broth to an OD_{600 nm} of 0.5. One milliliter (1 ml) of the suspension was centrifuged, and cells were suspended in 500 µl of sterile 0.22 µm filtered PBS. Twenty microliters (20 µl) of sample were added to Formvar-coated nickel grids and incubated for 5 minutes at room temperature (RT). The grids were washed once with 20 µl of PBS and subsequently fixed with 1% of paraformaldehyde (EM grade)/PBS for 30 minutes at RT. The grids were washed 3 times for 5 minutes at RT with PBS and incubated for 30 minutes at RT with blocking solution containing normal goat serum (AURION, Wageningen, The Netherlands). The grids were washed 3 times for 5 minutes at RT with AURION BSA-c incubation buffer (AURION, Wageningen, The Netherlands) and subsequently incubated for 60 minutes at RT with polyclonal rabbit antibodies directed against the putative pilus proteins diluted 1:5 in incubation buffer (AURION). The grids were washed 6 times for 5 minutes with incubation buffer and incubated for 60 minutes at RT with goat-anti rabbit gold-conjugated (10 nm) antibodies in a 1:20 dilution in incubation buffer. Samples were washed 6 times for 5 minutes in incubation buffer and 3 times for 5 minutes in PBS and subsequently fixed in 1% paraformaldehyde/PBS. The samples were washed 5 times for 5 minutes with distilled 0.22 µm filtered water and air dried. The grids were stained for 15 seconds with 1% uranyl acetate (in 0.22 µm filtered water) and washed 5 times for 5 minutes with distilled 0.22 µm filtered water and air dried. The grids were then analysed using a Philips CM10 electron microscope.

[0150] Detection of expressed proteins. Proteins were denatured as described by the manufacturer (Invitrogen) and subsequently separated on a 4-12% Bis-Tris gel using MOPS buffer (NuPAGE, Invitrogen). The proteins in gels were visualized using the Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO) stain.

[0151] Western blot analysis. Proteins separated by NuPAGE were transferred to nitrocellulose membranes as described by the manufacturer (Invitrogen). The membranes were blocked in TBS containing 0.05% Tween 20 and 4% skim milk (Difco) at room temperature for 1 hour. The membranes were subsequently incubated with a rabbit polyclonal antibody generated against the purified pilus proteins (BioGenes) in a 1: 5000 dilution in TBS containing 0.05% Tween 20 and 4% skim milk (Difco) for 1 hour at room temperature. This incubation was followed by an incubation with alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) in a 1: 1000 dilution in TBS containing 0.05% Tween 20 and 4 skim

milk (Difco) for 1 hour at room temperature. Reactivity with the expressed proteins was visualized using nitro blue tetrazolium (Merck, Darmstadt, Germany)-bromochloroindolyl phosphate (Sigma, St. Louis, MO) as a substrate.

B. Results

5 [0152] Generation of rabbit anti-pilus sera. Sera were generated in rabbits (two rabbits/antigen) against the purified pilus proteins 3, 11, 12, 16, 25, 35-1 and 35-2 of Example 1 above at BioGenes (Berlin) using a high speed protocol. From each rabbit 20 ml of pre-immune serum were obtained as well as 60 ml of specific antiserum. The purified proteins and the reactivity of the sera generated against the purified proteins on an immunoblot are shown in 10 FIGs. 5A and 5B respectively. Although the blot in FIG. 5B is heavily overloaded, the results show reactivity of the sera at the expected molecular sizes of the purified proteins. In addition, reactivity to proteins at other molecular sizes is observed. This additional reactivity may be caused by contaminations present in the purified protein samples.

[0153] Detection of pilus-like structures by western blot analysis. In other Streptococcal 15 species pilus-like structures show a high-molecular-mass ladder pattern on immunoblots when detecting cell wall extracts with sera specifically directed against the pilus proteins (Barocchi et. al. 2006). To confirm the role of the putative pilus proteins in pilus formation in *S. suis*, cell wall extracts of various *S. suis* isolates were screened with the antisera directed against the proteins 3, 11, 12, 16, 25, 35-1, and 35-2.

20 [0154] The results (FIGs. 6A and 6B) show a ladder of high-molecular-mass bands in cell wall extracts of the *S. suis* serotype 2 isolates 040920-01, 040910-2, 235/02, and 17/03 using antisera directed against pilus proteins 3 (rabbits 3806, 3807) and 11 (rabbit 3808), demonstrating expression of pili based on proteins 3 and 11 in these *S. suis* isolates. High-molecular-mass bands were not observed in the cell wall extracts of the same isolates when 25 serum obtained from rabbit 3809 (directed against protein 11) was used. The reason for the differences in reactivity between sera obtained from rabbits 3808 and 3809 (both immunized with protein 11) is unknown. High-molecular-mass banding patterns were neither observed in cell wall extracts of the serotype 2 isolates 4005, 3881, P1/7, and 89/1591 nor in cell wall extracts of the serotype 1, 7, and 9 isolates (FIGs. 6A and 6B) using serum specifically directed 30 against proteins 3 and 11.

[0155] Antiserum directed against protein 12 obtained from rabbit 3811 (FIG. 6C) showed a ladder of high-molecular-mass bands on cell wall extracts of the serotype 9 isolates 7997, 8067, and 8017. High molecular-mass bands also were observed in cell wall extracts of isolates 4501, 040920-01, 040910-2, 235/02, 17/03, 7711, 7917, and 8039. However, similar reaction patterns were obtained for the cell wall extract of these isolates when pre-immune sera from rabbit 3811 was used, suggesting that the observed reactivity is the result of a non-specific reaction.

[0156] Antiserum directed against protein 12 obtained from rabbit 3810 (FIG. 6C) did not show a ladder of high-molecular-mass bands for any of the isolates. Again, the reason for the differences in reactivity between sera obtained from two rabbits (3810 and 3811) immunized with protein 12 is unknown.

[0157] Antiserum directed against protein 16 showed bands around 97 kDa for cell wall extracts of isolates 89/1591, and 6254 (FIG. 6D). These bands were observed using sera obtained from both rabbits. As already discussed, in isolate 89/1591 the 5'-end and the 3'-end of gene 16 were found at two different locations on the chromosome. The bands observed in 89/1591 may represent expression of the N-terminal part of protein 16. Based on the immunoblot data obtained, the genetic organisation of gene 16 in isolate 6254 may be similar to the organisation found in 89/1591. In this respect, it is remarkable to note that isolates 89/1591 and 6254 were both originally isolated in North-America. As gene 16 is non-functional in P1/7, expression of gene 16 in P1/7 was not expected.

[0158] Antiserum directed against protein 25 did not show ladders of high-molecular-mass bands in any of the isolates used (FIG. 6E).

[0159] Gene 35 is a pseudogene in P1/7. Therefore, gene 35 was cloned into two fragments. The 5'-end and 3'-ends of the cloned gene were designated as 35-1 and 35-2, respectively. No clear reactivity was observed with the antisera directed against protein 35-2 and the cell wall extracts of any of the isolates used (FIG. 6F). The serum obtained from rabbit 3818 showed some reactivity to the serotype 7 isolates used. However, similar reactivity to the serotype 7 isolates was observed when pre-immune serum of rabbit 3818 was used, indicating that the observed reactivity may be the result of a non-specific reaction.

[0160] Antiserum obtained from rabbit 3817 (directed against protein 35-1) and pre-immune serum from rabbit 3817 showed similar patterns of reactivity on cell wall extracts of

serotype 7 isolates (FIG. 6G), indicating that the observed reactivity is the result of a non-specific reaction. In contrast, serum obtained from rabbit 3816 showed reactivity to high-molecular-mass bands in a considerable number of isolates used. However, the bands obtained seemed similar to bands obtained on blots using serum specifically directed against MRP. Therefore, the reactivity of serum 3816 was tested against purified MRP. In addition, a cell wall extract obtained from the isogenic MRP-EF mutant of strain 3881 (10M7E26) was tested for reactivity to serum 3816. The results show that serum of rabbit 3816 showed reactivity to purified MRP (FIG. 6H) and was negative on the isogenic MRP-EF mutant (FIG. 6G), indicating that serum of rabbit 3816 cross-reacted to MRP. Polyclonal antibodies (data not shown) and monoclonal antibodies directed against MRP did not bind to the purified protein 35-1 (FIG. 6H), indicating that the observed cross-reactivity seemed to be one-directional and that MRP and 35.1 did not share common antigenic sites.

[0161] Electron Micrograph (EM) analysis. To study the expression of pili in *S. suis* serotype 2 isolates EM examinations were performed. Representative pictures are shown in FIG. 7. For comparison, pictures obtained from the paper of Barocchi and co-workers are included (FIG. 7A). In accord with the data presented for *S. pneumoniae* (FIG. 7A), pili were clearly expressed on the surface of the *S. suis* isolate 040910-1 (FIG. 7B), as well as, on the surfaces of isolates P1/7 (FIG. 7C), 17/3 (FIG. 7D), 89/1591 (FIG. 7E), and 235/02 (not shown). In isolates P1/7, 89/1591, and 040910-1 the pili observed seemed to be long polymerized structures; whereas, the pili observed in isolate 17/3 seemed to have a more branched structure. No clear binding was observed between the expressed pili and the various immuno-gold labelled sera used. Although some of the gold particles seem to bind to the pilus structures (arrows in FIGs. 7C and 7E), most of the particles were found randomly distributed among the grid.

B. Results and conclusions

[0162] Electron microscopical (EM) examination revealed pilus structures on the surface of various *S. suis* serotype 2 isolates. Electron microscopy studies were performed on *S. suis* cells growing exponentially in TH broth indicating that pili are expressed under in vitro conditions and can be observed in logarithmically growing cells. These data are in accord with data observed for the expression of pili in other Streptococcal spp. (Barocchi et al., 2006). Western blot analysis of cell-wall extracts of various *S. suis* serotype 2 isolates clearly showed a clear ladder of high-molecular-mass bands in cell wall extracts of the *S. suis* isolates 040920-01,

040910-2, 235/02, and 17/03 using antisera directed against pilus proteins 3 and 11. The patterns observed were similar to those observed for pili identified in various Streptococcal spp., which strongly suggests that the expression of pili is based on proteins 3 and 11 in these *S. suis* isolates. Sera specific for the proteins 12, 16, 25, and 35 did not result in a high-molecular mass ladder pattern on any of the cell wall extracts used. Moreover, in immunoblot analyses of cell wall extracts from various *S. suis* isolates differences were observed between the antisera generated in two animals against the proteins 12, 16, 25, or 35. It was observed that one of the sera generated against protein 35-2 showed cross-reactivity to MRP. No specific binding was observed by EM examination between the pilus structures and the immuno-gold labelled sera specifically directed against the putative pilus proteins.

EXAMPLE 3: Generation of Mutant *S. suis* Isolates Having
Impaired Expression of Putative Pilus Genes

A. Materials and Methods

[0163] Bacterial strains and growth conditions. Wild type *S. suis* serotype 2 strains P1/7 and 89/1591 as well as *Escherichia coli* isolate Nova Blue (Novagen) were used in this study. The *S. suis* strains were grown on Colombia agar plates (Oxoid Ltd, London, United Kingdom) containing 6% horse blood at 5% CO₂, and 37°C. Liquid cultures were grown in Todd-Hewitt broth (THB; Oxoid Ltd, London, United Kingdom) for 18 h at 37°C without agitation. The *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth. When necessary, antibiotics were added to culture media at the following concentrations: for *E. coli*, ampicillin 100 µg/ml, chloramphenicol (Cm) 8 µg/ml and spectinomycin (Spc) 100 µg/ml; for *S. suis*, Cm 5 µg/ml and Spc 100 µg/ml.

[0164] Generation of knockout mutants. The generation of the mutant strains is schematically shown in FIGs. 8A-8C and 9A-9C. Primers used in this study are listed in Table 4. Briefly, to construct the mutant strains, the putative pilus genes as well as the flanking regions were cloned into an *E. coli* vector. The gene of interest(s) was inactivated by introducing a deletion as well as by inserting a spectinomycin resistance gene. See FIG. 9A. The entire cloned fragment was then cloned into a pSET5 vector. This vector contained the ColEI replication origin of pUC19 for efficient replication in *E. coli* as well as the temperature sensitive replication origin of pWVO1 for temperature sensitive replication in *S. suis* (Takatamusu et al). The plasmid constructs were introduced into *S. suis* by electroporation, and transformants were

selected at the permissive temperature. Subsequently, transformants were selected by growth at the restrictive temperature. See FIG. 9B. Insertion events were selected by growth on media containing Spc.. Double and single cross-over events were discriminated by screening colonies for the absence of Cm resistance. The CM resistance gene was associated with the plasmid. See FIG. 9C. Colonies were verified to have the expected genotype by polymerase chain reaction (PCR).

Table 4. Primers used to generate mutant knockout mutants

Primers for gene 3		
No.	Description	Sequence
1	3A Fw-NcoI	5' CAT GCC ATG GCA TGT CGG GTC TGT CTG ATT TA 3' SEQ ID NO: 29
2	3 A Rev-ApaI	5' TCC GGG CCC GGA CGT TAC TTA GAA AGCT 3' SEQ ID NO: 30
3	3B Fw-XmaI	5' CCC CCC GGG GGG TAC AGA AGT TAC AAG TGC 3' SEQ ID NO: 31
4	3B Rev-XhoI	5' CCG CTC GAG CCG AGT AAT CAC TGA CCG AT 3' SEQ ID NO: 32
5	3 complete Fw	5' CTG CTG ATA AAG TGA TTA CGA GTC C 3' SEQ ID NO: 33
6	3 complete Rev	5' GGC CAA TTC AAC CTC TGT CCA TAC TG 3' SEQ ID NO: 34
7	3 out Fw	5' CAA TTC TTT ACA TCC CTT GTG GCA G 3' SEQ ID NO: 35
8	3 out Rev	5' CCC AAG CCG CAA ATG CTT C 3' SEQ ID NO: 36
9	spec Fw-ApaI	5' TGC GGG CCC GCA GGT CGA TTT TCG TTC GT 3' SEQ ID NO: 37
10	spec Rev-XmaI	5' CCC CCC GGG GGG ATG CAA CGG TTT ATT GTT TTC TAA 3' SEQ ID NO: 38
Primers for gene 12		
No.	Description	Sequence
11	12A Fw-SalI	5' GTC GAC AGG CCC ACA TCA CGC AGT CTA AAC 3' SEQ ID NO: 39
12	12B Rev-NotI	5' GCG GCC GC AGC CCT GCA TCT TCG CTA CTT TC 3' SEQ ID NO: 40
13	12B Fw-XmaI	5' CCC CCC GGG GGG TGA GTT CTT CTA TGC GCG TAA CCC T 3' SEQ ID NO: 41
14	12A Rev-ApaI	5' CTG GGG CCC CAG TAG TGT CAG CAA TCA ATG TTT G 3' SEQ ID NO: 42
15	12 complete Fw	5' GGG CTT CGC ACG AGG ATG TTA 3' SEQ ID NO: 43

16	12 complete Rev	5' CAC GGT TAA TGG CCA GGG TCT T 3' SEQ ID NO: 44
17	12 out Fw	5' GTC AAT TTA AAA CTC AAG G 3' SEQ ID NO: 45
18	12 out Rev	5' GCC TTG TTG GCA TTT CCT G 3' SEQ ID NO: 46
19	spec Fw-ApaI	5' TGC GGG CCC GCA GGT CGA TTT TCG TTC GT 3' SEQ ID NO: 47
20	spec Rev-XmaI	5' CCC CCC GGG GGG ATG CAA CGG TTT ATT GTT TTC TAA 3' SEQ ID NO: 48
Primers for gene 35		
No.	Description	Sequence
21	35A Fw-SpeI	5' ACT AGT TGC GAA TAA TAA GCA AAT CTC AAA A 3' SEQ ID NO: 49
22	35B Rev-SacII	5' CCG CGG GAA GCC GAA GAC AGA ACT GGA GT 3' SEQ ID NO: 50
23	35B Fw-XmaI	5' CCC CCC GGG GGG GTA GGT GAA ATT AAA CTT GCA GCA G 3' SEQ ID NO: 51
24	35A Rev-ApaI	5' CAT GGG CCC ATG ATT CCA CGT ACA GTT TGT GTC A 3' SEQ ID NO: 52
25	35 complete Fw	5' AAT CGG TAA CAT CGA AAA TCC AGA G 3' SEQ ID NO: 53
26	35 complete Rev	5' ATG CCC TAC ACC TTT TTG AAG AAC G 3' SEQ ID NO: 54
27	35 out Fw	5' GGT GAA ATT GAA TAA TAA CGG 3' SEQ ID NO: 55
28	35 out Rev	5' CGT ACA CCA GTT CCT TTT TC 3' SEQ ID NO: 56
29	spec Fw-ApaI	5' TGC GGG CCC GCA GGT CGA TTT TCG TTC GT 3' SEQ ID NO: 57
30	spec Rev-XmaI	5' CCC CCC GGG GGG ATG CAA CGG TTT ATT GTT TTC TAA 3' SEQ ID NO: 58

[0165] Inactivation of gene 3 in P1/7. The Spc resistance cassette was amplified from pGA14-spc (Smith et al., 1995) using primers 9 and 10 and ligated to the blunt cloning vector pJET1.2 (Fermentas, St. LeonRot, Germany). The plasmid obtained was designated as pJET-spc and transformed into *E. coli*.

[0166] Primers 3 and 4 were used to amplify a 1.5 kb region containing the 3'-end of gene 3 using chromosomal DNA of strain P1/7. The amplified fragment was digested with the restriction enzymes XmaI and XhoI and then ligated to pJET-spc that had previously been digested with XmaI and XhoI. The resulting plasmid was designated as pJET-spc-3B and

transformed to *E. coli*. Primers 1 and 2 were used to amplify a 1.5 kb region containing the 5'-end of gene 3 using chromosomal DNA of strain P1/7. The amplified fragment was digested with the restriction enzymes NcoI and ApaI and then ligated to pJET-spc-3B digested with NcoI and ApaI. The plasmid obtained (pJET-3A-spc-3B) was subsequently digested with NcoI and XhoI, the resulting fragment (3A-spc-3B) was purified, made blunt using the blunting enzyme (Fermentas) and ligated to SmaI digested pSET5 (Takamatsu et al., 2001). The resulting plasmid was introduced into *E. coli*, and the transformants were selected on LB agar containing Cm and Spc. Purified plasmids were then transformed into *S. suis* strain P1/7 by electroporation (Smith et al, 1995). The transformants were selected on agar plates containing 100 µg/ml of Spc after growth at 30°C, the permissive temperature for replication of pSET5.

[0167] Several Spc and Cm resistant colonies were grown individually overnight in TH medium containing Spc at 30°C. The overnight cultures were then diluted 1:100 in TH medium without antibiotics and incubated for 4 h at 38°C, the non-permissive temperature of pSET5 replication. Cultures were serially diluted on Columbia agar plates containing Spc at 38°C to select for chromosomal integration. Double crossover events were selected for by screening individual colonies for sensitivity to chloramphenicol. The genotype of Cm sensitive and Spc resistant colonies was confirmed by PCR using primer pairs 5/ 6 and 7/8 as well as by sequence analysis.

[0168] Inactivation of gene 12 in 89/1591. Primers 11 and 12 were used to amplify a fragment containing gene 12 flanked on both sides by 1.5 kb regions using chromosomal DNA of strain 89/1591. The amplified fragment was ligated to the blunt cloning vector pJET1.2 and the ligation mixtures were transformed into *E. coli*. Plasmid DNA (designated pJET-12) obtained from transformants was then used to replace an internal fragment (173 bp) of gene 12 with the Spc resistance cassette.

[0169] To do this, an inverse PCR strategy was used on pJET-12 with primers 13 and 14. In addition, the Spc cassette was amplified from pGA14-spc using primers 19 and 20. The amplified fragment were then digested with ApaI and XmaI, ligated and transformed into *E. coli*. Purified plasmids (designated pJET-12-spc) were transformed into *S. suis* strain 89/1591 by electroporation (Smith et al., 1995). Integration events were selected for on agar plates containing 100 µg/ml of Spc after growth at 38°C. Double and single crossover events were

discriminated by PCR analysis using primer pairs 15/16 and 17/18. The genotype of the mutant strain was confirmed by sequence analysis.

[0170] Inactivation of gene 35 in P1/7. Primers 21 and 22 were used to amplify a fragment containing gene 35 flanked on both sides by 1.5 kb regions using chromosomal DNA of strain P1/7. The amplified fragment was ligated to the blunt cloning vector pJET1.2 and ligation mixtures were transformed into *E. coli*. Plasmid DNA (designated pJET-35) obtained from transformants was subsequently used to replace an internal fragment (287 bp) of gene 35 with the Spc resistance cassette.

[0171] To do this, an inverse PCR strategy was used on pJET-35 with primers 23 and 24. In addition, the Spc cassette was amplified from pGA14-spc using primers 29 and 30. The amplified fragments were digested with *Apal* and *Xmal*, ligated and transformed into *E. coli*. Subsequently, the entire insert fragment was cloned into vector pSET5. To do this, purified plasmid DNA was digested with *SpeI* and *SacII*, the insert fragment was purified, made blunt using the blunting enzyme (Fermentas) and ligated to *SmaI* digested pSET5. Ligation mixtures were introduced into *E. coli*. Purified plasmids were then transformed into *S. suis* strain P1/7 by electroporation. The transformants were selected on agar plates containing 100 µg/ml of Spc after growth at 30°C, the permissive temperature for replication of pSET. Several individual Spc and Cm resistant colonies were grown overnight in TH medium containing Spc at 30°C. The overnight cultures were then diluted 1:100 in TH medium without antibiotics and incubated for 3 h at 38°C, the non-permissive temperature of pSET replication. Cultures were serially diluted on Columbia agar plates containing Spc at 38°C to select for chromosomal integration. Double crossover events were selected for by screening individual colonies for sensitivity to Cm. Cm sensitive and Spc resistant colonies were confirmed to have the expected genotype by PCR using primer pairs 25/26 and 27/28 as well as by sequence analysis.

[0172] Sequencing. Sequence reactions were performed using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and analysed on the 3130 Genetic Analyser (Applied Biosystems). The sequence data obtained were analysed using the program DNASTar (Lasergene, Seqman).

B. Results

[0173] Genes 3, 12 and 35 were chosen as targets for inactivation as they code for the putative main pilus subunits. Agarose electrophoresis shows (FIG. 10A-10C) that the amplified

fragments comprising the inactivated genes are bigger than wild type isolate fragments. This size difference is due to the insertion of the Spc cassette. Moreover, no amplification is observed with the mutant isolates when using primers specific for the deleted region. Sequence analysis confirmed the genotype of the mutant isolates, and that mutant *S. suis* isolates impaired in the expression of putative pilus genes 3, 12 and 35 were generated.

EXAMPLE 4: Identification Of Antigens Providing Protection In Young Piglets
Against *S. suis* Infections Of Various Serotypes

[0174] Previous spot hybridization studies indicated that the genes encoding the cell wall anchored proteins 1, 4, 5, 8, 9, 10, 15, 20, 23 and 29 were conserved among all serotype 1, 2, 7 and 9 isolates tested. Here, the reactivity of these proteins expressed in *E. coli* with convalescent pig sera directed against various serotype 1, 2, 7 and 9 isolates is examined. In addition, the reactivity of the purified putative pilus proteins with the convalescent pig sera is described.

A. Materials and Methods.

[0175] Bacterial isolates and protein expression. The *E. coli* isolates BL21(DE3) (Novagen) expressing the cloned proteins 1, 5, 8, 9, 10, 15, 20, 23, and 29 were used in this study. *E. coli* strains were grown in Luria broth containing 100 ug/ml of ampicillin (Boehringer, Mannheim, Germany).

[0176] For protein expression, cells (10 ml) were grown to the exponential growth phase. IPTG (1 mM) was added and the cells were allowed to grow another 3-4 h at 37°C. Subsequently, cells were harvested by centrifugation and re-suspended in 2 ml water (super quality).

[0177] Detection of expressed proteins. Proteins were denatured and subsequently separated on a 4-12% Bis-Tris gel using MOPS buffer as recommended by the manufacturer (NuPAGE, Invitrogen). Proteins in gels were visualized using a Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO) staining.

[0178] Purification and quantification of proteins. Proteins were affinity purified from solubilized cell pellets using Ni-nitrilotriacetic acid (Ni²⁺-NTA) column chromatography as recommended by the manufacturer (Qiagen). Specifically, the IPTG induced cells were lysed following harvest. The cleared supernatants were then loaded onto Ni²⁺-NTA agarose columns. The columns were washed as suggested by the manufacturer (Qiagen) before eluting the His-tagged proteins. Protein concentrations were determined by using the BCA protein assay kit as

idirected by the manufacturer (Pierce). To calculate the protein concentration a known bovine serum albumin concentration range was used as a standard.

[0179] Western blot analysis. Proteins separated by NuPAGE were transferred to nitrocellulose membranes as directed by the manufacturer (Invitrogen). The membranes were
5 blocked in TBS containing 0.05% of Tween20 (TBST) and 5% of Skimmilk (Difco) at room temperature for 1 hour (h). The membranes were subsequently incubated with the convalescent sera directed against the various serotype 1, 2, 7, and 9 isolates in a 1:100 dilution in TBST containing 5% of Skimmilk (Difco) for 1 h at room temperature. Membranes were washed three
10 times for 5 minutes (min) in TBST and were subsequently incubated with alkaline phosphatase-conjugated goat anti-swine antibody (Jackson ImmunoResearch) in a 1:10,000 dilution in TBST containing 5% of Skimmilk (Difco) for 1 h at room temperature. Membranes were washed three times for 5 min in TBST, and the reactivity with the expressed proteins was visualized using Nitro Blue Tetrazolium (Merck, Darmstadt, Germany)-bromochloroindolyl phosphate (Sigma, St. Louis, MO) as a substrate.

15 B. Results.

[0180] Reactivity of cell wall anchored proteins to convalescent pig sera directed against various *S. suis* serotype isolates. To study the reactivity of proteins 1, 5, 8, 9, 10, 15, 20, 23, and 29 to convalescent pig sera directed against various serotype 1, 2, 7 and 9 isolates, lysates of IPTG induced and non-induced *E. coli* cells containing the various plasmid constructs were used
20 for western blot analysis. All convalescent sera reacted with the lysates of induced as well as with non-induced *E. coli* cells. As a result, the reactivity of the expressed proteins to the convalescent sera was difficult to judge. Therefore, the proteins were purified using a one-step purification procedure. (See Example 1.) Target gene 4 was excluded from this study.

[0181] A Coomassie stained gel (FIG. 11) shows that substantial amounts of purified
25 proteins were obtained for proteins 1, 5, 8, 10, 15, 20, and 23. Proteins 9 and 29 were obtained at very low concentrations. The purified proteins were subsequently used to study the reactivity to the convalescent pig sera by western blot analysis.

[0182] The data (FIGs. 12-14 and Table 5A) show that proteins 1, 5, 8, 10, and 20 were recognised by all convalescent sera available against serotype 1, 2, 7, and 9 isolates. Protein 9
30 showed weak reactivity to all sera except to the one directed against the serotype 1 strain 6112. Protein 23 was recognized by all sera except for the sera against the two serotype 1 isolates as

well as the strain 10 (serotype 2) specific serum. Protein 29 was recognized by sera directed against the serotype 2 isolates 3 (taken at 25 days after challenge) and P1/7 and showed a weak reactivity to serum directed against the serotype 2 isolate 10. No clear reactivity of protein 29 was observed with any of the other sera used. Protein 15 reacted strongly with sera obtained
5 from piglets before challenge (day 0) as well as the convalescent sera.

Table 5A. Reactivity to convalescent sera.

type	isolate	Protein 1	Protein 5	Protein 8	Protein 10	Protein 20	Protein 9	Protein 15	Protein 23	Protein 29
1	6112	+	+	+	+	+	-	reacted with day 0 samples	-	-
1	6388	+	+	+	+	+	(+)	reacted with day 0 samples	-	-
2	3	+	+	+	+	+	(+)	reacted with day 0 samples	+	+
2	10	+	+	+	+	+	(+)	reacted with day 0 samples	-	(+)
2	P1/7	+	+	+	+	+	(+)	reacted with day 0 samples	+	+
7	7711	+	+	+	+	+	(+)	reacted with day 0 samples	+	-
7	7917	+	+	+	+	+	(+)	reacted with day 0 samples	+	-
7	8039	+	+	+	+	+	(+)	reacted with day 0 samples	+	-
9	8067	+	+	+	+	+	(+)	reacted with day 0 samples	+	-
9	8017	+	+	+	+	+	(+)	reacted with day 0 samples	+	-

Table 5B. Reactivity to convalescent sera.

type	isolate	Protein 3	Protein 11	Protein 12	Protein 16	Protein 25	Protein 35.1	Protein 35.2
1	6112	+	-	-	-	-	-	-
1	6388	+	-	-	-	(+)	-	(+)
2	3	+	-	-	-	-	-	-
2	10	+	-	-	-	-	+	(+)
2	P1/7	+	-	-	-	-	-	-
7	7711	(+)	-	-	-	-	-	-
7	7917	(+)	(+)	(+)	-	-	-	-
7	8039	(+)	-	-	-	-	(+)	-
9	8067	-	-	-	-	-	-	-
9	8017	-	-	-	-	-	-	-

[0183] Reactivity of putative pilus proteins to convalescent pig sera directed against various *S. suis* serotype isolates. Putative pilus proteins were purified similarly as described

5 in Example 1. The reactivity of the purified proteins with convalescent pig sera was examined using western blot analysis. The data (FIGs. 12-14 and Table 5B) show that protein 3 was clearly recognized by convalescent sera directed against the serotype 1 and 2 isolates. A weak reactivity of protein 3 to convalescent sera directed against the serotype 7 isolates was observed.

10 **[0184]** Protein 35.1 showed reactivity to convalescent serum directed against the serotype 2 strain 10. Proteins 11 and 12 showed weak reactivity to convalescent serum directed against the serotype 7 isolate 7917. Weak reactivity was also observed for protein 25 against convalescent serum directed against the serotype 1 strain 6388, for protein 35.1 against convalescent serum directed against the serotype 7 strain 8039, and for protein 35.2
15 against convalescent sera directed against the serotype 1 strain 6388 and the serotype 2 strain 10. Putative pilus proteins 16 was not recognized by the convalescent sera used.

C. Discussion and conclusions.

[0185] Cell wall anchored proteins. In the present study proteins 1, 5, 8, 10 and 20 were recognised by all convalescent sera available against serotype 1, 2, 7 and 9 isolates.

20 These results indicate that the proteins 1, 5, 8, 10 and 20 are expressed during infection, are immunogenic, and are sufficiently conserved among the various isolates and serotypes to be recognized. These proteins are suggested as candidates for a vaccine providing protecting against *S. suis* serotypes 1, 2, 7, and 9.

[0186] Protein 9 only showed comparatively weak reactivity with all sera except for the serum directed against the serotype 1 strain 6112. It is unclear why the reactivity was comparatively weak.

5 [0187] Protein 23 was recognized by all sera except for the sera against the two serotype 1 isolates as well as the strain 10 (serotype 2) specific serum. The cause of this variation is unknown, but protein 23 may be expressed at different levels in the various isolates.

10 [0188] Protein 29 was recognized by sera directed against the serotype 2 isolates 3, 10, and P1/7. No clear reactivity was observed with any of the other sera used. Previous CGH data showed sequence diversity between the genes encoding proteins 29 in serotype 7 and 9 isolates compared to the serotype 1 and 2 isolates. This sequence variation may explain the negative results obtained using the sera directed against the serotype 7 and 9 isolates.

15 [0189] Protein 15 reacted strongly with sera obtained from piglets before challenge (day 0) as well as the convalescent sera. Apparently, protein 15 is either able to bind antibodies present in sera taken before challenge or interferes with the enzymatic detection method.

[0190] Pilus proteins. Convalescent sera directed against the serotype 1 and 2 isolates recognized the purified protein 3, which is encoded by the corresponding putative pilus gene. This result indicates that gene 3 is expressed during infection, is immunogenic, and is sufficiently conserved among the serotype 1 and 2 isolates to be recognized. Expression of gene 3 in serotype 1 and 2 isolates could be expected based on the data presented in FIG. 15, where gene 3 (being part of the cluster 1 pilus genes) was exclusively found in serotype 1 and 2 isolates. Recently, Holden and co-workers suggested gene 3 to be the gene encoding the main protein subunit of the putative pilus in the serotype 2 isolate P1/7. However, it is unclear from these current results whether gene 3 is able to form functional pilus structures in serotype 1 and 2 isolates. As serotype 7 isolates do not contain a gene homologous to gene 3, reactivity of the protein encoded by gene 3 with serotype 7 specific convalescent sera was not expected. It is possible that the observed weak signal is the result of non-specific binding.

30 [0191] In the pilus cluster 1, the gene encoding protein 3 is genetically linked to the gene encoding protein 16, which is in P1/7 and believed to encode an ancillary pilus protein to the protein 3 main subunit. However, in P1/7 gene 16 was identified as being a pseudogene (Holden et al.). Based on this data, expression of gene 16 in P1/7 was not expected. Western blot analysis confirmed that P1/7 derived convalescent sera did not react

with protein 16. In addition, data showed that none of the sera examined contained antibodies against protein 16, suggesting that gene 16 is also a pseudogene in the other serotype 1 and 2 isolates.

[0192] Based on the data, see FIG. 15, proteins 11 and 12 were not expected to be expressed by the serotype 1 and 2 isolates used. Western blot data are in accordance with this expectation. Genes 11 and 12 were only found in the non-virulent isolate 891591 and in two isolates obtained from Japan.

[0193] Previous hybridization studies showed that serotype 7 isolates do not contain genes homologous to the genes encoding the proteins 11 and 12, suggesting that the weak western blot signal observed with serotype 7 isolate 7917 is the result of some non specific binding.

[0194] Most of the convalescent sera used did not recognize proteins 25, and 35.1 and 35.2. As previously indicated gene 35 is a pseudogene in strain P1/7 and was cloned into two parts (35.1 containing the 5'-end of the gene and 35.2 containing the 3'-end of the gene).

Based on these data, binding of P1/7 specific convalescent sera to protein 35.1 and 35.2 was not expected. However, the data presented in FIG. 15 suggested that expression of proteins 25, 35.1 and 35.2 could have been expected for the remaining serotype 2 isolates as well as the type 1 and 9 isolates. But, no consistent pattern of recognition was observed with these isolates. The reason for the lack of antibodies against these putative pilus proteins is unknown.

[0195] The overall data generated with the putative pilus proteins seems inconsistent with the EM examinations. Here, the presence of pilus-like structures was demonstrated on the surface of serotype 1, 2, 7 and 9 isolates. The reasons for this discrepancy are unknown. One explanation could be that pilus proteins are only expressed *in vitro* but not (or only at a low level) *in vivo*. Alternatively, the lack of antibodies against the putative pilus proteins could indicate that the putative pilus genes, as shown in FIG. 15, do not encode the pilus-like structures observed by EM.

D. Summary.

[0196] Cell wall anchored proteins. The present study shows that proteins 1, 5, 8, 10, and 20 (as numbered herein) were recognised by all convalescent sera available against serotype 1, 2, 7, and 9 isolates—indicating that the proteins 1, 5, 8, 10, and 20 are expressed during infection—are immunogenic and are sufficiently conserved among the various isolates and serotypes to be recognized. Protein 9 showed weak reactivity to all sera except for the

serum directed against the serotype 1 strain 6112. Protein 23 was recognized by all sera except for the sera against the two tested serotype 1 isolates as well as the strain 10 (serotype 2) specific serum. Protein 29 was recognized by sera directed against the serotype 2 isolates 3 (taken at 25 days after challenge) and P1/7 and showed a weak reactivity to serum directed against the serotype 2 isolate 10. No clear reactivity was observed with any of the other sera used. Protein 15 reacted with sera obtained from non-challenged animals.

[0197] Pilus proteins. Convalescent sera directed against the serotype 1 and 2 isolates recognized the purified protein 3, which is encoded by one of the identified putative pilus genes, indicating that gene 3 is expressed during infection, is immunogenic and is sufficiently conserved among the serotype 1 and 2 isolates to be recognized. Proteins 11, 12, 16, 25, and 35.1 and 35.2 did not show a consistent pattern of recognition. The reason for the lack of detectable antibodies against these putative pilus proteins is unknown. However, the data suggest that the corresponding genes do not encode the pilus like structures that were observed by EM.

EXAMPLE 5: Western Blot Analysis of Wild Type and Pilus Mutant Strains

[0198] In this study western blot analysis was used to compare wild type and pilus protein mutant isolates using newly prepared batches of antisera generated with *E. coli* derived putative pilus proteins.

A. Materials and Methods

[0199] Bacterial isolates and growth conditions. The *S.suis* isolates P1/7, 89/1591, 235/02 and 040910-1 as well as the mutant isolates P1/7Δ3, P1/7Δ35 and 891591Δ12 were used in this study. *S. suis* cells were grown in Todd-Hewitt broth (TH) (Code CM189, Oxoid) and incubated overnight under aerobiosis at 37°C. Overnight cultures were diluted 10-fold in TH broth and grown to an optical density of 0.5 at 600nm.

[0200] Generation of rabbit-anti sera. Pilus proteins expressed in *E. coli* were purified from solubilized cell pellets using Ni²⁺-NTA column chromatography using non-denaturing as well as a denaturing purification procedures as described by the manufacturer (Qiagen). Rabbit antisera against the purified pilus proteins were generated at BioGenes GmbH (Berlin, Germany) using a 28 day schedule. Pre-immune serum (20 ml) as well as 60 ml of antiserum were delivered.

[0201] Preparation of cell wall extracts from *S. suis* cells. Cell wall extract were prepared as described by Fittipaldi et al. Briefly, *S. suis* cultures were grown in TH broth to

an OD₆₀₀ nm of 0.8. The cells (45 ml) were then collected by centrifugation and resuspended in 1 ml of a raffinose solution (20 mM Tris-HCl pH 8.0; 10 mM MgCl₂ and 25% of raffinose). Protease inhibitors (Roche) and mutanolysin (500U/ml; Sigma, L6876) were added to the suspension. After incubation for 1 h at 37°C, protoplasts were collected by centrifugation. Supernatants containing the cell wall proteins were collected and frozen at -20°C until used.

[0202] Detection of expressed proteins. Proteins were denatured as described by the manufacturer (Invitrogen) and subsequently separated on a 4-12% Bis-Tris gel using MOPS buffer (NuPAGE, Invitrogen).

[0203] Western blot analysis. Proteins separated by NuPAGE were transferred to nitrocellulose membranes as described by the manufacturer (Invitrogen). The membranes were blocked in TBS containing 0.05% Tween80 and 4% milk powder (Campina) at room temperature for 1 h. The membranes were subsequently incubated with a rabbit polyclonal antibody generated against the purified pilus proteins (BioGenes). The sera were used at a 1:100 dilution in TBS containing 0.05% Tween80 and 4% milk powder (Campina); the membranes were incubated for 1 h at room temperature, which was followed by an incubation with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:1000 in TBS containing 0.05% Tween20 and 4% milk powder (Difco); the membranes were incubated for 1 h at room temperature. Reactivity with the expressed proteins was visualized using Nitro Blue Tetrazolium (Merck, Darmstadt, Germany)-bromochloroindolyl phosphate (Sigma, St. Louis, MO) as a substrate.

B. Results.

[0204] Detection of pilus-like structures by Western blot analysis. The results (FIGs. 16A-16D) show a ladder of high-molecular-mass bands in cell wall extracts of the wild type P1/7, 235/02 and 040910-1 isolates as well in the mutant P1/7Δ35 isolate using antisera directed against pilus proteins 3. The reactivity is observed with antisera raised against protein purified using a native purification procedure as well as with a denatured purification procedure (compare FIGs. 16A and 16B to FIGs. 16C and 16D). This high-molecular-mass banding pattern is absent in the P1/7Δ3 mutant isolate as well in 891581 and 891591Δ12 (FIGs. 16A-16D), which indicates that the observed reactivity with the wild type P1/7 is specific for the protein encoded by gene 3 and that gene 3 encodes the major pilus subunit in P1/7.

[0205] Isolate 891591 was previously shown to contain a gene encoding putative second major pilus subunit (gene 12). High-molecular-weight banding patterns were observed in cell wall extracts of 891591 using serum directed against protein 12 (FIGs. 17A-17D). These high-molecular-weight banding patterns were not observed in the mutant
5 891591 Δ 12 isolate. These results suggest a role for gene 12 in pilus formation in isolate 891591.

[0206] A high-molecular-mass banding pattern could not be detected after screening the cell wall extracts with antisera directed against protein 35-1 (FIGs. 18A-18D). In addition, similar patterns of reactivity were observed on cell wall extract of P1/7 and P1/7 Δ 35. Based
10 on these data a role of protein 35 in pilus formation could not be established.

C. Discussion and conclusions.

[0207] The data in this study indicate that proteins 3 and 12 are major pilus subunits of strains P1/7 and 891591, respectively.

[0208] High molecular weight banding patterns were observed in wild type P1/7 using
15 sera directed against protein 3. This result is in contrast to previous research, where high molecular banding patterns could not be observed in the wild type P1/7 isolate using protein 3 specific sera.

[0209] Here, new batches of antisera to detect the pilus structures, and cell wall extracts were prepared according to the procedure described by Fittipaldi et al. In contrast to the
20 procedure previously used for the preparation of the cell wall extracts, the procedure used by Fittipaldi et al. makes use of raffinose instead of glucose to stabilize the protoplasts. In addition, protease inhibitors are added during the incubation period. It is unclear which modification(s) resulted in the different outcome (i.e. protection of the integrity of the high molecular weight structures by the protease inhibitor or better quality antisera). In any case,
25 the data showed that the newly prepared sera were able to detect the *E. coli* expressed pilus subunits (proteins 3 and 12), as well as, the high molecular weight structures in the cell extract preparations. No difference was observed between the sera directed against proteins purified using a native or a denatured purification procedure.

[0210] A high-molecular-mass banding pattern could not be detected after screening the
30 cell wall extracts with antisera directed against protein 35-1. In addition, similar patterns of reactivity were observed on cell wall extract of P1/7 and P1/7 Δ 35. Based on these data a role of protein 35 in pilus formation could not be established. These data are in accordance with the fact that gene 35 in P1/7 has been annotated as being a pseudogene.

EXAMPLE 6: Vaccination of Piglets Against *S. suis* Infection

[0211] Three-week-old conventional piglets, PCR negative for *S. suis* type 2 and with no history of *S. suis* associated disease, were used in this study. The piglets were either vaccinated intramuscularly (IM) or intranasally (IN) using conventional techniques.

5 [0212] The IM piglets were vaccinated on days 0 and 28 with either 250µg of protein 3 or 500µg of a combination of proteins 3 and 12 adjuvanted with Stimune (Prionics) at a vol/vol ratio of 4/5 (antigen/adjuvant). The IM piglets were challenged intraperitoneally on day 35 with challenge isolate ATCC700794 at a dose of 2×10^9 . Positive, negative and strict control animals were included in this study. All groups consisted of 15 piglets with the exception of the strict control group that consisted of 5 piglets. The piglets were observed for 7 days following challenge. Clinical signs observed included lameness, changes in behavior, CNS signs as well as mortality. Severely affected animals were euthanized. The study is outlined in Table 6.

Table 6. Vaccination of different groups of piglets.

Group	# Pigs	Vaccine	Adjuvant	Antigen Concentration	Days of Vacc.	Vacc. Route	Chall. Dose	Chall. Day	Chall. Route
1	15	protein 3	Stimune	250ug protein	0,21	<i>i.m.</i>	2×10^9	35	<i>i.p.</i>
2	15	protein 3 & 12	Stimune	125ug of each protein	0,21	<i>i.m.</i>	2×10^9	35	<i>i.p.</i>
3	15	Type 2 Bacterin (positive control)	Stimune	2×10^{10} cells	0,21	<i>i.m.</i>	2×10^9	35	<i>i.p.</i>
4	15	Placebo	Stimune	N/A	0.21	<i>i.m.</i>	2×10^9	35	<i>i.p.</i>
5	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

15

[0213] Groups 1, 2, and 3 resulted in a statistically significant reduction of the frequency of mortality, lameness and abnormal behavior as compared to the placebo group.

[0214] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More

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specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the following claims.

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[0215] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

What is claimed is:

1. An immunogenic composition comprising at least one *Streptococcus suis* pilus peptide and a physiologically-acceptable vehicle, wherein the *S. suis* pilus peptide is immunoreactive to *S. suis*.
5
2. The immunogenic composition of claim 1, wherein the *S. suis* pilus peptide is encoded by a *S. suis* serotype gene or contiguous fragment thereof, wherein the *S. suis* serotype is selected from the group consisting of serotypes 1, 2, 7, and 9.
3. The immunogenic composition of claim 1 or 2, wherein the *S. suis* pilus peptide is selected from the group consisting of
10
 - a) a peptide having the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7;
 - b) a peptide have at least 85% homology to and/or identity with one or more peptides of a);
15
 - c) a peptide encoded by a polynucleotide having the sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13;
 - d) a contiguous fragment of a peptide of a), b) or c); and
20
 - e) any combination thereof.
4. The immunogenic composition of any one of claims 1 to 3, wherein the physiologically-acceptable vehicle is selected from the group consisting of a pharmaceutically or veterinarily acceptable carrier, adjuvant, or combination thereof.
5. A method of provoking an immune response against *Streptococcus suis* infection comprising the administration of the immunogenic composition of claim 1 to a subject.
25
6. A method of reducing the incidence or severity of a clinical sign associated with *Streptococcus suis* infection comprising the administration of the immunogenic composition of claim 1 to a subject in need thereof, wherein the reduction of the incidence of or the severity of a clinical sign is at least 10% relative to a subject not receiving the immunogenic composition.
30

7. The method of claim 6, wherein the clinical sign is selected from the group consisting of meningitis, septicemia, pneumonia, endocarditis, arthritis, endophthalmitis, and deafness.
8. The method of claim 6 or 7, wherein the subject is an animal selected from the group consisting of a porcine, a murid, an equid, a lagomorph, and a bovid.
9. The method of claim 6 or 7, wherein the subject is a human.
10. A method of preparing the immunogenic composition of any one of claims 1 to 4, comprising mixing at least one *Streptococcus suis* pilus peptide with a physiologically-acceptable vehicle.
- 10 11. A method of diagnosing *Streptococcus suis* infection in a subject, comprising providing at least one *Streptococcus suis* pilus peptide, contacting the *Streptococcus suis* pilus peptide with a sample obtained from the subject, and identifying the subject as having a *Streptococcus suis* infection if an antibody in the sample that is capable of binding the *Streptococcus suis* pilus peptide is detected.
- 15 12. The method of claim 11, wherein the *Streptococcus suis* pilus peptide is selected from the group consisting of:
 - a) a peptide having the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 7;
 - 20 b) a peptide have at least 85% homology to and/or identity with one or more peptides of a);
 - c) a peptide encoded by a nucleic acid having the sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13;
 - 25 d) a fragment of the peptides of a), b) or c); and
 - e) any combinations thereof.
13. The method of claim 11 or 12, wherein the binding is detected using a second antibody capable of binding the antibody in the sample.
14. A kit comprising at least one *Streptococcus suis* pilus peptide, an immunogenic carrier, a container for packaging the *Streptococcus suis* pilus peptide and immunogenic carrier, a set of printed instructions; and a dispenser capable of administering a vaccine to an animal.
- 30

15. A kit for preparing the immunogenic composition of any one of claims 1 to 4, comprising (i) the *Streptococcus suis* pilus peptide and (ii) the physiologically-acceptable vehicle, wherein (i) and (ii) are packaged separately.
16. The kit of claim 15, further comprising instructions for use of the kit.
- 5 17. The kit of claim 15 or 16, further comprising a dispenser.
18. The kit of any one of claims 15 to 17, wherein the *Streptococcus suis* pilus peptide is selected from the group consisting of:
- 10 a) a peptide having the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7;
- b) a peptide have at least 85% homology to and/or identity with one or more peptides of a);
- c) a peptide encoded by a nucleic acid having the sequence of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO:11, SEQ ID NO: 12, or SEQ ID NO: 13;
- 15 d) a fragment of the peptides of a), b) or c); and
- e) any combination thereof.

FIG. 1A

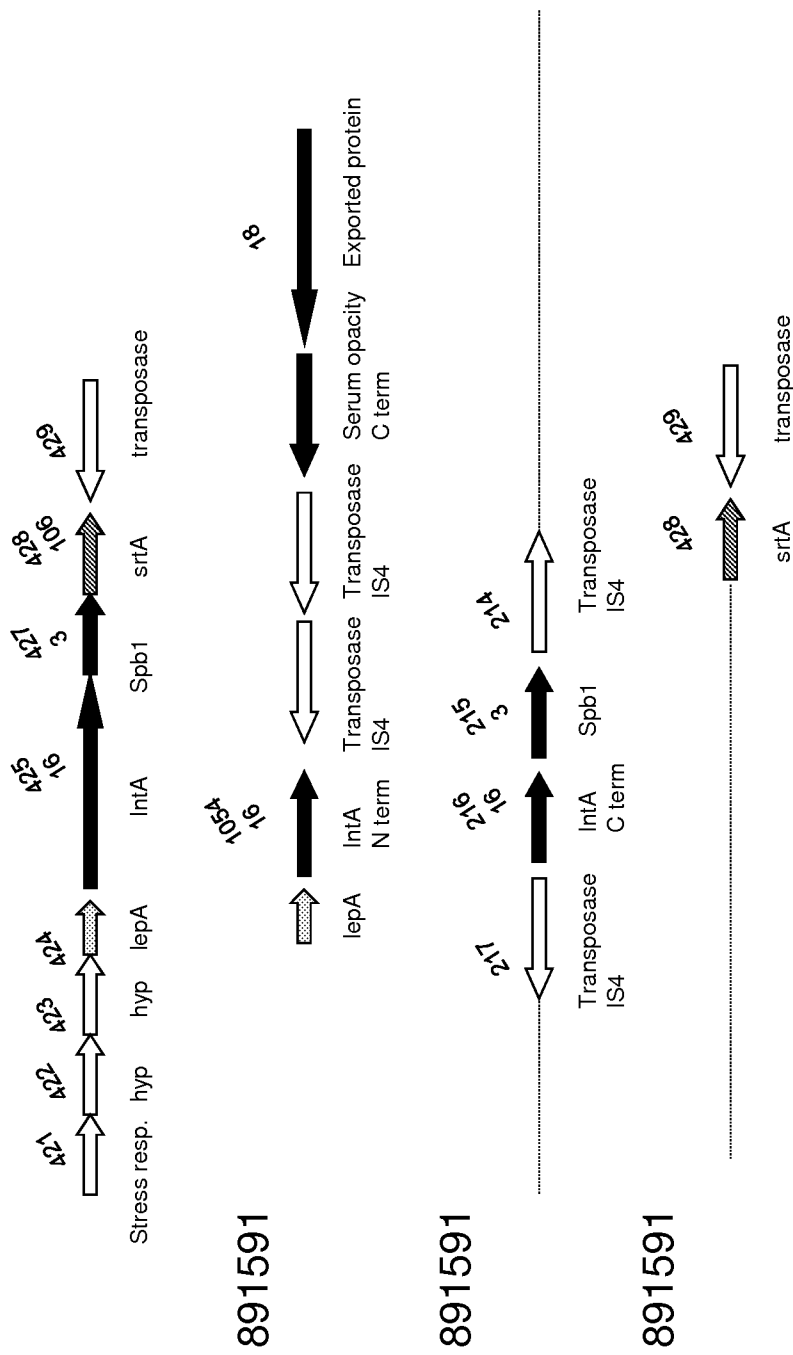


FIG. 1B

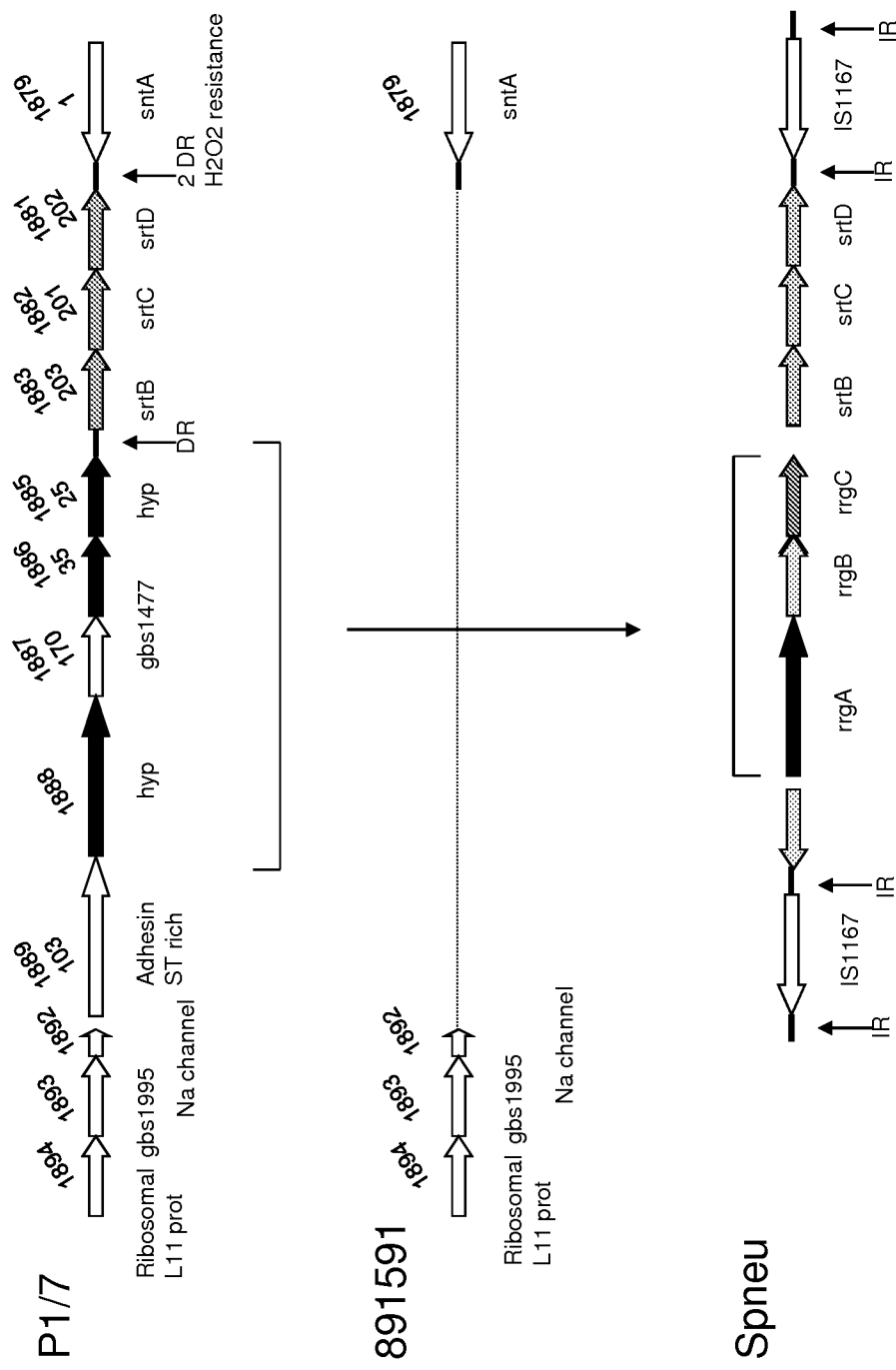


FIG. 1C

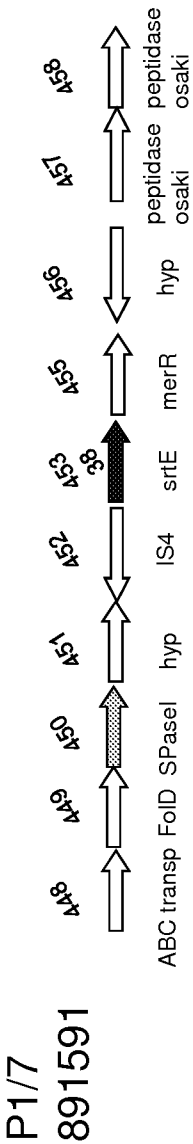


FIG. 1D

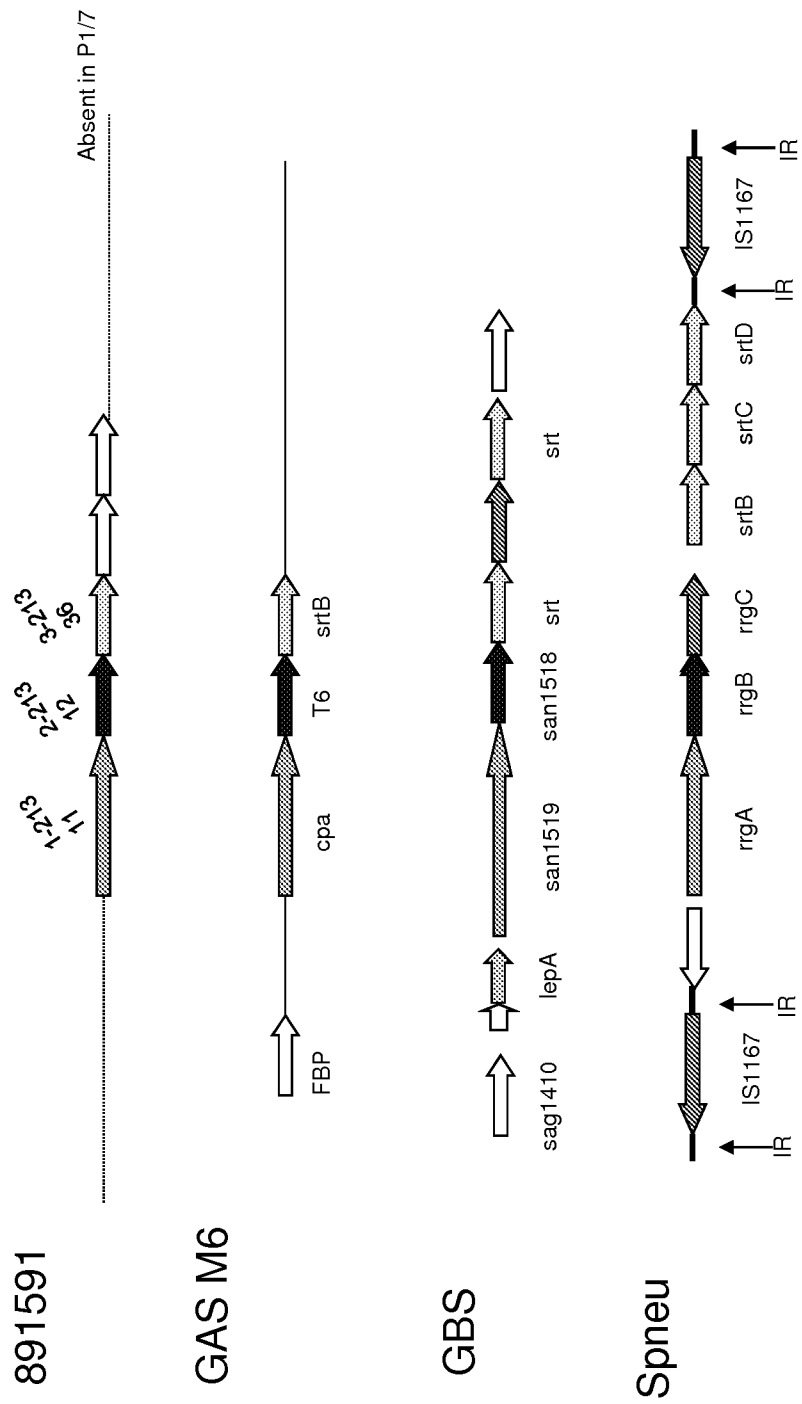
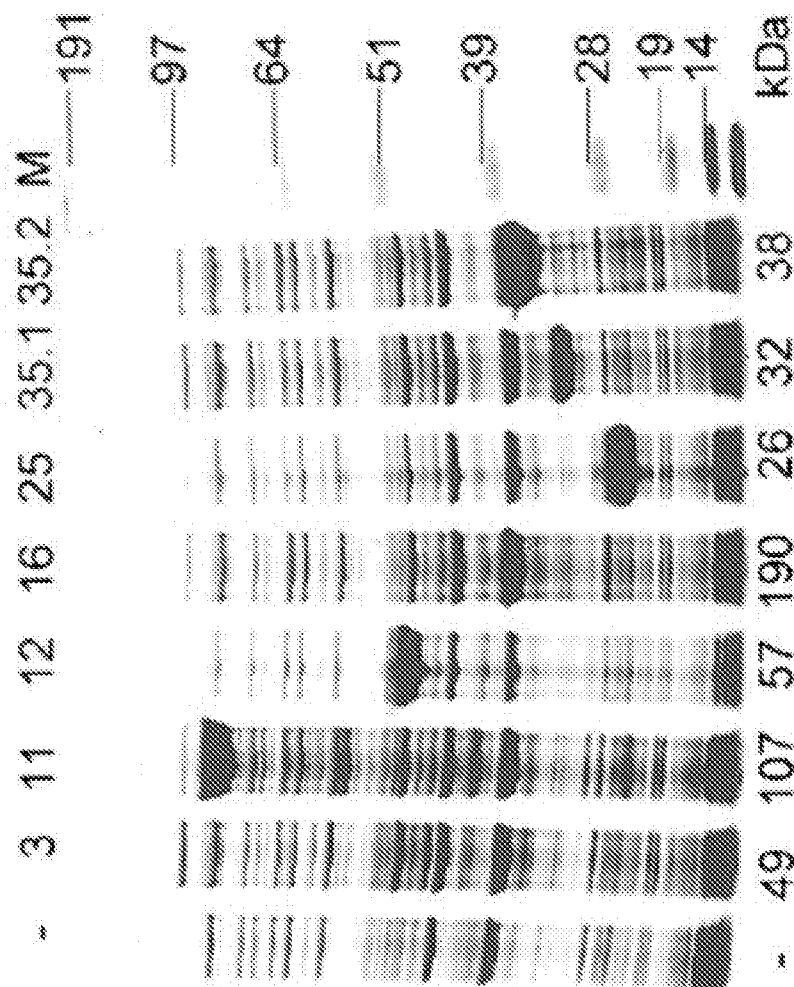
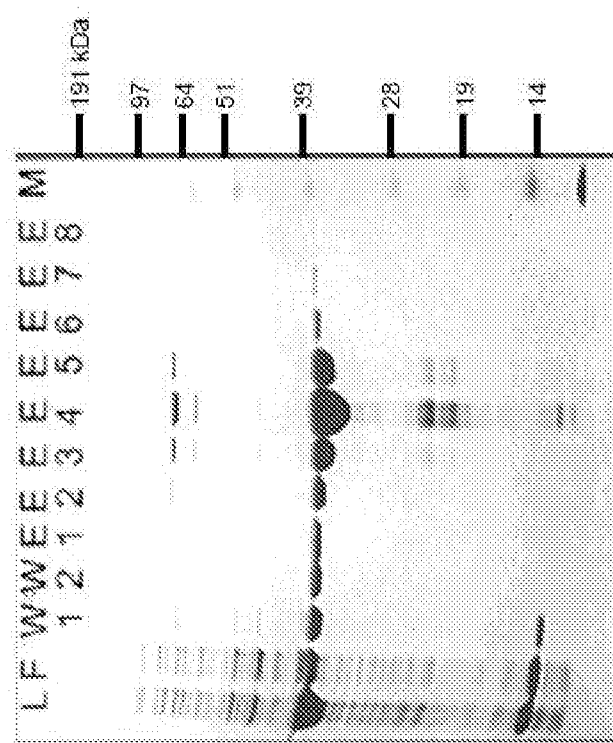


FIG. 2



6/38

FIG. 3



7/38

FIG. 4

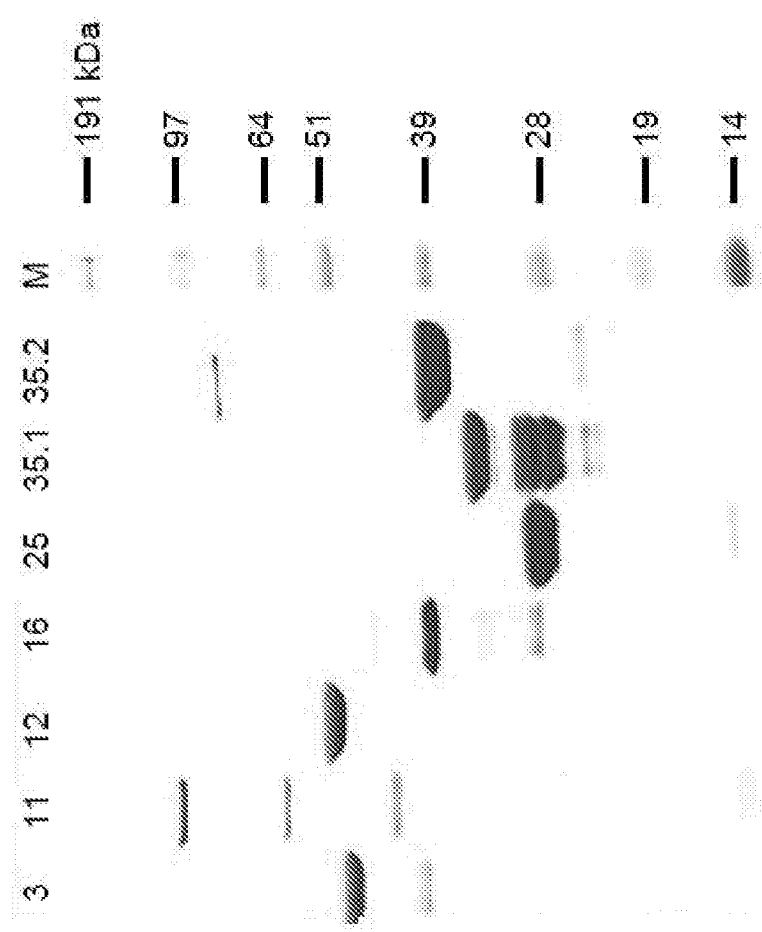


FIG. 5B

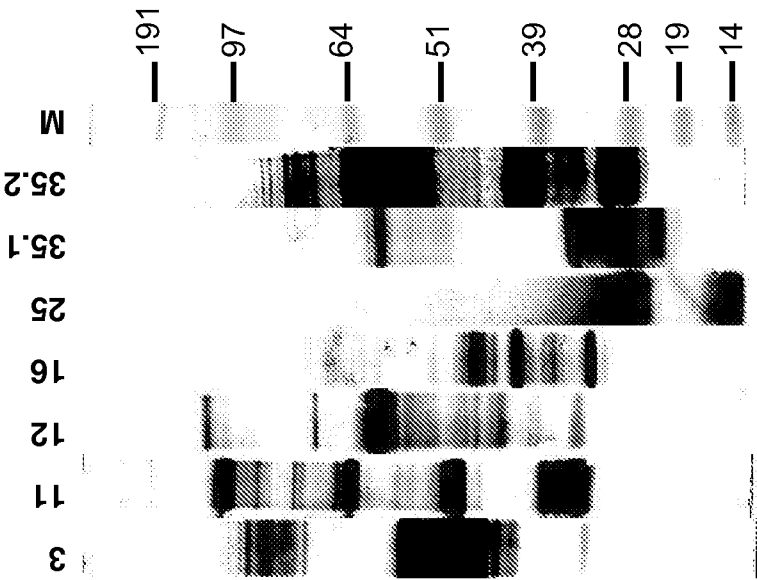
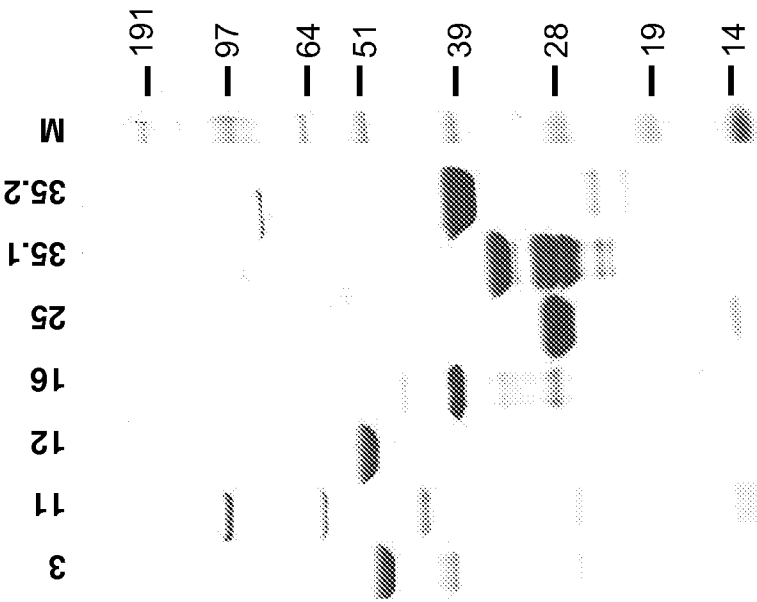
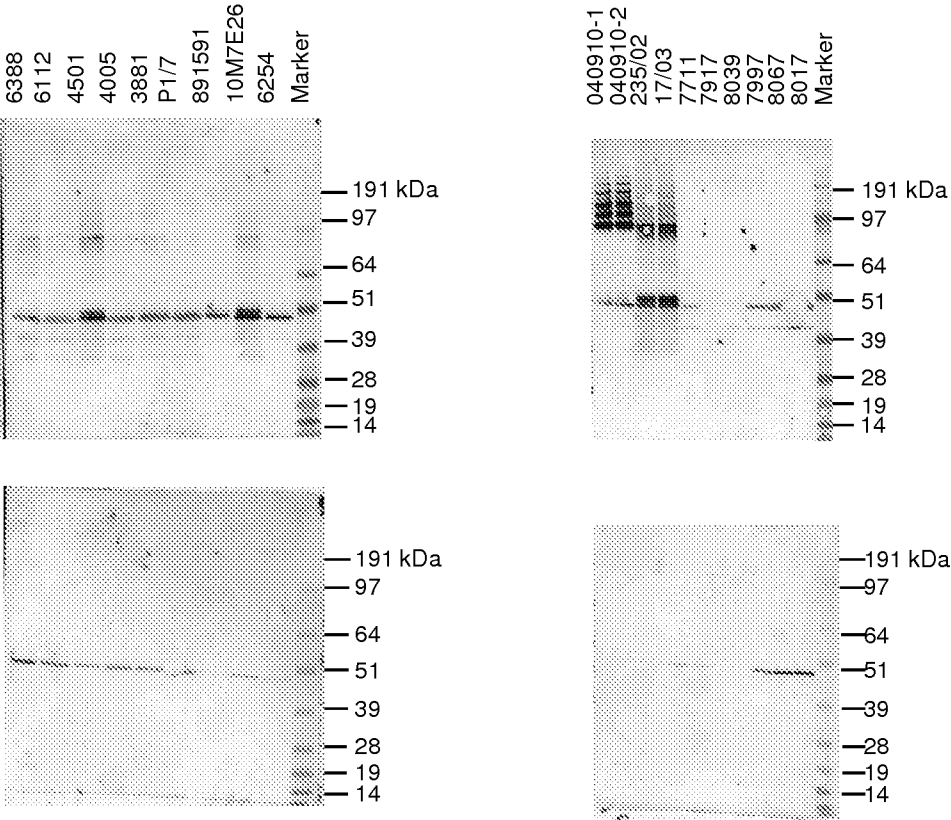


FIG. 5A



9/38

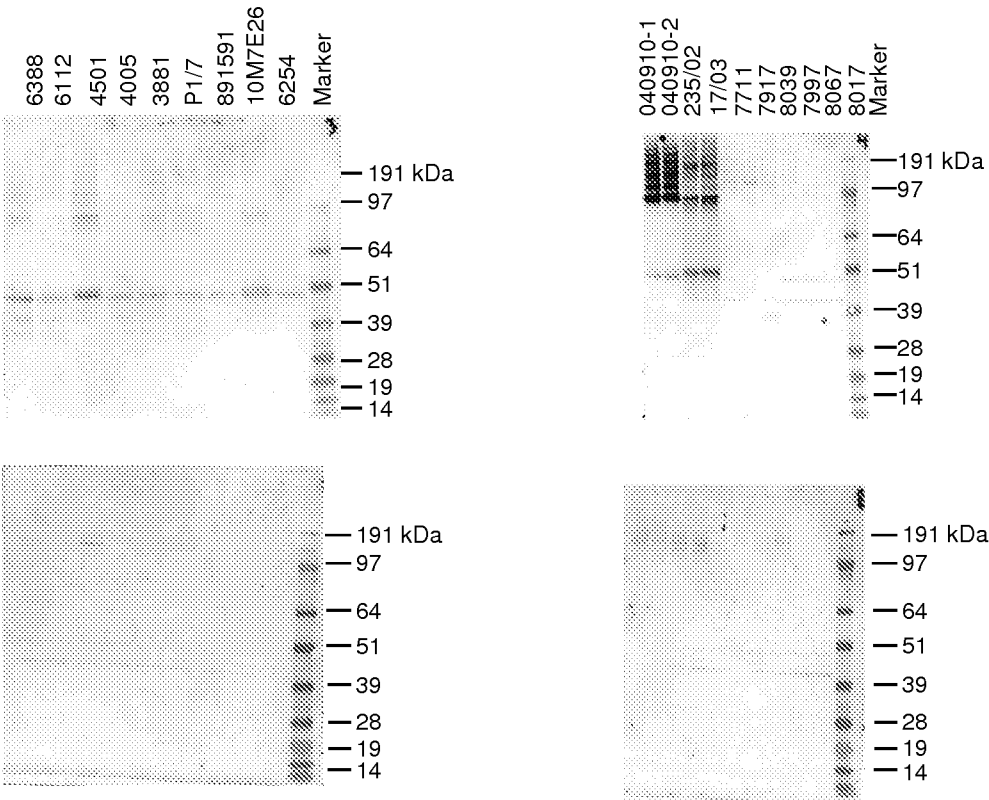
FIG. 6A



Rabbit #3806

10/38

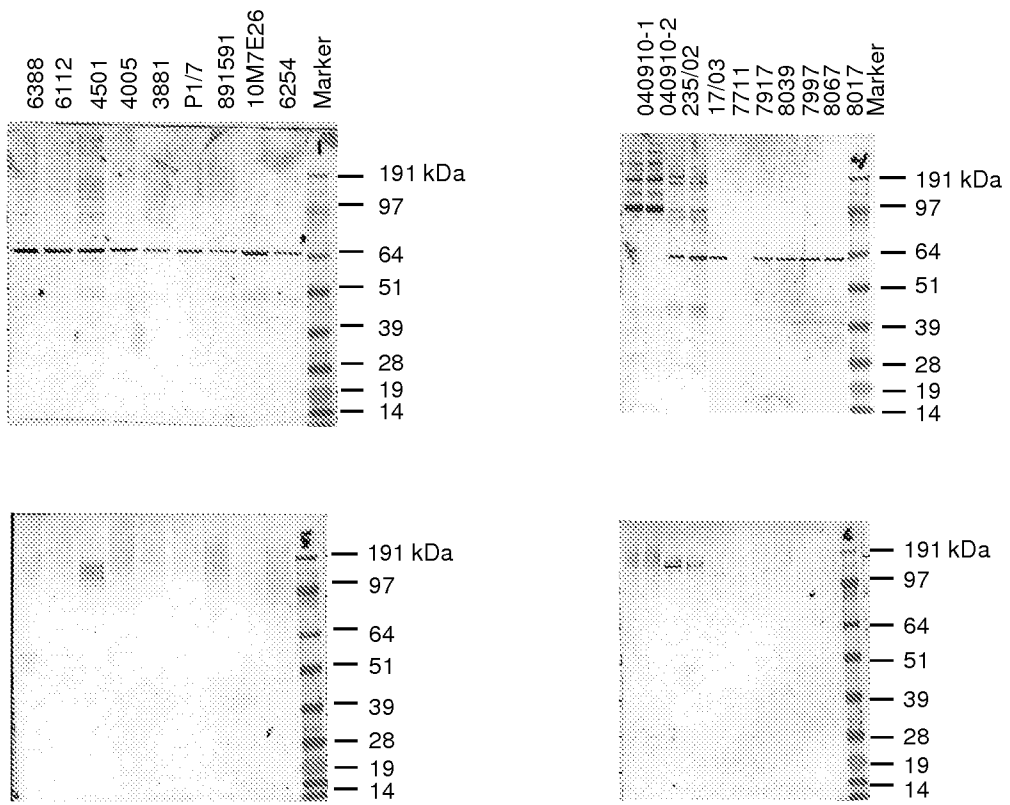
FIG. 6A Cont.



Rabbit #3807

11/38

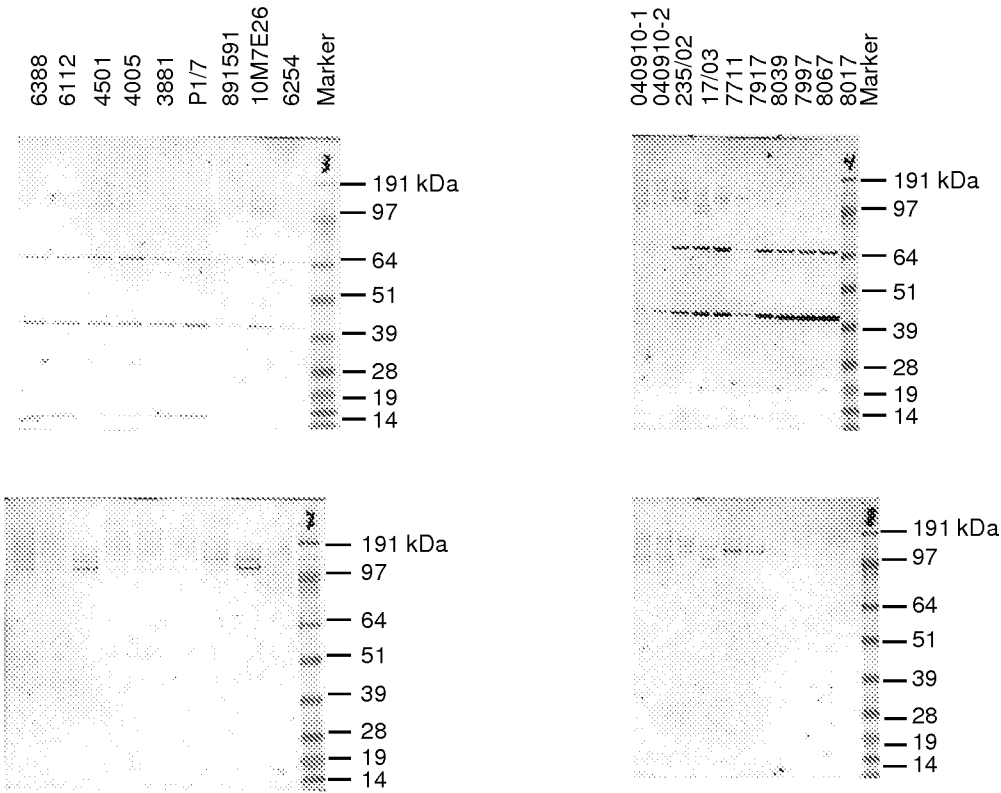
FIG. 6B



Rabbit #3808

12/38

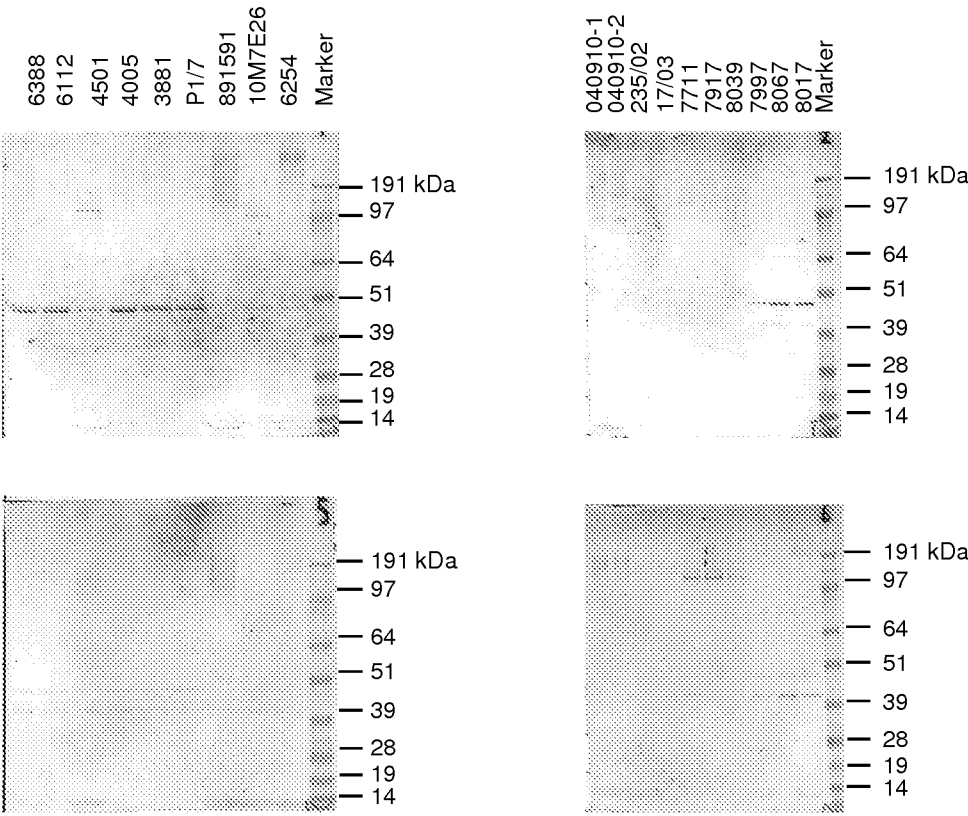
FIG. 6B Cont.



Rabbit #3809

13/38

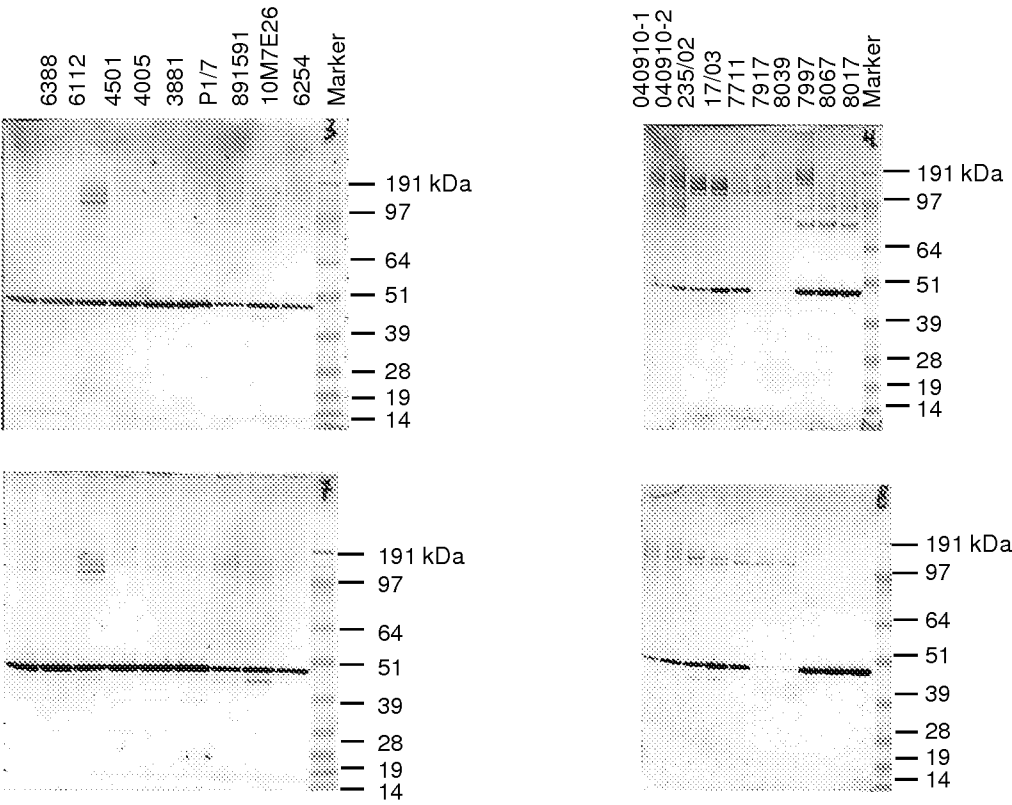
FIG. 6C



Rabbit #3810

14/38

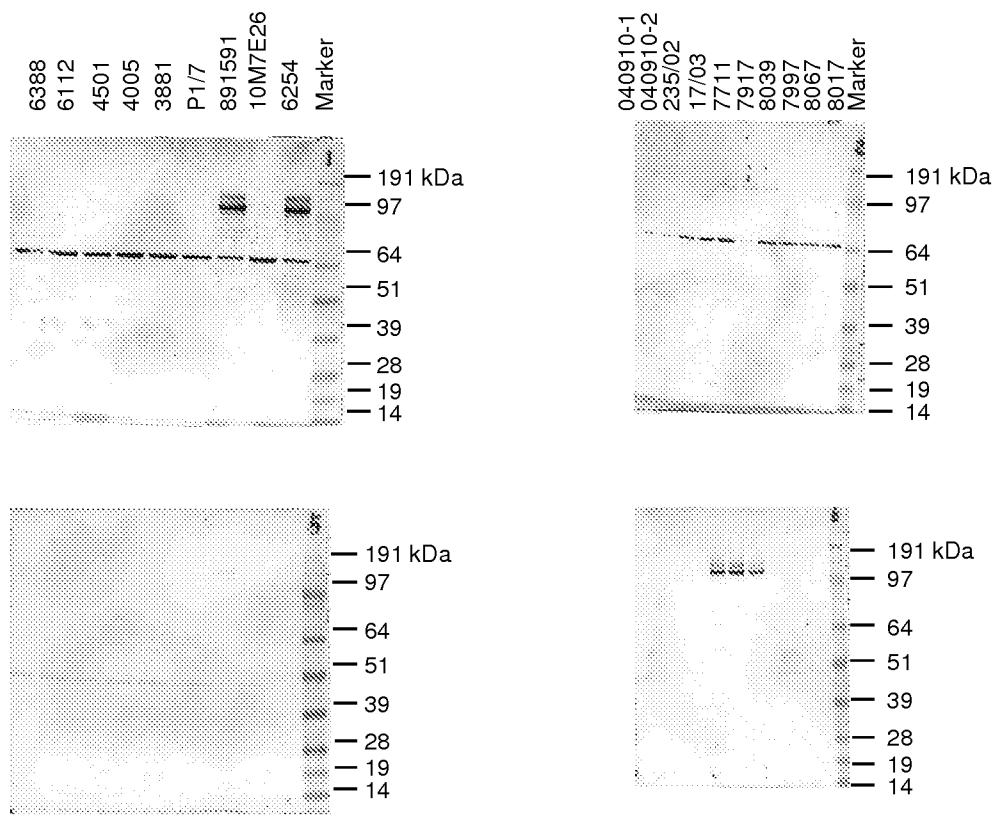
FIG. 6C Cont.



Rabbit #3811

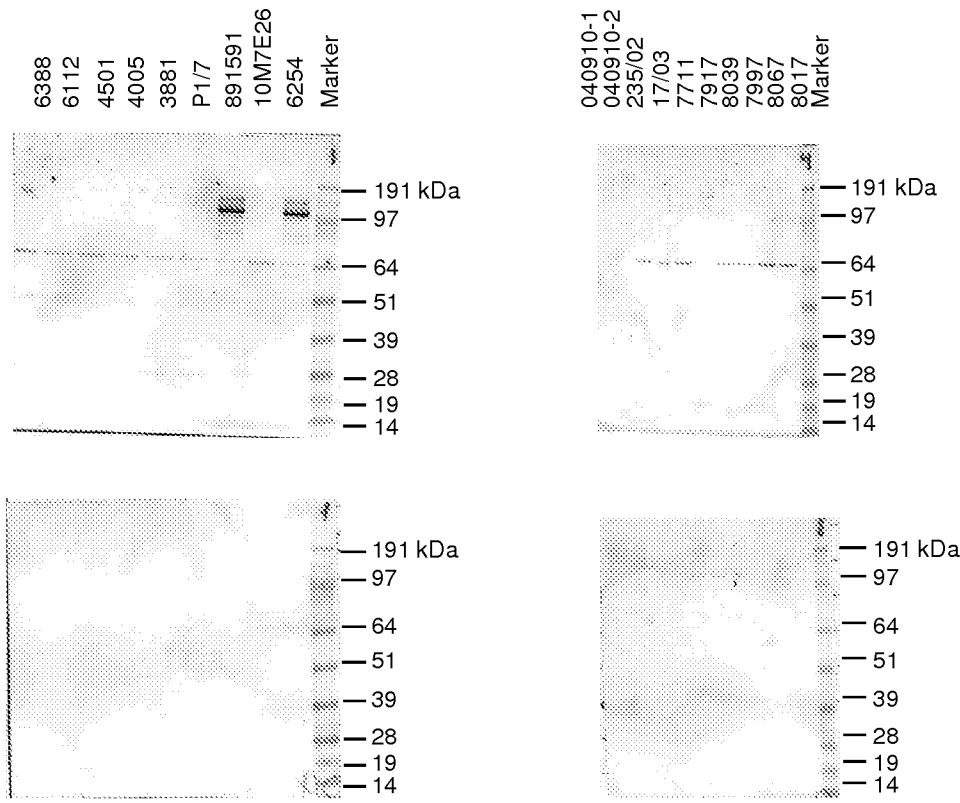
15/38

FIG. 6D



Rabbit #3812

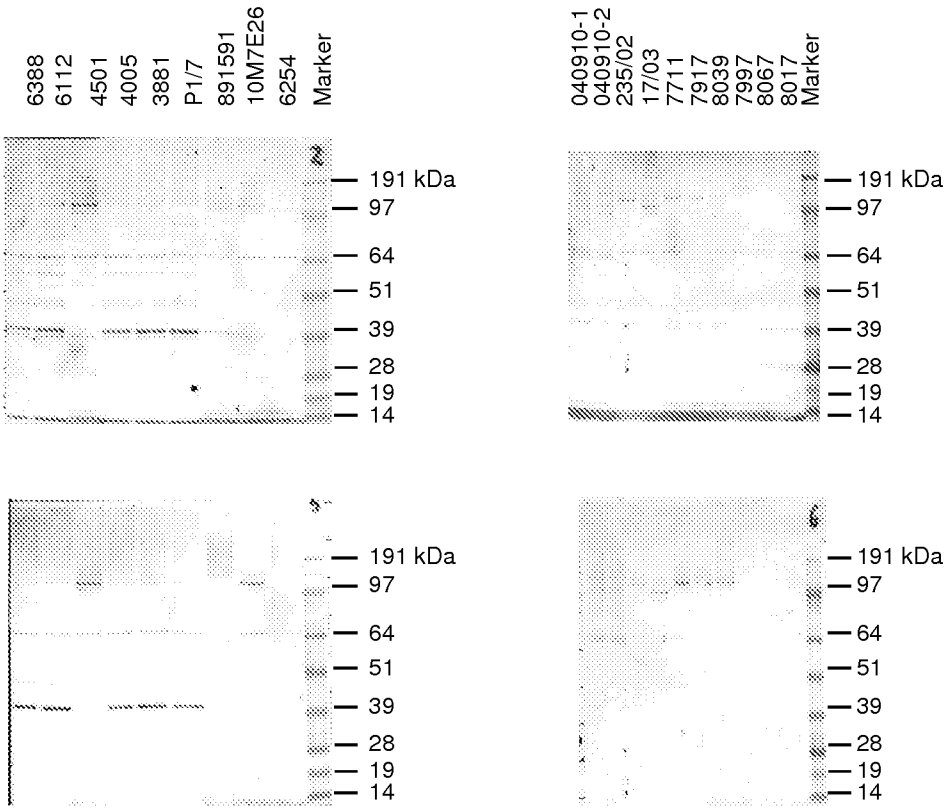
16/38
FIG. 6D Cont.



Rabbit #3813

17/38

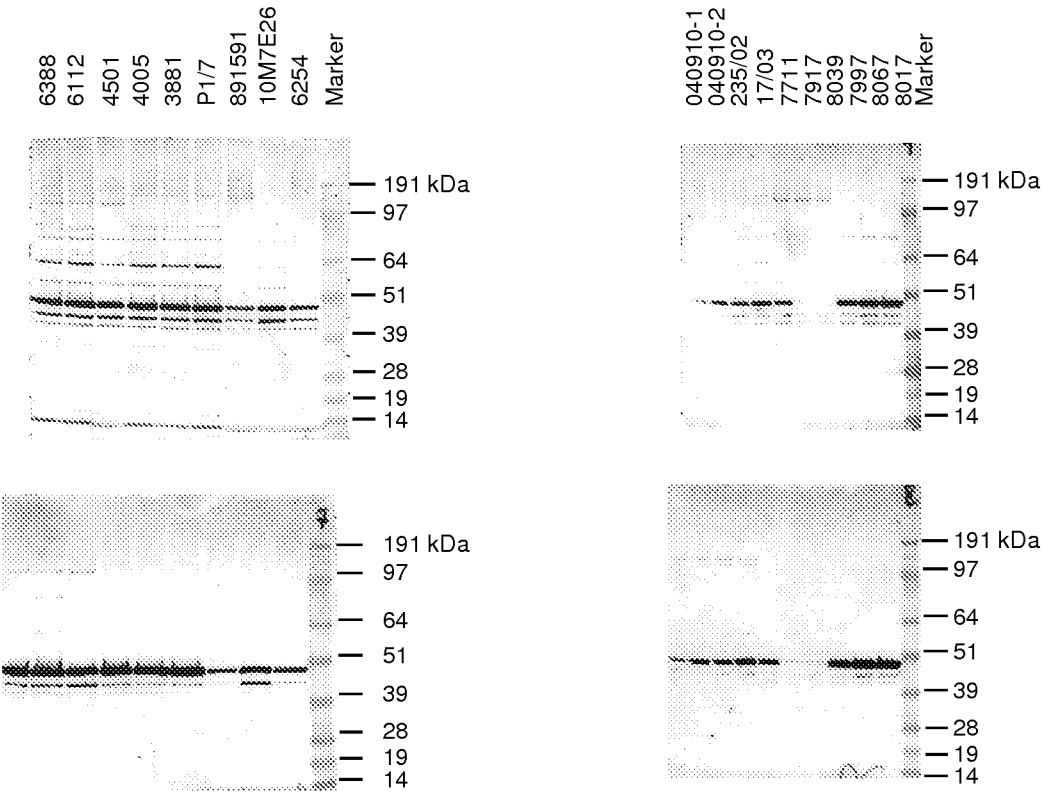
FIG. 6E



Rabbit #3814

18/38

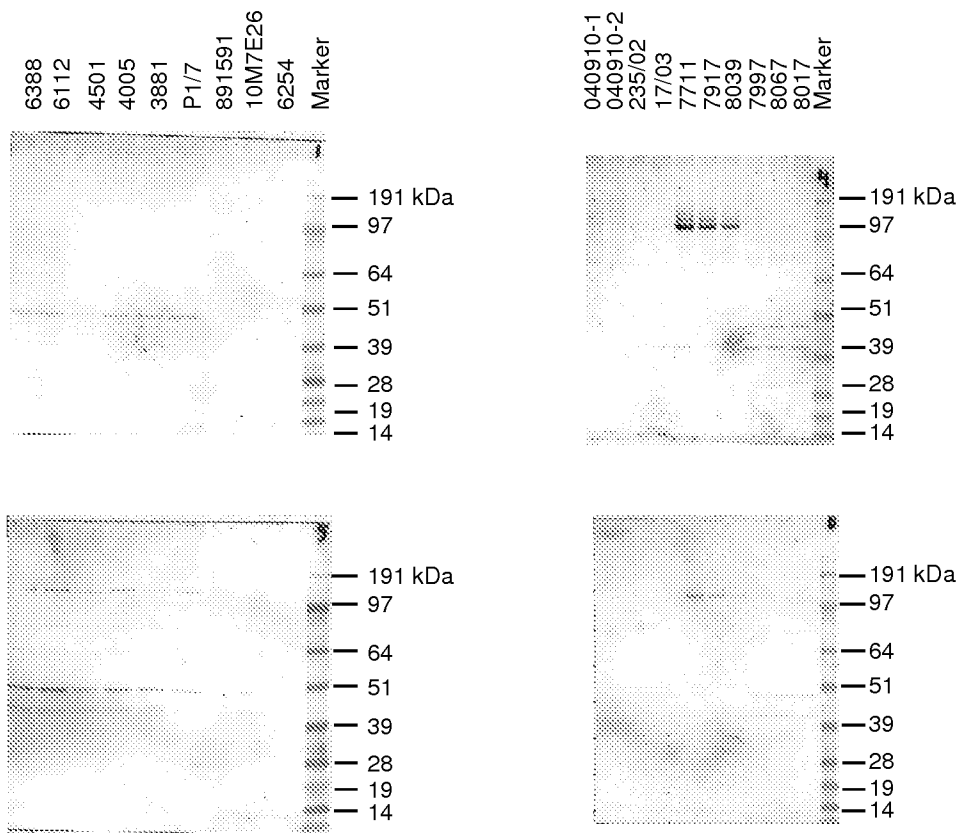
FIG. 6E Cont.



Rabbit #3815

19/38

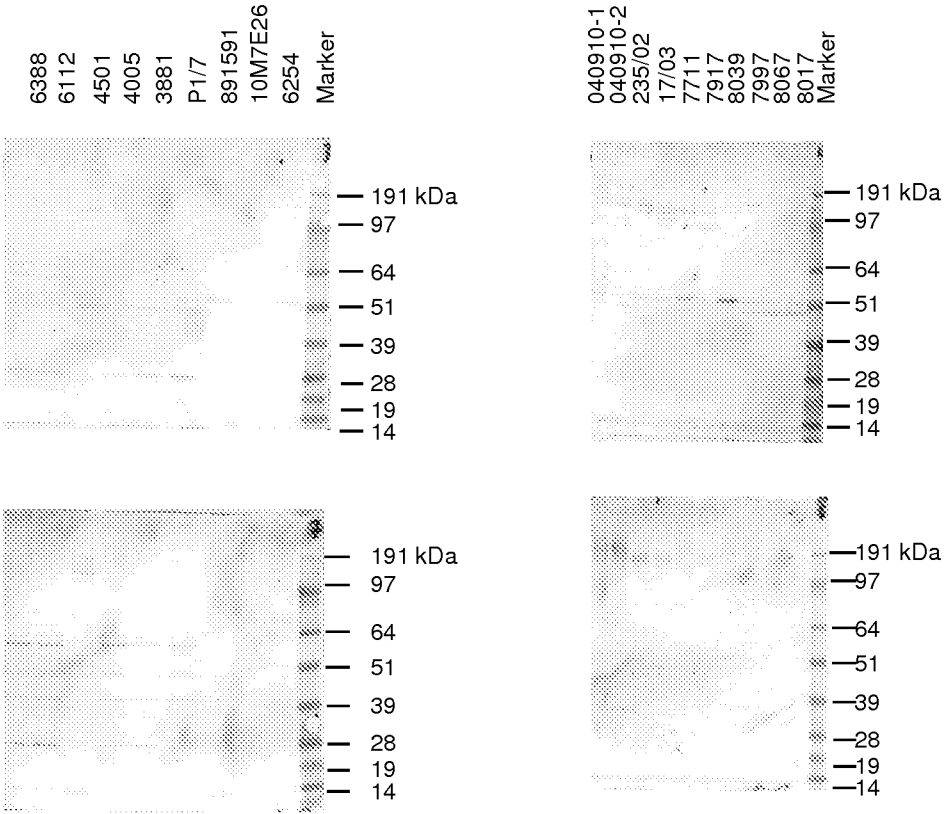
FIG. 6F



Rabbit #3818

20/38

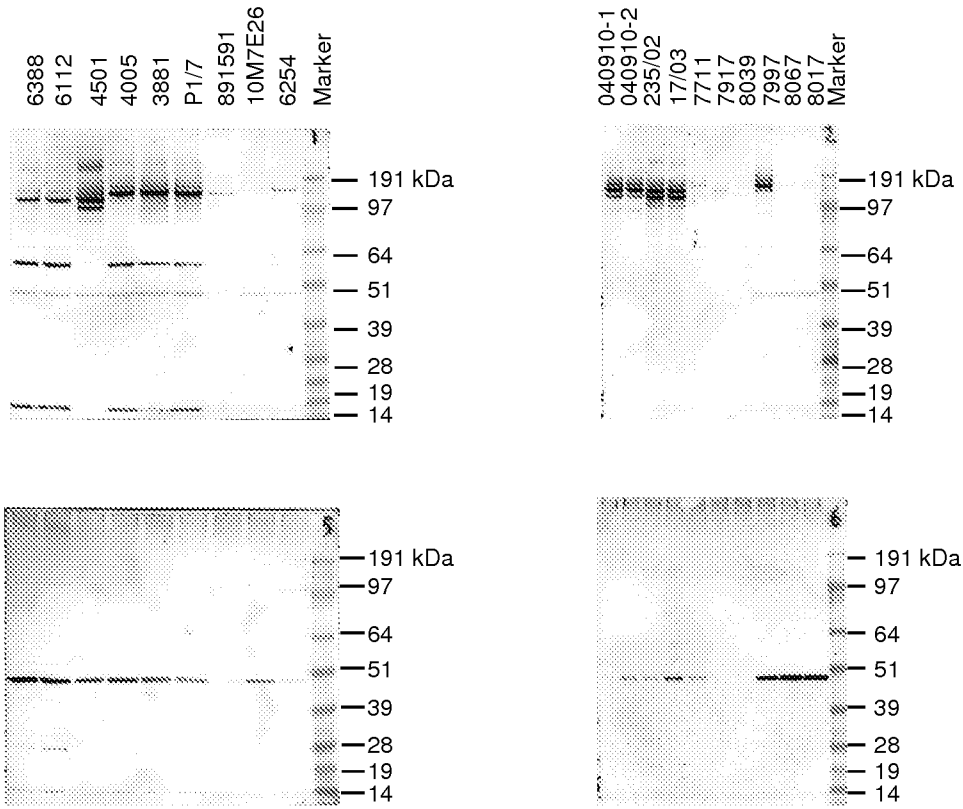
FIG. 6F Cont.



Rabbit #3819

21/38

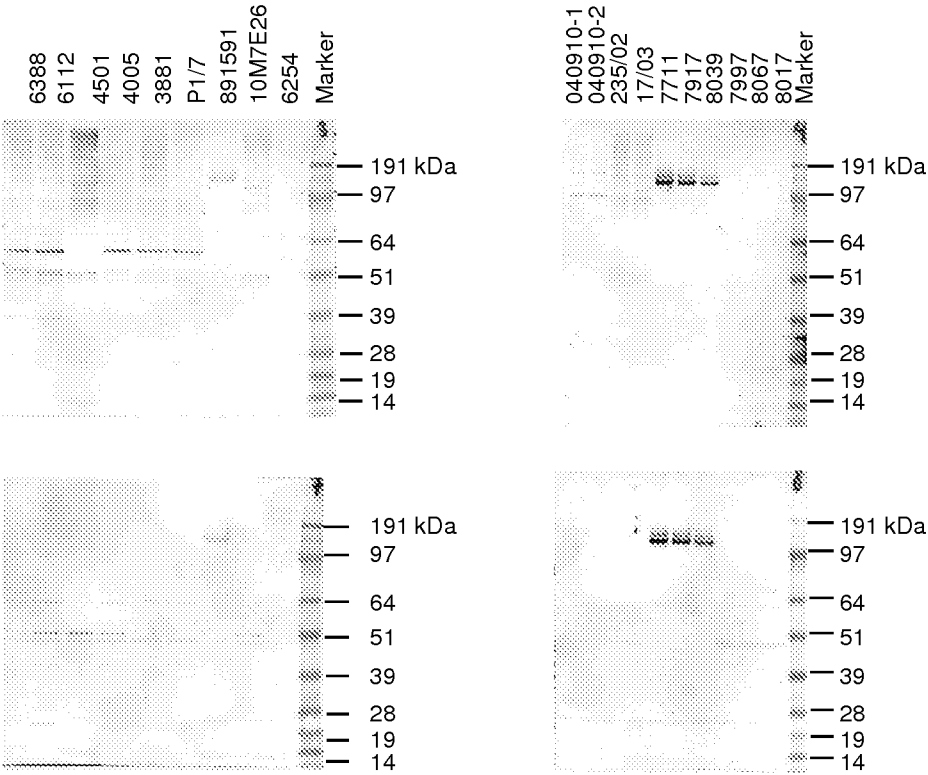
FIG. 6G



Rabbit #3816

22/38

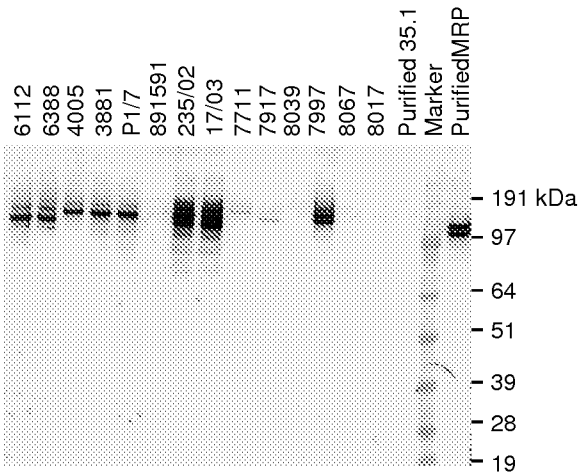
FIG. 6G Cont.



Rabbit #3817

23/38

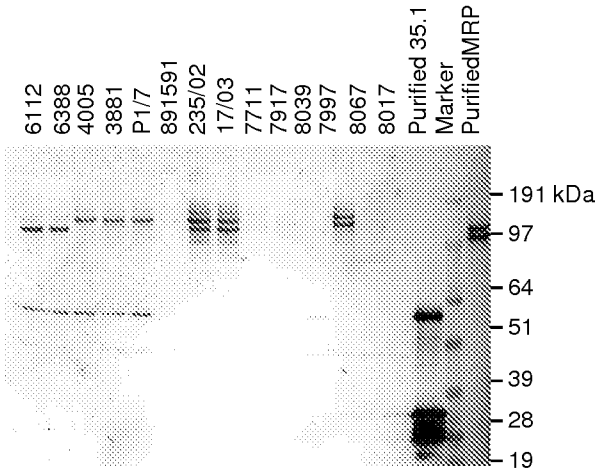
FIG. 6H



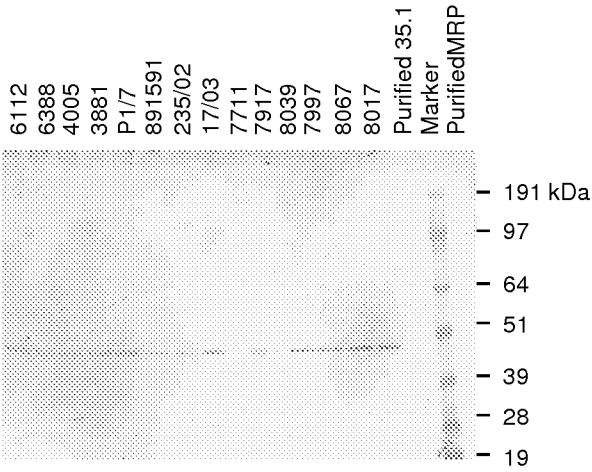
Mab anti-MRP

24/38

FIG. 6H Cont.



Anti 35.1 - Rabbit #3816
Final bleed sera



Anti 35.1 - Rabbit #3816
Pre-immune sera

FIG. 7A

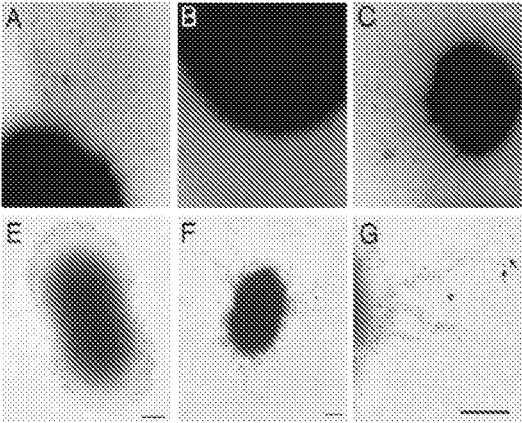


FIG. 7B

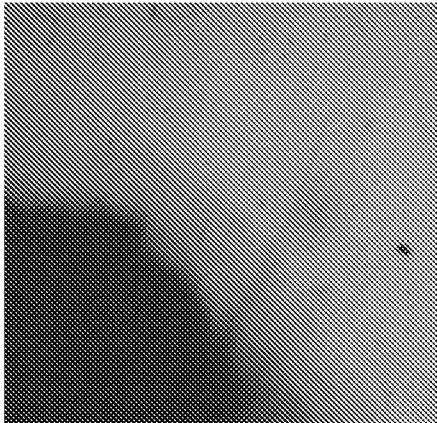


FIG. 7C

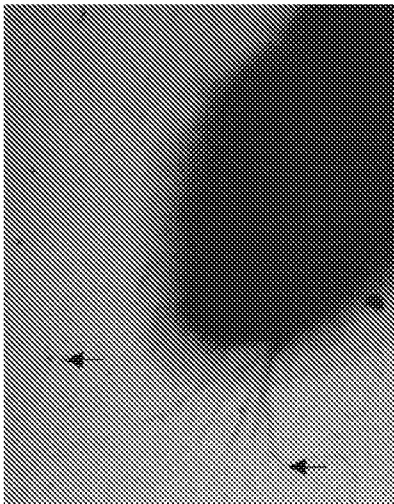


FIG. 7D

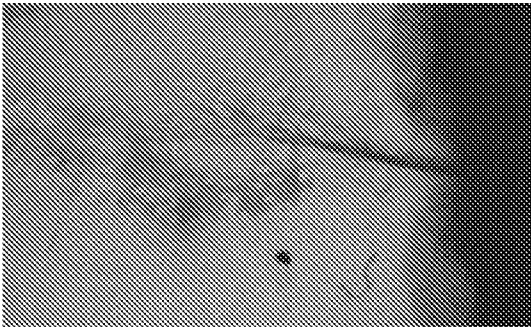
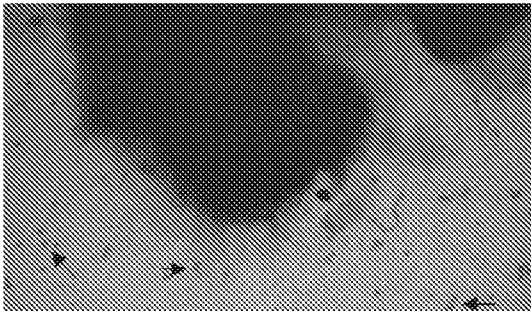


FIG. 7E



26/38

FIG. 8A

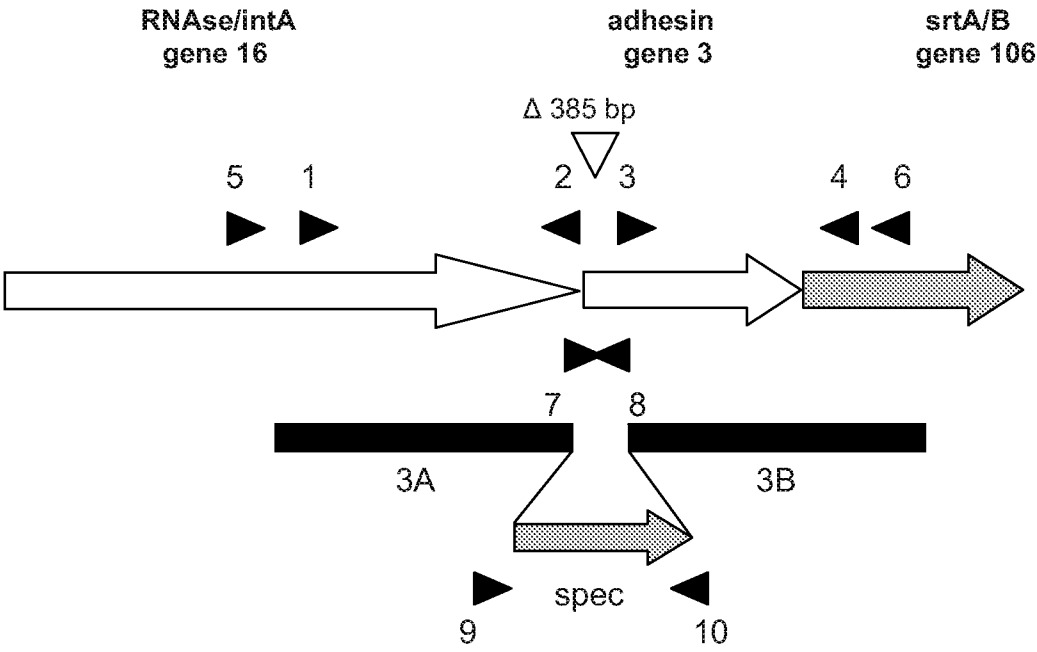
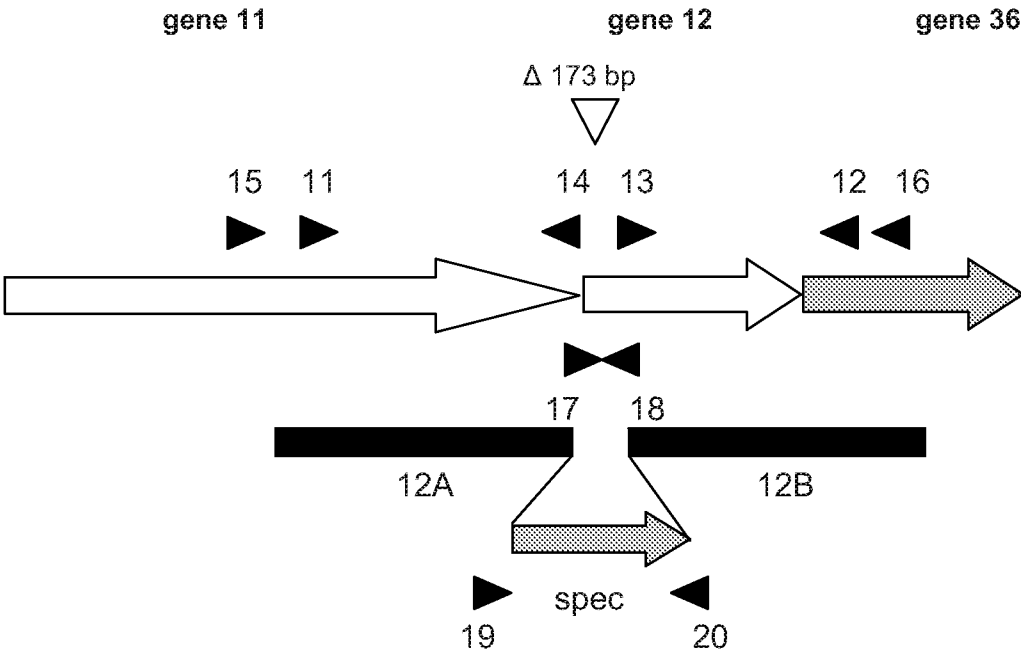
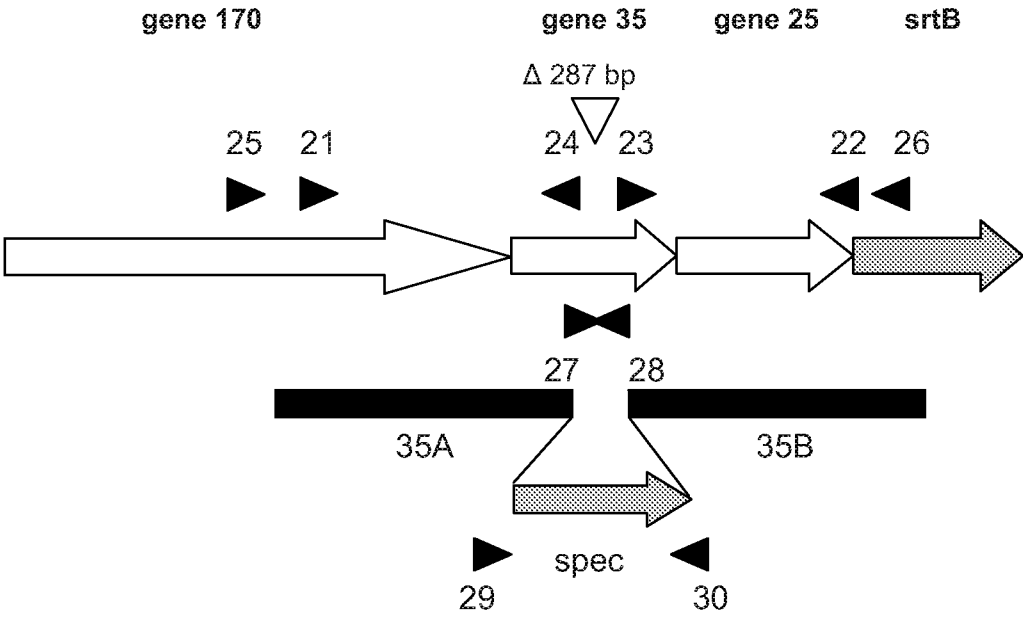


FIG. 8B



27/38

FIG. 8C



28/38

FIG. 9A

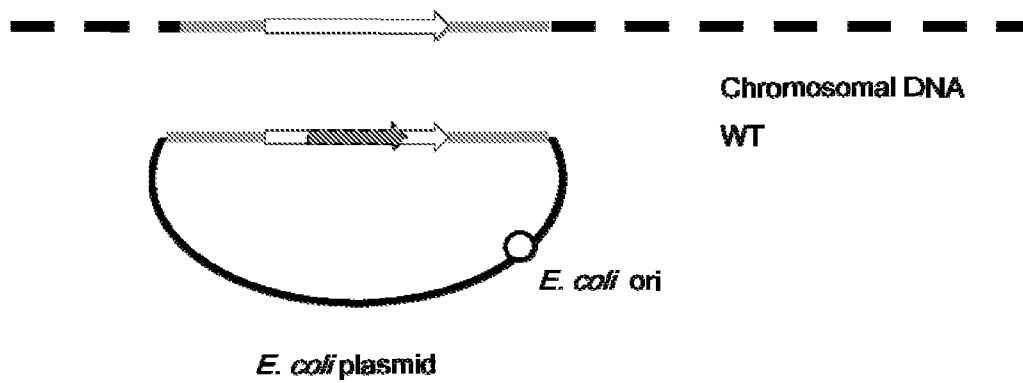


FIG. 9B

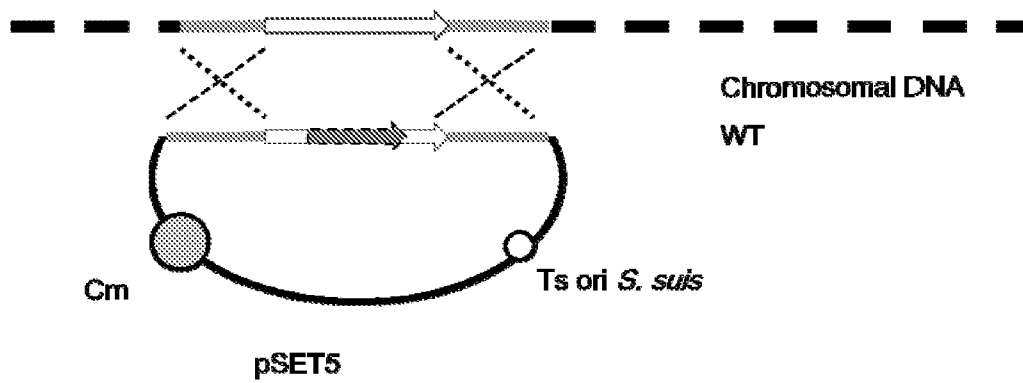
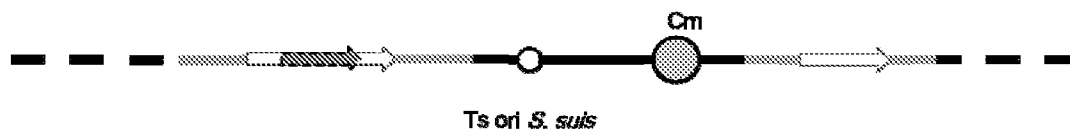


FIG. 9C

Single cross-over; SpcR/CmR



Double cross-over; SpcR/CmS



FIG. 10A

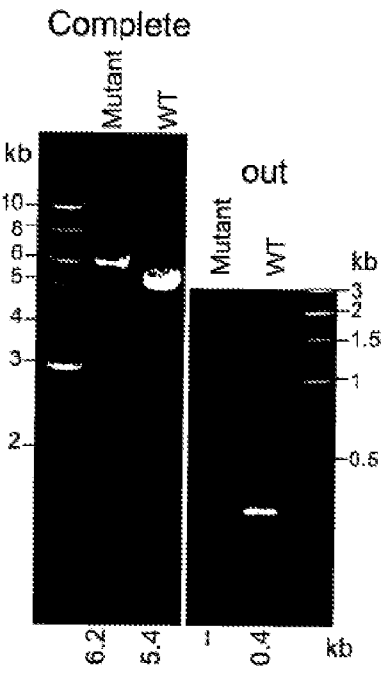


FIG. 10B

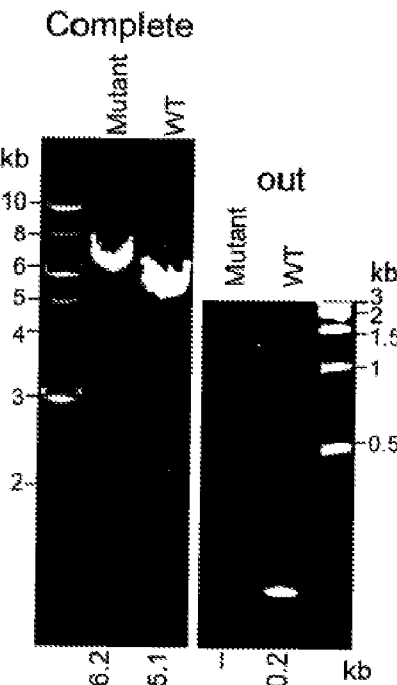
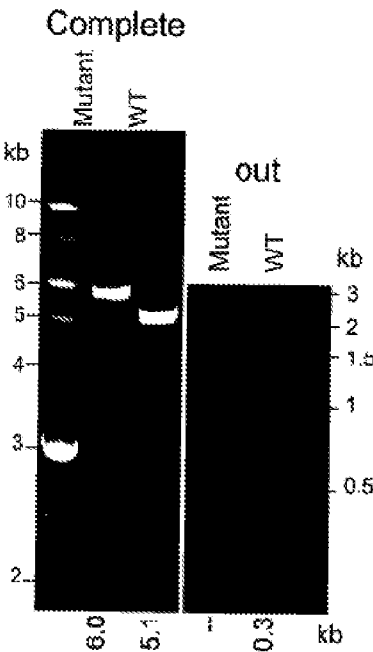


FIG. 10C



30/38

FIG. 11

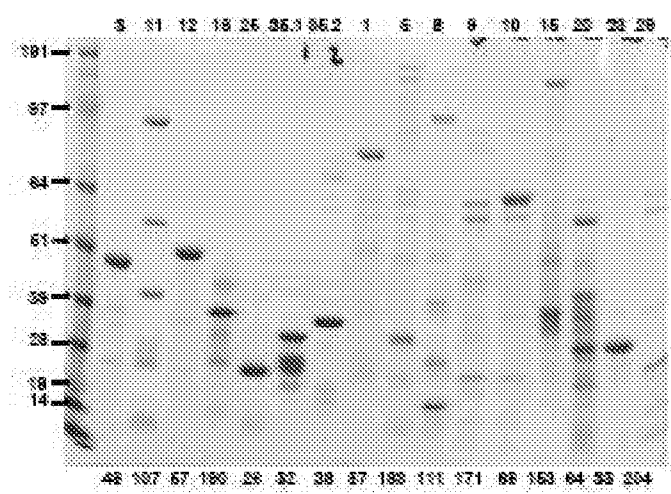


FIG. 12A

Titer 3.7 0

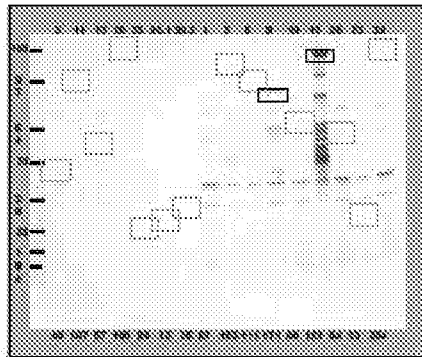
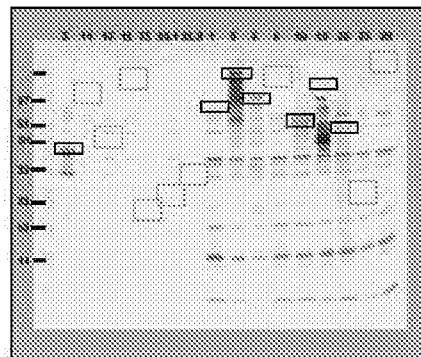
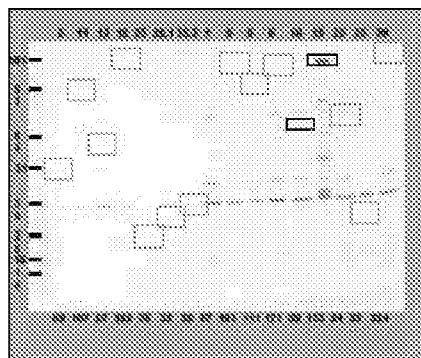


FIG. 12B

Titer 8.2 35



Titer 4.4 0 days



Titer 10.7 44 days

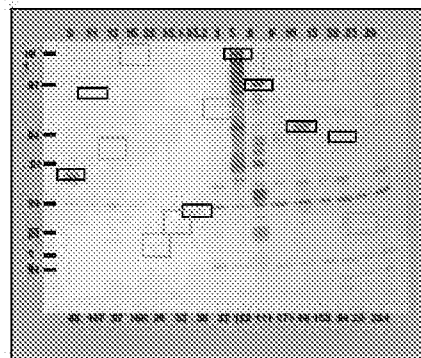


FIG. 12C

FIG. 12D

FIG. 13C

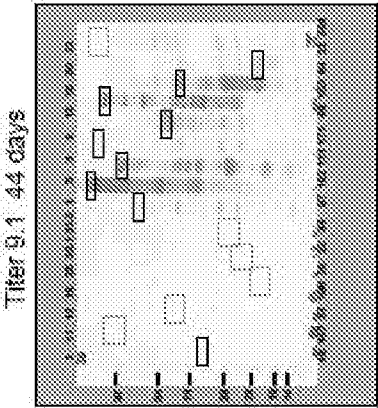


FIG. 13F

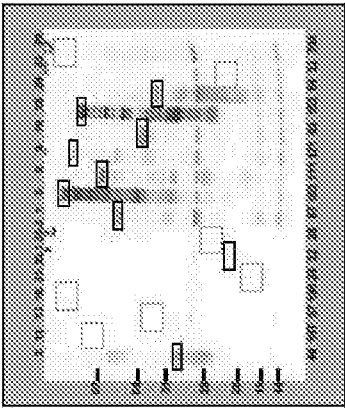


FIG. 13B

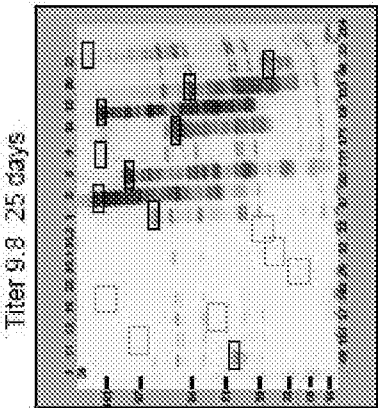


FIG. 13E

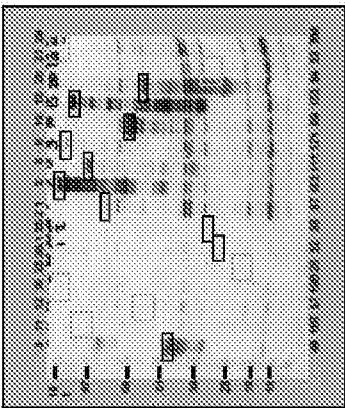


FIG. 13A

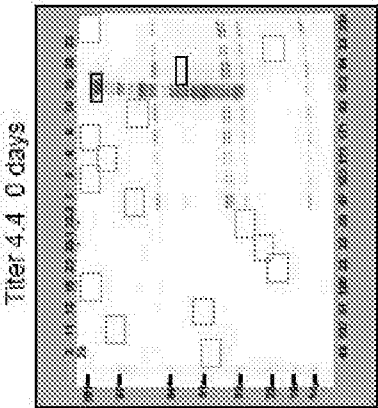


FIG. 13D

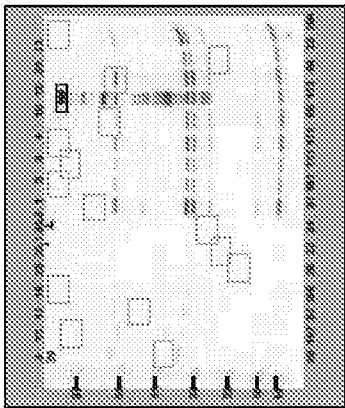


FIG. 13I

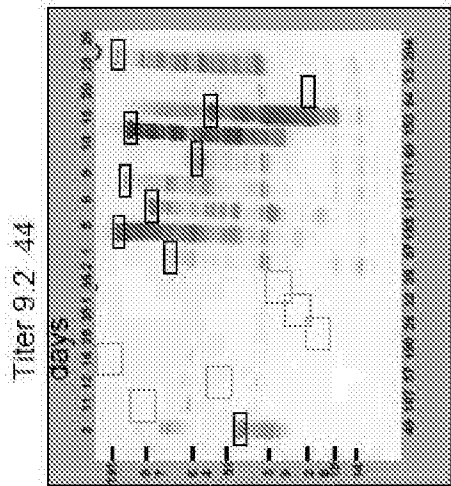


FIG. 13H

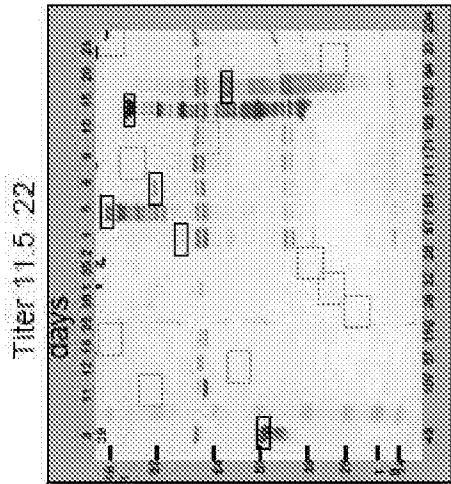


FIG. 13G

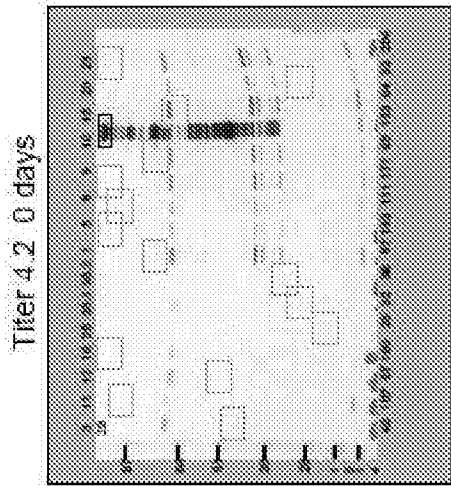


FIG. 14A

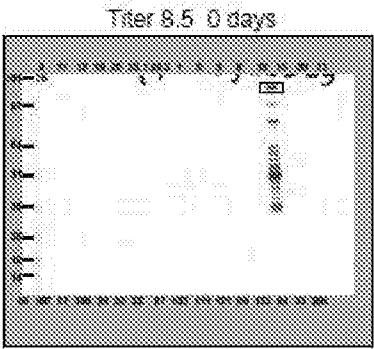


FIG. 14B

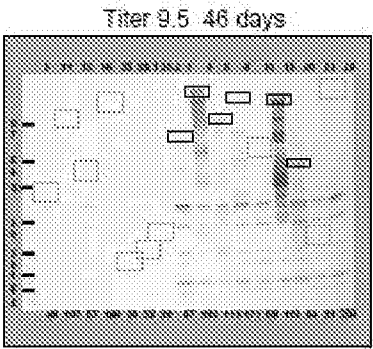


FIG. 14C

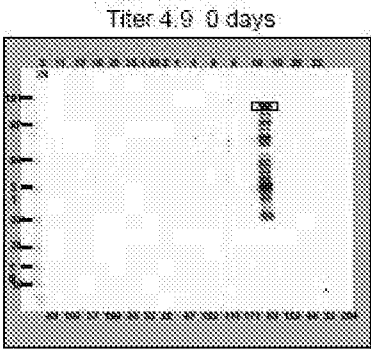


FIG. 14D

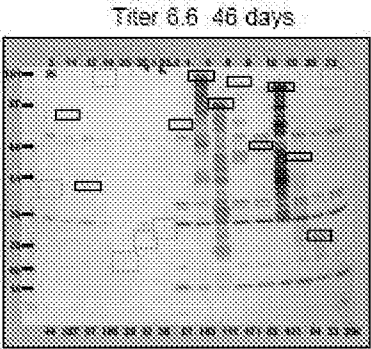


FIG. 14E

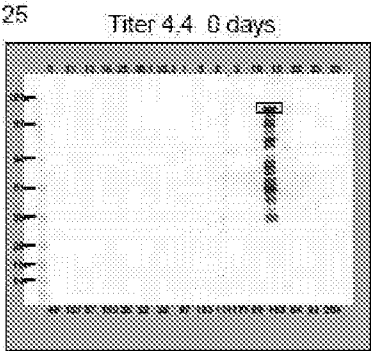
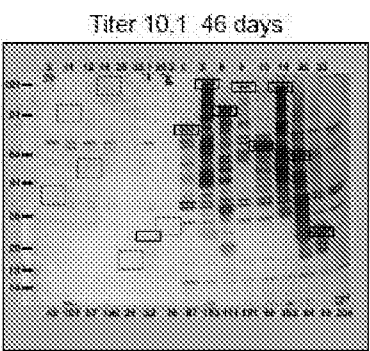


FIG. 14F



35/38

FIG. 15A

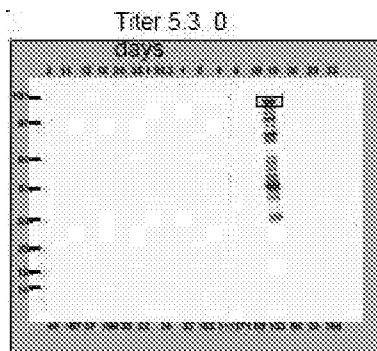
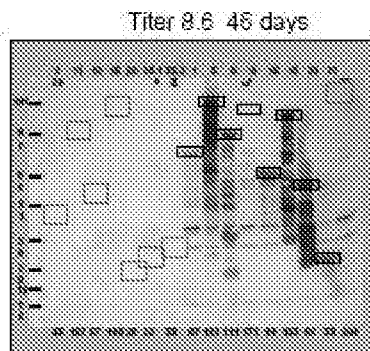
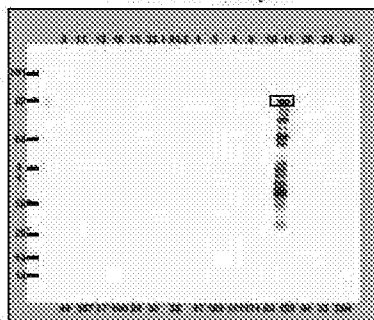


FIG. 15B



4

Titer 5.1 0 days



Titer 9.4 48 days

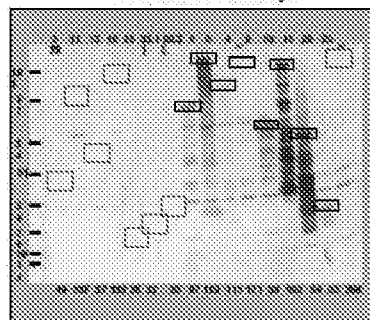


FIG. 15C

FIG. 15D

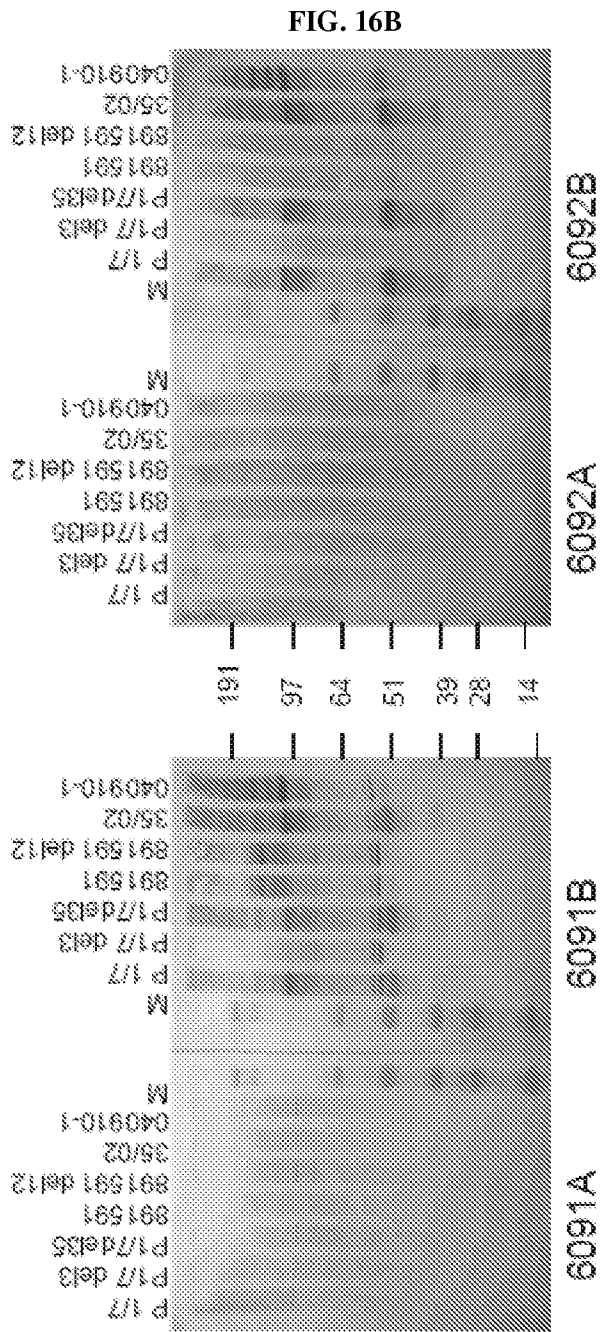


FIG. 16A

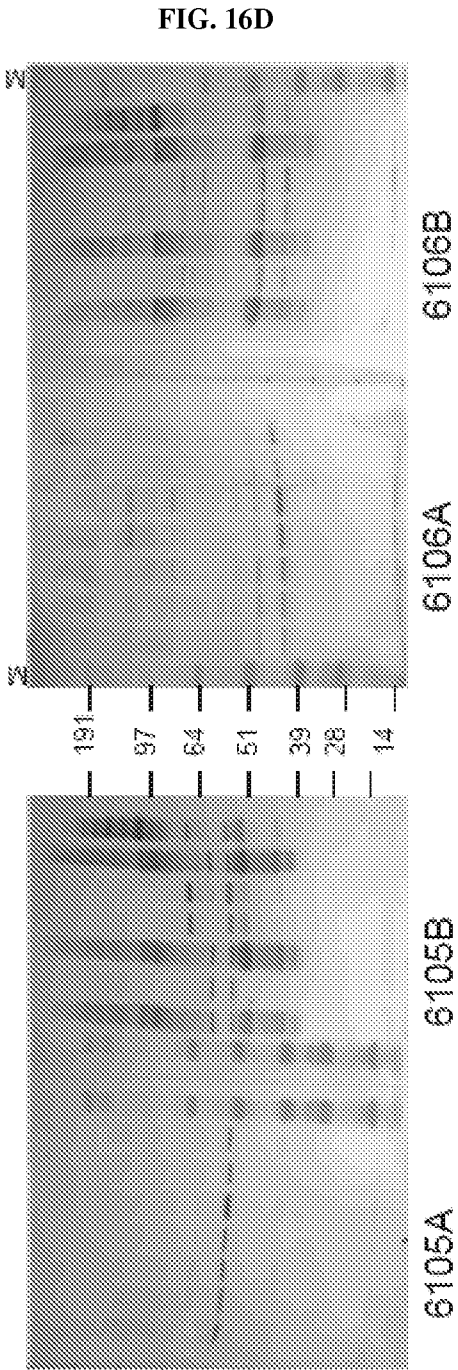


FIG. 16C

FIG. 17B

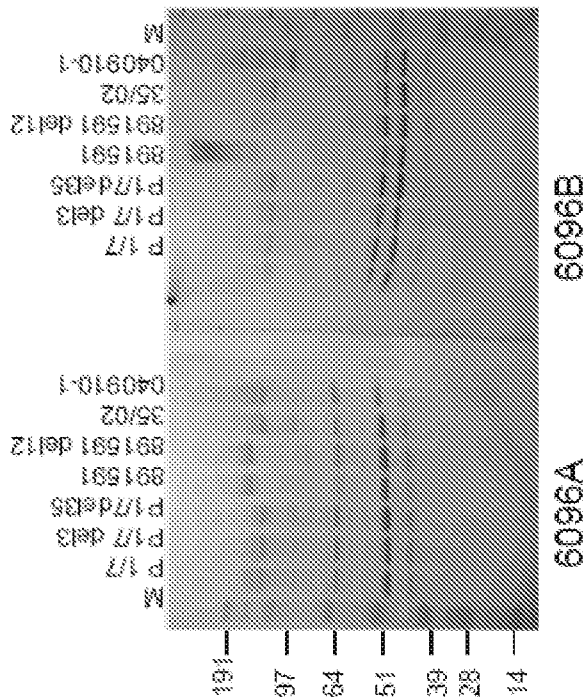


FIG. 17D

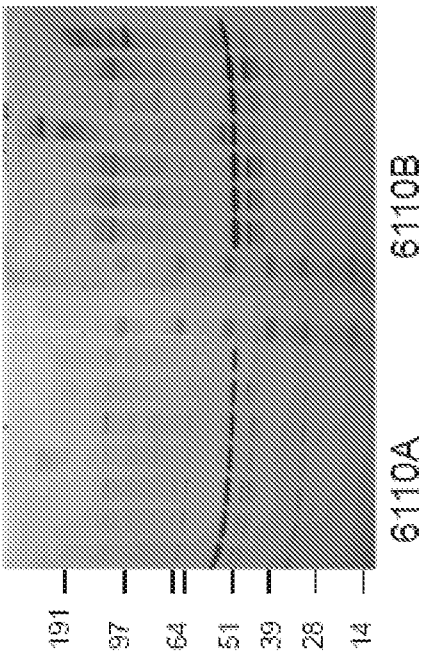


FIG. 17A

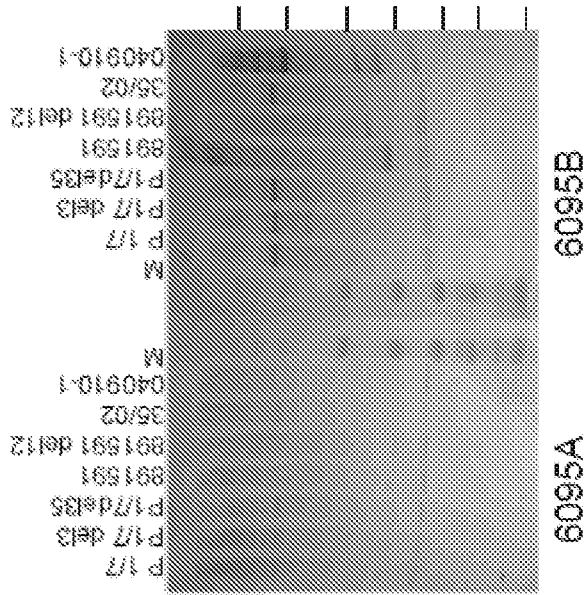


FIG. 17C

