



(86) Date de dépôt PCT/PCT Filing Date: 2007/01/18  
 (87) Date publication PCT/PCT Publication Date: 2007/02/14  
 (85) Entrée phase nationale/National Entry: 2008/07/17  
 (86) N° demande PCT/PCT Application No.: US 2007/060720  
 (87) N° publication PCT/PCT Publication No.: 2008/019162  
 (30) Priorités/Priorities: 2006/01/18 (US60/760,008);  
 2006/08/31 (US60/841,521)

(51) Cl.Int./Int.Cl. *A61K 39/00* (2006.01)  
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(54) Titre : COMPOSITIONS ET PROCEDES SE RAPPORTANT A DES PROTEINES DE BACTERIES  
 STAPHYLOCOCCIQUES  
 (54) Title: COMPOSITIONS AND METHODS RELATED TO STAPHYLOCOCCAL BACTERIUM PROTEINS

(57) **Abrégé/Abstract:**

The present invention concerns methods and compositions for treating or preventing a bacterial infection, particularly infection by a Staphylococcus bacteria. The invention provides methods and compositions for stimulating an immune response against the bacteria. In certain embodiments, the methods and compositions involve a secreted virulence factor, which may be EsxA and/or EsxB, and/or peptide processed by sortase, which may be SdrD, SdrE, IsdA, IsdB SdrC, Spa, IsdC, ClfA, ClfB, SasF, or combinations thereof.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
14 February 2008 (14.02.2008)

PCT

(10) International Publication Number  
**WO 2008/019162 A3**(51) International Patent Classification:  
A61K 39/00 (2006.01)(21) International Application Number:  
PCT/US2007/060720

(22) International Filing Date: 18 January 2007 (18.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/760,008 18 January 2006 (18.01.2006) US  
60/841,521 31 August 2006 (31.08.2006) US(71) Applicant (for all designated States except US): UNIVER-  
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78701 (US).(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS,  
JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS,  
LT, LU, LV, LY, MA, MD, ME, MG, MK, MN, MW, MX,  
MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO,  
RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,  
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,  
GN, GQ, GW, ML, MR, NE, SN, TD, TG).**Published:**

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments
- with sequence listing part of description published sepa-  
rately in electronic form and available upon request from  
the International Bureau

(88) Date of publication of the international search report:  
24 July 2008(15) Information about Correction:  
**Previous Correction:**  
see Notice of 17 April 2008

(54) Title: COMPOSITIONS AND METHODS RELATED TO STAPHYLOCOCCAL BACTERIUM PROTEINS

(57) Abstract: The present invention concerns methods and compositions for treating or preventing a bacterial infection, particu-  
larly infection by a Staphylococcus bacteria. The invention provides methods and compositions for stimulating an immune response  
against the bacteria. In certain embodiments, the methods and compositions involve a secreted virulence factor, which may be EsxA  
and/or EsxB, and/or peptide processed by sortase, which may be SdrD, SdrE, IsdA, IsdB SdrC, Spa, IsdC, CifA, CifB, SasF, or  
combinations thereof.

WO 2008/019162 A3

## DESCRIPTION

### COMPOSITIONS AND METHODS RELATED TO STAPHYLOCOCCAL BACTERIUM PROTEINS

This application claims priority to U.S. Provisional Patent applications serial  
5 number 60/760,008 filed January 18, 2006 and serial number 60/841,521 filed August  
31, 2006, each of which is incorporated herein by reference in its entirety.

This invention was made with government support under grant number R01  
AI38897 awarded by the National Institutes of Health (NIH). The government has  
certain rights in the invention.

10

## BACKGROUND OF THE INVENTION

### I. FIELD OF THE INVENTION

The present invention relates generally to the fields of immunology,  
microbiology, and pathology. More particularly, it concerns methods and  
compositions involving extracellular bacterial proteins, which can be used to invoke  
15 an immune response against the bacteria. Extracellular proteins include proteins of  
the Ess pathway and/or peptides or proteins processed by the sortase pathway,  
including proteins or polypeptides of Staphylococcal and other gram-positive bacteria.

### II. BACKGROUND

*Staphylococcus aureus* and many other Gram-positive bacteria are known to  
20 secrete virulence factors or exotoxins into the extracellular milieu. These factors are  
secreted via the Sec translocon across the membrane by a mechanism that requires N-  
terminal signal peptides for recognition. Recent advances in *Streptococcus pyogenes*  
and *Bacillus subtilis* revealed that the translocon may be part of a larger complex  
(ExPortal), that organizes transport of signal peptide-bearing precursor proteins to  
25 dedicated sites within the bacterial cell envelope (Rosch and Caparon, 2004; Campo  
*et al.*, 2004). Sec-independent translocation of virulence factors has been observed in  
group A streptococci and other Gram-positive species (Chaatwal, 2002). The  
mechanism of substrate recognition and transport for these “signal peptide less”  
polypeptides has thus far not been revealed (Pancholi and Fischetti, 1992). In group  
30 A streptococci, secretion across the plasma membrane and injection of polypeptides

into the cytosol of host cells uses a cytolysin-mediated transport mechanism (Madden *et al.*, 2001), that functionally resembles type III secretion of Gram-negative bacteria (Galan and Collmer, 1999).

*Mycobacterium tuberculosis* secretes ESAT-6, a virulence factor that triggers  
5 cell-mediated immune responses and interferon- $\gamma$  production during tuberculosis. ESAT-6 and its relative, CFP-10 encoded by genes *esxA* (Rv3875) and *esxB* (Rv3874), respectively, are transported across the membrane of mycobacteria by a specialized secretion system that requires multiple membrane proteins. Some of these proteins carry one or more FtsK-SpoIIIE-like domain(s) (FSD), suggesting that ATP  
10 hydrolysis may be required for ESAT-6 and CFP-10 transport. Although ESAT-6 secretion system (Ess) genes have been identified in the sequenced genomes of other microbes, the possibility that this pathway represents a general virulence strategy has hitherto not been addressed.

Mycobacteria are equipped with the typical Sec machinery. However, much  
15 attention has been drawn to the secretion of two virulence factors, ESAT-6 (early secreted antigen target 6 kDa) and CFP-10 (culture filtrate antigen 10 kDa), encoded by *Mycobacterium tuberculosis* H37Rv *esxA* (Rv3875) and *esxB* (Rv3874). *M. tuberculosis* variants lacking *esxA* display defects in the establishment of tuberculosis and EsxA induces a strong T-cell response during infection (Sorenson *et al.*, 1995).  
20 Comparative genomics of mycobacteria related to *M. tuberculosis* H37Rv identified multiple regions of difference that have been deleted from both virulent and avirulent species (Mahairas *et al.*, 1996; Cole *et al.*, 1998; Pym *et al.*, 2002). *esxA* (Rv3875) is located within region of difference 1 (RD1). RD1 is the only locus specifically deleted in the attenuated *Mycobacterium bovis* bacillus Calmette–Gue´rin (bacille  
25 Calmette-Gue´rin) vaccine strain and avirulent *Mycobacterium microi* strain (Mahairas *et al.*, 1996; Pym *et al.*, 2002; Calmette, 1927; Harboe *et al.* 1996; Philipp *et al.*, 2003). Although RD1 is certainly not the only virulence trait of mycobacteria, it appears to be a prominent factor in attenuating vaccine strains (Pym *et al.*, 2002; Pym *et al.*, 2003; Hsu *et al.*, 2003).

30 A survey of *M. tuberculosis* H37Rv genome sequence revealed at least 23 ESAT-6 homologues (Cole *et al.*, 1998), 10 of which are encoded within five gene clusters that specify large soluble and membrane-bound ATPases with two or more

FtsK/SpoIIIE-like domains (FSDs) (Gey Van Pittius *et al.*, 2001; Pallen *et al.*, 2002). Pallen *et al.* (2002) discovered ESAT-6 homologues in the sequenced genomes of other Gram-positive bacteria including *Bacillus subtilis*, *Bacillus anthracis*, *Clostridium acetobutylicum*, *Listeria monocytogenes*, and *S. aureus*. The genes for  
5 the ESAT-6-like proteins are clustered with at least one gene encoding an FSD-type membrane protein, leading Pallen to propose that FSD ATPases may represent a universal portal for excretion of ESAT-6-like proteins (Pallen *et al.*, 2002).

Support for this hypothesis was garnered when deletions of FSD-like genes (Rv3870, Rv3871) prevented the secretion of *M. tuberculosis* ESAT-6 and CFP-10  
10 (Pym *et al.*, 2003; Hsu *et al.*, 2003; Stanley *et al.*, 2003; Guinn *et al.*, 2004). A third membrane protein encoded by Rv3877/snm4 was found to be required for secretion of ESAT-6 and CFP-10 (Stanley *et al.*, 2003). Therefore, in mycobacteria secretion of ESAT-6 and CFP-10 requires a specialized mechanism encoded by genes flanking *esxA* and *esxB* on the chromosome. This specialized secretion system has been  
15 referred to as Snm for secretion in mycobacteria (Stanley *et al.*, 2003). Most importantly, this dedicated secretion pathway may explain the absence of processing of ESAT-6 or CFP-10 during secretion (Sorenson *et al.*, 1995). Although it has been surmised that FSD proteins may represent a general Gram-positive secretion system (Pallen *et al.*, 2002), this hypothesis has only recently received experimental  
20 verification (Burts *et al.*, 2005).

Moreover, pathogenic bacteria continue to pose a global health problem. Few vaccines and other preventative measures have been developed to combat bacterial infections. The *Staphylococcus aureus* pathogen is the most common food-borne infection and it is responsible for causing a number of other infections, including  
25 infections of open wounds and toxic shock syndrome. There is a continued need for therapeutic and preventative agents for infection by staphylococci, as well as other bacterial pathogens.

### **SUMMARY OF THE INVENTION**

Therefore, the present invention provides methods and compositions that can  
30 be used to treat or prevent bacterial pathogen infection. The invention is based on data that an immune response can be generated against one or more extracellular

proteins in bacteria, which includes secreted virulence factors and cell surface proteins.

In some cases, methods for stimulating an immune response involve administering to the subject an effective amount of a composition including an  
5 extracellular protein, which include i) secreted virulence factor, and/or a cell surface protein or peptide, or ii) a recombinant nucleic acid molecule encoding a secreted virulence factor, and/or a cell surface protein or peptide. Compositions of the present invention include immunogenic compositions wherein the antigen(s) or epitope(s) are contained in an amount effective to achieve the intended purpose. More specifically,  
10 an effective amount means an amount of active ingredients effective to stimulate or elicit an immune response, or provide resistance to or amelioration of infection. In more specific aspects, an effective amount prevents, alleviates or ameliorates symptoms of disease or infection, or prolongs the survival of the subject being treated. Determination of an effective amount is well within the capability of those skilled in  
15 the art, especially in light of the detailed disclosure provided herein. For any preparation used in the methods of the invention, an effective amount or dose can be estimated initially from *in vitro*, cell culture, and/or animal model assays. For example, a dose can be formulated in animal models to achieve a desired immune response or circulating antibody concentration or titer. Such information can be used  
20 to more accurately determine useful doses in humans.

In certain embodiments a secreted virulence factor is an Esx protein, for instance, all or part of an EsxA or EsxB protein. In certain aspects, a cell surface protein is a sortase substrate, including, but not limited to an isolated SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, SasF or combinations thereof. In certain  
25 embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more of EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, or SasF can be specifically excluded from a formulation of the invention. In certain aspects, the composition is not a Staphylococci bacterium or does not contain Staphylococci bacteria. In a particular embodiment a composition comprises either an isolated EsxA or EsxB protein, or  
30 both an isolated EsxA and an isolated EsxB proteins. In still further aspects, the isolated EsxA and EsxB proteins are multimerized and preferably dimerized. In certain aspects of the invention, a composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or

more isolated cell surface proteins or segments thereof. In a further aspect the cell surface protein is a sortase substrate, including, but not limited to SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, or SasF. In certain embodiments, methods and compositions of the invention will include a second, third, fourth, fifth, sixth or more  
5 sortase substrate. Alternatively, the composition may be or include a recombinantly engineered Staphylococcus bacterium that has been altered in a way that comprises specifically altering the bacterium with respect to the relevant secreted virulence factor or cell surface protein. For example, the bacteria may be recombinantly modified to express more of the relevant virulence factor or cell surface protein than it  
10 would express if unmodified.

The term "isolated" can refer to a nucleic acid or polypeptide that is substantially free of cellular material, bacterial material, viral material, or culture medium (when produced by recombinant DNA techniques) of their source of origin, or chemical precursors or other chemicals (when chemically synthesized). Moreover,  
15 an isolated compound refers to one that can be administered to a subject as an isolated compound; in other words, the compound may not simply be considered "isolated" if it is adhered to a column or embedded in an agarose gel. Moreover, an "isolated nucleic acid fragment" or "isolated peptide" is a nucleic acid or protein fragment that is not naturally occurring as a fragment and/or is not typically in the functional state.  
20 Moieties of the invention, such as antigens or immunogens, may be conjugated or linked covalently or noncovalently to other moieties such as adjuvants, proteins, peptides, supports, fluorescence moieties, or labels. The term "conjugate" or "immunoconjugate" is broadly used to define the operative association of one moiety with another agent and is not intended to refer solely to any type of operative  
25 association, and is particularly not limited to chemical "conjugation." Recombinant fusion proteins are particularly contemplated. A nucleic acid or polypeptide composition can be at least of a purity of 60, 65, 70, 75, 80, 85, 90, 95, 98, or 100% based on the amount other contaminating substances.

The term EsxA protein refers to a protein that includes isolated wild-type  
30 EsxA polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria EsxA proteins in nature. Similarly, the term EsxB protein refers to a protein that includes isolated wild-type EsxB

polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria EsxB proteins in nature. Further, the term SdrD protein refers to a protein that includes isolated wild-type SdrD polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria SdrD proteins in nature. Still further, the term SdrE protein refers to a protein that includes isolated wild-type SdrE polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria SdrE proteins in nature. Yet still further, the term IsdA protein refers to a protein that includes isolated wild-type IsdA polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria IsdA proteins in nature. The term IsdB protein refers to a protein that includes isolated wild-type IsdB polypeptides from staphylococcus bacteria, as well as variants that stimulate an immune response against staphylococcus bacteria IsdB proteins in nature. An immune response refers to a humoral response, a cellular response, or both a humoral and cellular response in an organism. An immune response can be measured by assays that include, but are not limited to, assays measuring the presence or amount of antibodies that specifically recognize an Esx protein or cell surface protein, assays measuring T-cell activation or proliferation, and/or assays that measure modulation in terms of activity or expression of one or more cytokines.

Compositions of the invention may further comprise an adjuvant. An adjuvant may be covalently or non-covalently coupled to a polypeptide or peptide of the invention. In certain aspects, the adjuvant is chemically conjugated to a protein, polypeptide, or peptide.

Embodiments of the present invention include methods for eliciting an immune response against a staphylococcus bacterium or staphylococci in a subject comprising providing to the subject an effective amount of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more secreted virulence factors and/or cell surface proteins. A secreted virulence factor includes, but is not limited to an EsxA or EsxB protein. A cell surface protein includes sortase substrates, which include, but are not limited to SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and SasF. The term "providing" is used according to its ordinary meaning to indicate "to supply or furnish for use." In some



embodiments, the protein is provided directly by administering the protein, while in other embodiments, the protein is effectively provided by administering a nucleic acid that encodes the protein. In certain aspects the invention contemplates compositions comprising various combinations of antigens, peptides, and/or epitope, for example  
5 compositions can comprise

EsxA, EsxB, or both EsxA and EsxB in combination with, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, or SasF; IsdA, IsdB, Spa, ClfA, ClfB, IsdC, or SasF; IsdB, Spa, ClfA, ClfB, IsdC, or SasF; Spa, ClfA, ClfB, IsdC, or SasF; ClfA, ClfB, IsdC, or SasF; ClfB, IsdC, or SasF; IsdC, or SasF; SasF; SdrC, SdrD, SdrE, IsdA, IsdB, Spa,  
10 ClfA, ClfB, or IsdC; SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, or ClfB; SdrC, SdrD, SdrE, IsdA, IsdB, Spa, or ClfA; SdrC, SdrD, SdrE, IsdA, or IsdB; SdrC, SdrD, SdrE, or IsdA; SdrC, SdrD, or SdrE; SdrC, or SdrD; or SdrC.

In certain aspects, a composition can comprise SdrE and IsdA, IsdB, Spa, ClfA, ClfB, IsdC, and SasF; IsdB, Spa, ClfA, ClfB, IsdC, and SasF; Spa, ClfA, ClfB,  
15 IsdC, and SasF; ClfA, ClfB, IsdC and SasF; ClfB, IsdC, and SasF; IsdC and SasF; SasF; IsdA, IsdB, Spa, ClfA, ClfB, and IsdC; IsdA, IsdB, Spa, ClfA, and ClfB; IsdA, IsdB, Spa, and ClfA; IsdA, IsdB, and Spa; IsdA and IsdB; IsdA; IsdB; Spa; ClfA; ClfB; IsdC; and so forth.

In certain aspects, a composition can comprise IsdA and SdrE, IsdB, Spa,  
20 ClfA, ClfB, IsdC, and SasF; IsdB, Spa, ClfA, ClfB, IsdC, and SasF; Spa, ClfA, ClfB, IsdC, and SasF; ClfA, ClfB, IsdC, and SasF; ClfB, IsdC, and SasF; IsdC and SasF; SasF; SdrE, IsdB, Spa, ClfA, ClfB, and IsdC; SdrE, IsdB, Spa, ClfA, , and ClfB; SdrE, IsdB, Spa, , and ClfA; SdrE, IsdB, and Spa; SdrE and IsdB; SdrE; IsdB; Spa; ClfA; ClfB; IsdC; and so forth.

In certain aspects, a composition can comprise IsdB and SdrE, IsdA, Spa,  
25 ClfA, ClfB, IsdC, and SasF; IsdA, Spa, ClfA, ClfB, IsdC, and SasF; Spa, ClfA, ClfB, IsdC, and SasF; ClfA, ClfB, IsdC, and SasF; ClfB, IsdC, and SasF; IsdC and SasF; SasF; SdrE, IsdA, Spa, ClfA, ClfB, and IsdC; SdrE, IsdA, Spa, ClfA, and ClfB; SdrE, IsdA, Spa, and ClfA; SdrE, IsdA, and Spa; SdrE, and IsdA; SdrE; IsdA; Spa; ClfA;  
30 ClfB; IsdC; and so forth.

In certain aspects, a composition can comprise Spa and SdrE, IsdA, IsdB, ClfA, ClfB, IsdC, and SasF; IsdA, IsdB, ClfA, ClfB, IsdC, and SasF; IsdB, ClfA, ClfB, IsdC, and SasF; ClfA, ClfB, IsdC, and SasF; ClfB, IsdC, and SasF; IsdC and SasF; SasF; SdrE, IsdA, IsdB, ClfA, ClfB, and IsdC; SdrE, IsdA, IsdB, ClfA, and ClfB; SdrE, IsdA, IsdB, and ClfA; SdrE, IsdA, and IsdB; SdrE and IsdA; SdrE; IsdA; IsdB; ClfA; ClfB; IsdC; and so forth.

In certain aspects, a composition can comprise ClfA and SdrE, IsdA, IsdB, Spa, ClfB, IsdC, and SasF; IsdA, IsdB, Spa, ClfB, IsdC, and SasF; IsdB, Spa, ClfB, IsdC, and SasF; Spa, ClfB, IsdC, and SasF; ClfB, IsdC, and SasF; IsdC and SasF; SasF; SdrE, IsdA, IsdB, Spa, ClfB, and IsdC; SdrE, IsdA, IsdB, Spa, and ClfB; SdrE, IsdA, IsdB, and Spa; SdrE, IsdA, and IsdB; SdrE and IsdA; SdrE; IsdA; IsdB; Spa; ClfB; IsdC; and so forth.

In certain aspects, a composition can comprise ClfB and SdrE, IsdA, IsdB, Spa, ClfA, IsdC, and SasF; IsdA, IsdB, Spa, ClfA, IsdC, and SasF; IsdB, Spa, ClfA, IsdC, and SasF; Spa, ClfA, IsdC, and SasF; ClfA, IsdC, and SasF; IsdC and SasF; SasF; SdrE, IsdA, IsdB, Spa, ClfA, and IsdC; SdrE, IsdA, IsdB, Spa, and ClfA; SdrE, IsdA, IsdB, and Spa; SdrE, IsdA, and IsdB; SdrE, and IsdA; SdrE; IsdA; IsdB; Spa; ClfA; IsdC; and so forth.

In certain aspects, a composition can comprise IsdC and SdrE, IsdA, IsdB, Spa, ClfA, ClfB, and SasF; IsdA, IsdB, Spa, ClfA, ClfB, and SasF; IsdB, Spa, ClfA, ClfB, and SasF; Spa, ClfA, ClfB, and SasF; ClfA, ClfB, and SasF; ClfB and SasF; SdrE, IsdA, IsdB, Spa, ClfA, and ClfB; SdrE, IsdA, IsdB, Spa, and ClfA; SdrE, IsdA, IsdB, and Spa; SdrE, IsdA, and IsdB; SdrE, and IsdA; SdrE; IsdA; IsdB; Spa; ClfA; ClfB; and so forth.

In certain aspects, a composition can comprise SasF and SdrE, IsdA, IsdB, Spa, ClfA, ClfB, and IsdC; IsdA, IsdB, Spa, ClfA, ClfB, and IsdC; IsdB, Spa, ClfA, ClfB, and IsdC; Spa, ClfA, ClfB, and IsdC; ClfA, ClfB, and IsdC; ClfB and IsdC; SdrE, IsdA, IsdB, Spa, ClfA, and ClfB; SdrE, IsdA, IsdB, Spa, and ClfA; SdrE, IsdA, IsdB, and Spa; SdrE, IsdA, and IsdB; SdrE, and IsdA; SdrE; IsdA; IsdB; Spa; ClfA; ClfB; IsdC and so forth.

Embodiments of the invention include compositions that may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to a secreted virulence protein or a surface protein. In a further embodiment of the invention a composition may include  
5 a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an EsxA protein, preferably the EsxA protein will have an amino acid sequence of SEQ ID NO:2. Similarity or identity, with identity being preferred, is known in the art, a number of different programs can be used to identify whether a protein (or nucleic acid) has sequence  
10 identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman (1981), by the sequence identity alignment algorithm of Needleman & Wunsch (1970), by the search for similarity method of Pearson & Lipman (1988), by computerized implementations of  
15 these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux *et al.* (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by using alignment tools known to and readily ascertainable to those of skill in the art.

20 In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an EsxB protein. In certain aspects the EsxB protein will have the amino acid sequence of SEQ ID NO:4.

In yet still further embodiments of the invention a composition may include a  
25 polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an SdrD protein. In certain aspects the SdrD protein will have the amino acid sequence of SEQ ID NO:6.

In further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%,  
30 96%, 97%, 98%, or 99% identity or similarity to an SdrE protein. In certain aspects the SdrE protein will have the amino acid sequence of SEQ ID NO:8.

In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an IsdA protein. In certain aspects the IsdA protein will have the amino acid sequence of SEQ ID NO:10.

5 In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an IsdB protein. In certain aspects the IsdB protein will have the amino acid sequence of SEQ ID NO:12.

10 Embodiments of the invention include compositions that include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to a Spa protein. In certain aspects the Spa protein will have the amino acid sequence of SEQ ID NO:14.

15 In a further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to a ClfB protein. In certain aspects the ClfB protein will have the amino acid sequence of SEQ ID NO:16.

20 In still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an IsdC protein. In certain aspects the IsdC protein will have the amino acid sequence of SEQ ID NO:18.

In yet further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to a SasF protein. In certain aspects the SasF protein will have the amino acid sequence of SEQ ID NO:20.

25 In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an SdrC protein. In certain aspects the SdrC protein will have the amino acid sequence of SEQ ID NO:22.

In yet still further embodiments of the invention a composition may include a polypeptide, peptide, or protein that is or is at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity or similarity to an ClfA protein. In certain aspects the ClfA protein will have the amino acid sequence of SEQ ID NO: 24.

5 In the context of a polypeptide or protein, the term "identity" refers to a polypeptide having a sequence that is at least 85%, preferably at least 90%, more preferably at least 95%, and most preferably at least 98% or 99% or more identical to the amino acid sequence of the reference polypeptide. The term "similarity" refers to  
10 either identical with the reference polypeptide or constitute conservative substitutions with the reference polypeptides.

The EsxA protein, EsxB protein, or other protein transported by the Ess pathway, and/or SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, and SasF or other cell surface proteins may include the following, or at least or at most 3, 4, 5, 6,  
15 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116,  
20 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201,  
25 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250 contiguous amino acids, or any range derivable therein of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, and/or SEQ ID NO:24, respectively.  
30

The compositions may be formulated in a pharmaceutically acceptable composition. In certain aspects of the invention the staphylococcus bacterium is an *S. aureus* bacterium.

In further aspects of the invention a composition may be administered more than one time to the subject, and may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or more times. The administration of the compositions include, but is not limited to oral, parenteral, subcutaneous, intramuscular, intravenous administration, or various combinations thereof.

Embodiments of the invention include administering to the subject a composition comprising a non-EsxA or non-EsxB Ess protein. The Ess protein may be in the same composition as EsxA protein or EsxB protein, but need not be.

In still further embodiments, a composition comprises a recombinant nucleic acid molecule encoding an EsxA protein or a recombinant nucleic acid molecule encoding an EsxB protein, or both a nucleic acid molecule encoding an EsxA protein and a nucleic acid molecule encoding an EsxB protein. In certain aspects a nucleic acid molecule encodes both an EsxA protein and an EsxB protein. Typically a recombinant nucleic acid molecule encoding an EsxA or EsxB protein contains a heterologous promoter. In certain aspects, a recombinant nucleic acid molecule of the invention is a vector, in still other aspects the vector is a plasmid. In certain embodiments the vector is a viral vector. Aspects of the invention include compositions that further comprise a nucleic acid encoding another Ess protein. In certain aspects a composition includes a recombinant, non-staphylococcus bacterium containing the EsxA or EsxB protein. In particular aspects the recombinant non-staphylococcus bacteria is *Salmonella* or another gram-positive bacteria. A composition is typically administered to human subjects, but administration to other animals that are capable of eliciting an immune response is contemplated. In further aspects the staphylococcus bacterium is a *Staphylococcus aureus*. In further embodiments the immune response is a protective immune response.

In further embodiments a composition comprises a recombinant nucleic acid molecule encoding a cell surface protein such as an SdrD protein, a recombinant nucleic acid molecule encoding an SdrE protein, a recombinant nucleic acid molecule

encoding an IsdA protein, and/or a recombinant nucleic acid molecule encoding an IsdB protein,. Alternatively, a composition may include nucleic acid molecules encoding two or more cell surface proteins. Cell surface proteins include, but are not limited to a SdrD, SdrE, IsdA, or IsdB protein. In certain aspects a nucleic acid molecule encodes two or more cell surface proteins, such as SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF protein. Typically a recombinant nucleic acid molecule contains a heterologous promoter. In certain aspects, a recombinant nucleic acid molecule of the invention is a vector, in still other aspects the vector is a plasmid. In certain embodiments the vector is a viral vector. Aspects of the invention include compositions that further comprise a nucleic acid encoding another sortase substrate protein. In certain aspects a composition includes a recombinant, non-staphylococcus bacterium containing the SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF protein. In particular aspects the recombinant non-staphylococcus bacteria is Salmonella or another gram-positive bacteria. A composition is typically administered to human subjects, but administration to other animals that are capable of eliciting an immune response is contemplated, particularly cattle, horses, goats, sheep and other domestic animals. In further aspects the staphylococcus bacterium is a *Staphylococcus aureus*. In further embodiments the immune response is a protective immune response.

In still further aspects, the methods and compositions of the invention can be used to prevent, ameliorate, reduce, or treat infection of glands, *e.g.*, mammary glands, particularly mastitis and other infections. Other methods include, but are not limited to prophylactically reducing bacterial burden in a subject not exhibiting signs of infection, particularly those subjects suspected of or at risk of being colonized by a target bacteria, *e.g.*, patients that are or will be at risk or susceptible to infection during a hospital stay, treatment, and/or recovery.

Still further embodiments include methods for stimulating in a subject a protective or therapeutic immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition including (i) an EsxA and/or EsxB protein or peptide thereof; or, (ii) a nucleic acid molecule encoding an EsxA and/or EsxB protein or peptide thereof, or (iii) any combination or permutation of bacterial proteins described herein. In a preferred

embodiment the composition is not a staphylococcus bacteria. In certain aspects the subject is a human or a cow. In a further aspect the composition is formulated in a pharmaceutically acceptable formulation. The staphylococci may be *Staphylococcus aureus*.

5 Yet still further embodiments include vaccines comprising a pharmaceutically acceptable composition having an isolated EsxA and/or EsxB, or any other combination or permutation of protein(s) or peptide(s) described, wherein the composition is capable of stimulating an immune response against a staphylococcus bacterium. The vaccine may comprise an isolated EsxA and an isolated EsxB, or any  
10 other combination or permutation of protein(s) or peptide(s) described. In certain aspects of the invention the isolated EsxA and isolated EsxB, or any other combination or permutation of protein(s) or peptide(s) described are multimerized, *e.g.*, dimerized. In a further aspect, the vaccine composition is contaminated by less than about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, 0.25, 0.05% (or any range derivable therein)  
15 of other Staphylococcal proteins. A composition may further comprise an isolated non-EsxA or non-EsxB Ess protein. Typically the vaccine comprises an adjuvant. In certain aspects a protein or peptide of the invention is linked (covalently or non-covalently coupled) to the adjuvant, preferably the adjuvant is chemically conjugated to the protein.

20 In still yet further embodiments, a vaccine composition is a pharmaceutically acceptable composition having a recombinant nucleic acid encoding all or part of an EsxA or EsxB protein, or any other combination or permutation of protein(s) or peptide(s) described, wherein the composition is capable of stimulating an immune response against a staphylococcus bacteria. The vaccine composition may comprise a  
25 recombinant nucleic acid encoding all or part of an EsxA protein, all or part of an EsxB protein, or all or part of both an EsxA protein and EsxB protein, or any other combination or permutation of protein(s) or peptide(s) described. A recombinant nucleic acid of the invention may encode all or part of both EsxA and EsxB proteins in the same or a separate nucleic acid. In certain embodiments the recombinant  
30 nucleic acid contains a heterologous promoter. Preferably the recombinant nucleic acid is a vector. More preferably the vector is a plasmid or a viral vector. A vaccine may also comprise a nucleic acid encoding another member of the Ess proteins. In



some aspects the vaccine includes a recombinant, non-staphylococcus bacterium containing the nucleic acid. The recombinant non-staphylococci may be Salmonella or another gram-positive bacteria. The vaccine may comprise an adjuvant.

5 Still further embodiments include methods for stimulating in a subject a protective or therapeutic immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition including i) a SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF protein or peptide thereof; or, ii) a nucleic acid molecule encoding a SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF protein or peptide thereof. In a preferred embodiment the composition is not a staphylococcus bacteria. In certain aspects the subject is a human or a cow. In a further aspect the composition is formulated in a pharmaceutically acceptable formulation. The staphylococci may be *Staphylococcus aureus*. Methods of the invention also include compositions that contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more secreted virulence factors and/or cell surface proteins, such as EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF in various combinations. In certain aspects a vaccine formulation includes SdrD, SdrE, IsdA and IsdB; or SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, and SasF.

Yet still further embodiments include vaccines comprising a pharmaceutically acceptable composition having an isolated SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF protein or peptide thereof, wherein the composition is capable of stimulating an immune response against a staphylococcus bacterium. The vaccine may comprise two or more isolated SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF proteins or peptides thereof. In certain aspects of the invention the isolated SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF proteins are multimerized, e.g., dimerized. In a further aspect, the vaccine composition is contaminated by less than about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, 0.25, 0.05% (or any range derivable therein) of other Staphylococcal proteins. Typically the vaccine comprises an adjuvant. In certain aspects a protein or peptide of the invention is linked (covalently or non-covalently coupled) to the adjuvant, preferably the adjuvant is chemically conjugated to the protein. Vaccines of the invention may include 1, 2, 3, 4, 5, 6, or more secreted virulence factors and/or cell surface proteins, such as

EsxA, EsxB, SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF in various combinations.

In still yet further embodiments, a vaccine composition is a pharmaceutically acceptable composition having a recombinant nucleic acid encoding all or part of an SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC or SasF protein, wherein the composition is capable of stimulating an immune response against a staphylococcus bacteria. The vaccine composition may comprise a recombinant nucleic acid encoding all or part of a SdrD protein, all or part of an SdrE protein, all or part of an IsdA protein, all or part of an IsdB or all or part of two or more of SdrD, SdrE, IsdA, or IsdB protein. A recombinant nucleic acid of the invention may encode all or part of SdrD, SdrE, IsdA, IsdB, Spa, ClfB, IsdC and/or SasF proteins in the same or a separate nucleic acid. In certain embodiments the recombinant nucleic acid contains a heterologous promoter. Preferably the recombinant nucleic acid is a vector. More preferably the vector is a plasmid or a viral vector. A vaccine may also comprise a nucleic acid encoding another member of the sortase substrate proteins. In some aspects the vaccine includes a recombinant, non-staphylococcus bacterium containing the nucleic acid. The recombinant non-staphylococci may be Salmonella or another gram-positive bacteria. The vaccine may comprise an adjuvant. Aspects of the invention also include nucleic acids encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more surface proteins or secreted virulence factors. In certain aspects the proteins or peptides may be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more expression vectors (*e.g.*, one or more nucleic acid or plasmid) and/or delivery vectors (*e.g.*, one or more bacteria or virus).

Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well. In particular, any embodiment discussed in the context of an Esx protein or nucleic acid may be implemented with respect to other secreted virulence factors, and/or cell surface proteins, such as SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF proteins (or nucleic acids), and vice versa.

The embodiments in the Example section are understood to be embodiments of the invention that are applicable to all aspects of the invention.

The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.”

5 Throughout this application, the term “about” is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

Following long-standing patent law, the words “a” and “an,” when used in conjunction with the word “comprising” in the claims or specification, denotes one or  
10 more, unless specifically noted.

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 specific embodiments of the invention, are given by way of illustration only, since various  
15 changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

### **DESCRIPTION OF THE DRAWINGS**

So that the matter in which the above-recited features, advantages and objects of the invention as well as others which will become clear are attained and can be  
20 understood in detail, more particular descriptions and certain embodiments of the invention briefly summarized above are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate certain embodiments of the invention and therefore are not to be considered limiting in their scope.

25 **FIGs. 1A-1C.** *S. aureus* ess locus encoding ESAT-6-like proteins. (FIG. 1A) Protein sequence alignment of *S. aureus* (S.a.) EsxA and EsxB with *M. tuberculosis* (M.t.) EsxA. *M. tuberculosis* and *S. aureus* EsxA display 20.8% identity and 25% similarity, whereas *S. aureus* EsxB and *M. tuberculosis* EsxA are 17.8% identical and 35% similar. All three proteins contain the WXG motif. (FIG. 1B) Comparison of the  
30 *M. tuberculosis* H37Rv ESAT-6 locus with the *S. aureus*, *L. monocytogenes* ess loci

and the *B. subtilis* yuk locus. Genes and proteins indicate FSD factors, ESAT-6-like, mycobacterial genes, and staphylococcal (also in *Listeria* or bacilli). (FIG. 1C) Membrane topology or soluble character of proteins encoded by the *S. aureus* *ess* locus.

5           **FIGs. 2A-2B.** Subcellular location of EsxA and EsxB. (FIG. 2A) *S. aureus* strain Newman cultures were separated into medium (MD), total (T), and loosely wall associated (L) fractions. Proteins were precipitated with TCA, separated on SDS-PAGE, and detected by immunoblotting with specific antibodies [ $\alpha$ -EsxA,  $\alpha$ -EsxB,  $\alpha$ -Spa (protein A),  $\alpha$ -IsdG, and  $\alpha$ -IsdE]. (FIG. 2B) *S. aureus* strain Newman cultures  
10 were fractionated into medium (MD), cell wall (CW), cytoplasm (C), and membrane (M) compartments. TCA-precipitated proteins in each compartment were revealed by SDS-PAGE and immunoblotting.

**FIGs. 3A-3B.** Factors affecting the production and secretion of EsxA and EsxB. (FIG. 3A) Schematic drawing of the *Ess* cluster with the position of *bursa aurealis* insertions (filled triangles) and *esxA24* mutation (open triangles). (FIG. 3B)  
15 Medium (MD) and total protein fractions (T) were separated on SDS-15% PAGE and immunoblotted with  $\alpha$ -EsxA and  $\alpha$ -EsxB.  $\alpha$ -SrtA is used as a control for total protein loaded.

**FIGs. 4A-4B.** Role of *EssC* in EsxA and EsxB secretion. (FIG. 4A)  
20 Predicted topology of *EssC* with insertion sites of *bursa aurealis* in each strain (filled triangles). *EssC* contains two FstK-SpoIIIE-like domains (FSD). (FIG. 4B) Immunoblotting of total cellular protein (T) or culture medium(MD) with  $\alpha$ -EsxA and  $\alpha$ -EsxB antibodies. Insertional mutations at codon 264 (mutant 1) and codon 618 (mutant 2) prevented production/secretion of EsxA and EsxB. Mutants 1–4 are  
25 strains  $\Phi$ NE04464 (insertion at codon 264),  $\Phi$ NE02191 (insertion at codon 618),  $\Phi$ NE02832 (insertion at codon 721), and  $\Phi$ NE13038 (insertion at codon 1279), respectively.  $\alpha$ -SrtA was used as a control for the total protein loaded.

**FIG. 5.** *S. aureus* *esxA*, *esxB*, and *essC* mutants are defective in the pathogenesis of murine abscesses. Ten 6-week-old BALB-c mice were injected retro-orbitally with  $10^6$  cfu for each strain. Mice were killed 4 days after infection. Kidneys and liver were removed and homogenized. Viable bacteria were counted  
30

after dilution and colony formation on tryptic soy agar. Statistical significance was examined with the Student t test, and P values were recorded. The limit of detection (solid line) was determined to be 10 cfu ( $10^1$ ).

**FIG. 6.** Serum IgG titers of animals acutely infected with *S. aureus*. Three-week old BALB/c mice were infected by intravenous inoculation with  $1 \times 10^7$  cfu of *S. aureus* Newman or PBS buffer (mock control). Mice were bled on day 0 and 30 and various dilutions of serum samples were analyzed for EsxA and EsxB specific IgG by custom ELISA.

**FIG. 7.** Purified EsxA confers partial immunity to *S. aureus* infection in the mouse model of disease.

**FIG. 8.** Protection conferred by surface proteins of *S. aureus*, as assessed by the renal abscess model. BALB/c mice received two doses of 100  $\mu$ g protein or phosphate-buffered saline (PBS) adsorbed to CFA (day 0) or IFA (day 11). Mice were challenged with *S. aureus* strain Newman ( $\sim 10^6$  CFU) and 96 hours after infection the animals were killed and the kidneys excised, homogenized and plated. The dashed line indicates the limit of detection of staphylococci in renal tissues.

**FIGs. 9A-9D.** Immunization with the combined vaccine (IsdA, IsdB, SdrD and SdrE) generates protective immunity against *S. aureus* abscess formation in mice. BALB/c mice were mock immunized (FIG. 9A and FIG. 9B) or immunized with the combined vaccine (FIG. 9C and FIG. 9D) and challenged by retro-orbital infection with *S. aureus* Newman. Four days after infection, animals were killed and kidneys removed. Organ tissue was fixed in formalin, thin sectioned and stained with hematoxylin/eosin. Microscopic images of whole kidneys (FIG. 9A and FIG. 9C) or organ tissue at higher magnification (FIG. 9B and FIG. 9D) revealed abscess formation only in mock immunized animals. Similar results were obtained for six organ tissues in each group. Bars indicate 1 mm (FIG. 9A and FIG. 9C) or 0.1 mm (FIG. 9B and FIG. 9D). Arrows identify a staphylococcal abscess with a central concentration of staphylococci (FIG. 9B) as well as polymorphonuclear cell infiltrate (FIG. 9D).

**FIG. 10.** Immunization with the combined vaccine (IsdA, IsdB, SdrD and SdrE) generates protective immunity against lethal *S. aureus* challenge. Mice ( $n = 8$ -

10) were immunized with individual surface protein antigens (IsdA, IsdB, SdrD or SdrE), with the combined vaccine (IsdA, IsdB, SdrD and SdrE) or with PBS. Animals were challenged by intra-peritoneal injection of *S. aureus* Newman ( $2 \times 10^{10}$  CFU) and monitored over seven days. When compared to animals receiving mock immunization (PBS), the significance of protective immunity generated by various antigens was recorded with the Fisher's exact test as follows: IsdA (P= 0.34372), IsdB (P= 0.22049), SdrD (P= 0.24006), SdrE (P=0.31508) and Combined Vaccine (P= 0.02941). When compared to animals receiving the combined vaccine, significance of protective immunity was recorded with the Fisher's exact test as follows: PBS (P= 0.02941), IsdA (P= 0.02941), IsdB (P= 0.05294), SdrD (P= 0.00377) and SdrE (P= 0.01131).

**FIGs. 11A-11E.** Immunization with the combined vaccine generates protective immunity against lethal challenge with five different clinical *S. aureus* isolates. Mice (n = 10) were immunized with the combined vaccine (IsdA, IsdB, SdrD and SdrE) or with PBS, challenged by intra-peritoneal injection with clinical *S. aureus* isolates ( $3-10 \times 10^9$  CFU) and monitored over seven days for survival. When compared to animals receiving mock immunization (PBS), the significance of protective immunity generated by the combined vaccine was recorded with the Fisher's exact test as follows: FIG. 11A, N315 (P= 0.06502); FIG. 11B, NRS248 (P= 0.00036); FIG. 11C, NRS252 (P= 0.03215); FIG. 11D, USA100 (P= 0.00542); and FIG. 11E, USA400 (P= 0.00542).

**FIG. 12.** Antibodies against IsdA, IsdB, SdrD, and SdrE mediate complement-dependent opsonophagocytosis. Phagocytic assays were performed to determine the mechanism of the protection afforded by these surface proteins. Rabbit antibodies, rabbit complement, freshly isolated human PMNs, and *S. aureus* were incubated and then plated on agar medium to measure bacterial survival as colony forming units (CFU). Percentage killing was calculated. Error bars represent the SEM.

**FIG. 13.** ELISA showing the titers of EsxA, EsxB, EsaC antigens in the sera of mice infected with either one of the following staphylococcal strains Mu50, MW2, Newman (NM), USA300, USA700. PBS is used as a control (uninfected animals). The last panel shows the same ELISA for SrtA (Sortase A). Sortase A is a protein of

the plasma membrane of staphylococci and animals do not develop anti-SrtA IgG during infection. As a positive control, the serum of a rabbit immunized with recombinant SrtA is shown (SrtA+, last panel on the right).

**FIG. 14.** Serum IgG titers of patients infected with *S. aureus*. Sera of two non infected individuals were used as a control. Various dilutions of sera were analyzed for EsxA and EsxB specific IgG by custom ELISA.

### **DETAILED DESCRIPTION OF THE INVENTION**

The pathogenesis of staphylococcal infections relies on many different virulence factors such as secreted exotoxins, exopolysaccharides, and surface adhesins. However, deletion of single genes encoding such factors causes either no defect or results in only modest reduction of virulence. The development of staphylococcal vaccines is hindered by the multifaceted nature of staphylococcal invasion strategies. It is well established that live attenuated micro-organisms are highly effective vaccines; immune responses elicited by such vaccines are often of greater magnitude and of longer duration than those produced by non-replicating immunogens. One explanation for this may be that live attenuated strains establish limited infections in the host and mimic the early stages of natural infection. Embodiments of the invention are directed to immunogenic extracellular proteins, polypeptides, and peptides (including both secreted and cell surface proteins or peptides) of gram positive bacteria for the use in mitigating or immunizing against infection. In particular embodiments the bacteria is a staphylococcus. Extracellular proteins, polypeptides, or peptides include, but are not limited to secreted and cell surface proteins of the targeted bacteria.

The human pathogen *S. aureus* secretes EsxA and EsxB, two ESAT-6 like proteins, across the bacterial envelope (Burts *et al.*, 2005, which is incorporated herein by reference). Staphylococcal *esxA* and *esxB* are clustered with six other genes in the order of transcription: *esxA esaA essA esaB essB essC esaC esxB* (FIG. 1). The acronyms *esa*, *ess*, and *esx* stand for ESAT-6 secretion accessory, system, and extracellular, respectively, depending whether the encoded proteins play an accessory (*esa*) or direct (*ess*) role for secretion, or are secreted (*esx*) into the extracellular milieu. The entire cluster of eight genes is herein referred to as the Ess cluster. The inventors have shown that *esxA*, *esxB*, *essA*, *essB*, and *essC* are all required for

synthesis or secretion of EsxA and EsxB. Mutants that fail to produce EsxA, EsxB, and EssC display defects in the pathogenesis of *S. aureus* murine abscesses, suggesting that this specialized secretion system may be a general strategy of human bacterial pathogenesis.

5           The *Staphylococcus aureus* Ess pathway can be viewed as a secretion module equipped with specialized transport components (Ess), accessory factors (Esa) and cognate secretion substrates (Esx). EssA, EssB and EssC are required for EsxA and EsxB secretion. Because EssA, EssB and EssC are predicted to be transmembrane proteins (FIG. 1), it is contemplated that these proteins form a secretion apparatus.

10       Some of the proteins in the *ess* gene cluster may actively transport secreted substrates (acting as motor) while others may regulate transport (regulator). Regulation may be achieved, but need not be limited to, transcriptional or post-translational mechanisms for secreted polypeptides, sorting of specific substrates to defined locations (e.g., extracellular medium or host cells), or timing of secretion events during infection. At

15       this point, it is unclear whether all secreted Esx proteins function as toxins or contribute indirectly to pathogenesis. Two hypotheses may be considered. Hypothesis one: secreted EsxA and EsxB directly affect host function and hence encode bona fide toxins. Members of the ESAT-6 family only share between 16-20% identity at the protein level. Conservation of sequence identity may be due to cis-

20       acting elements within peptide sequence required for substrate recognition or secretion while the remainder of the polypeptide may encode toxin activity(ies). Hypothesis two: EsxA and EsxB could serve as chaperones for the secretion of other proteins (effectors) into the extra-cellular medium or into the cytosol of eukaryotic cells. In this model, while one of the machinery components (EssC) and EsxAB are

25       shared by mycobacteria and gram-positive bacteria, and may be identified by sequence similarity, the secreted effectors may be highly specific for a given bacterial species and implement infectious disease strategies unique to this bacterial species. For example, the accessory proteins EsaB and EsaC could be substrates for the EssABC-EsxAB secretion machinery.

30           Staphylococci rely on surface protein mediated-adhesion to host cells or invasion of tissues as a strategy for escape from immune defenses. Furthermore, *S. aureus* utilize surface proteins to sequester iron from the host during infection. The



majority of surface proteins involved in staphylococcal pathogenesis carry C-terminal sorting signals, *i.e.*, they are covalently linked to the cell wall envelope by sortase. Further, staphylococcal strains lacking the genes required for surface protein anchoring, *i.e.*, sortase A and B, display a dramatic defect in the virulence in several  
5 different mouse models of disease. Thus, surface protein antigens represent a validated vaccine target as the corresponding genes are essential for the development of staphylococcal disease and can be exploited in various embodiments of the invention. The sortase enzyme superfamily are Gram-positive transpeptidases responsible for anchoring surface protein virulence factors to the peptidoglycan cell  
10 wall layer. Two sortase isoforms have been identified in *Staphylococcus aureus*, SrtA and SrtB. These enzymes have been shown to recognize a LPXTG or NPQTN motif in substrate proteins, respectively. The SrtB isoform appears to be important in heme iron acquisition and iron homeostasis, whereas the SrtA isoform plays a critical role in the pathogenesis of Gram-positive bacteria by modulating the ability of the bacterium  
15 to adhere to host tissue via the covalent anchoring of adhesions and other proteins to the cell wall peptidoglycan. Embodiments of the invention include, but are not limited to compositions and methods related to SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC and/or SasF proteins.

## I. STAPHYLOCOCCAL ANTIGENS

20 Certain aspects of the invention include methods and compositions concerning proteinaceous compositions including polypeptides, peptides, or nucleic acid encoding such, of an Ess pathway, preferably of the Esx class and more preferably all or part of EsxA, EsxB, or other proteins transported by the Ess pathway, or sortase substrates including, but not limited to SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA,  
25 ClfB, IsdC, SasF or combinations thereof. These proteins may be modified by deletion, insertion, and/or substitution. In particular embodiments, modifications of these proteins are capable of eliciting an immune response in a subject.

The Esx polypeptides include the amino acid sequence of Esx proteins from bacteria in the *Staphylococcus* genus. The Esx sequence may be from a particular  
30 staphylococcus species, such as *Staphylococcus aureus*, and may be from a particular strain, such as Newman. In certain embodiments, the EsxA sequence is SAV0282 from strain Mu50 (which is the same amino acid sequence for Newman) and can be

accessed using Genbank Accession Number Q99WU4 (gi|68565539) (SEQ ID NO:2), which is hereby incorporated by reference. In other embodiments, the EsxB sequence is SAV0290 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number Q99WT7 (gi|68565532) (SEQ ID NO:4), which is hereby incorporated by reference. In further embodiments, other polypeptides transported by the Ess pathway may be used, the sequences of which may be identified by one of skill in the art using databases and internet accessible resources.

The sortase substrate polypeptides include, but are not limited to the amino acid sequence of SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC or SasF proteins from bacteria in the *Staphylococcus* genus. The sortase substrate polypeptide sequence may be from a particular staphylococcus species, such as *Staphylococcus aureus*, and may be from a particular strain, such as Newman. In certain embodiments, the SdrD sequence is from strain N315 and can be accessed using Genbank Accession Number NP\_373773.1 (gi|15926240) (SEQ ID NO:6), which is hereby incorporated by reference. In other embodiments, the SdrE sequence is from strain N315 and can be accessed using Genbank Accession Number NP\_373774.1 (gi|15926241) (SEQ ID NO:8), which is hereby incorporated by reference. In other embodiments, the IsdA sequence is SAV1130 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number NP\_371654.1 (gi|15924120) (SEQ ID NO:10), which is hereby incorporated by reference. In other embodiments, the IsdB sequence is SAV1129 from strain Mu50 (which is the same amino acid sequence for Newman) and can be accessed using Genbank Accession Number NP\_371653.1 (gi|15924119) (SEQ ID NO:12), which is hereby incorporated by reference. In further embodiments, other polypeptides transported by the Ess pathway or processed by sortase may be used, the sequences of which may be identified by one of skill in the art using databases and internet accessible resources.

Examples of various proteins that can be used in the context of the present invention can be identified by analysis of database submissions of bacterial genomes, including but not limited to Refseq accession numbers NC\_002951 (GI:57650036 and GenBank CP000046), NC\_002758 (GI:57634611 and GenBank BA000017),

NC\_002745 (GI:29165615 and GenBank BA000018), NC\_003923 (GI:21281729 and GenBank BA000033), NC\_002952 (GI:49482253 and GenBank BX571856), NC\_002953 (GI:49484912 and GenBank BX571857), NC\_007793 (GI:87125858 and GenBank CP000255), NC\_007795 (GI:87201381 and GenBank CP000253) each of  
5 which are incorporated herein by reference in their entirety.

As used herein, a “protein” or “polypeptide” refers to a molecule comprising at least ten amino acid residues. In some embodiments, a wild-type version of a protein or polypeptide are employed, however, in many embodiments of the invention, a modified protein or polypeptide is employed to generate an immune  
10 response. The terms described above may be used interchangeably herein. A “modified protein” or “modified polypeptide” refers to a protein or polypeptide whose chemical structure, particularly its amino acid sequence, is altered with respect to the wild-type protein or polypeptide. In some embodiments, a modified protein or polypeptide has at least one modified activity or function (recognizing that proteins or  
15 polypeptides may have multiple activities or functions). It is specifically contemplated that a modified protein or polypeptide may be altered with respect to one activity or function yet retain a wild-type activity or function in other respects, such as immunogenicity.

In certain embodiments the size of a protein or polypeptide (wild-type or  
20 modified) may comprise, but is not limited to, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150,  
25 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975, 1000, 1100, 1200, 1300, 1400, 1500, 1750, 2000, 2250, 2500 amino molecules or greater, and any range derivable therein, or derivative thereof. It is contemplated that polypeptides may be mutated by truncation, rendering them  
30 shorter than their corresponding wild-type form, but also they might be altered by fusing or conjugating a heterologous protein sequence with a particular function (*e.g.*,

for targeting or localization, for enhanced immunogenicity, for purification purposes, etc.).

As used herein, an "amino molecule" refers to any amino acid, amino acid derivative, or amino acid mimic known in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

Accordingly, the term "proteinaceous composition" encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid.

Proteinaceous compositions may be made by any technique known to those of skill in the art, including (i) the expression of proteins, polypeptides, or peptides through standard molecular biological techniques, (ii) the isolation of proteinaceous compounds from natural sources, or (iii) the chemical synthesis of proteinaceous materials. The nucleotide as well as the protein, polypeptide, and peptide sequences for various genes have been previously disclosed, and may be found in the recognized computerized databases. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (on the World Wide Web at [ncbi.nlm.nih.gov/](http://ncbi.nlm.nih.gov/)). The coding regions for these genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art.

Amino acid sequence variants of EsxA, EsxB, and other polypeptides transported by the Ess pathway ("other Ess pathway polypeptides"), and/or SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, SasF or other sortase substrates can be substitutional, insertional, or deletion variants. A modification in a polypeptide of the invention may affect 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88,

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474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490,  
25 491, 492, 493, 494, 495, 496, 497, 498, 499, 500 or more non-contiguous or  
contiguous amino acids of the polypeptide, as compared to wild-type. A polypeptide  
processed or secreted by the Ess pathway, and/or SdrC, SdrD, SdrE, IsdA, IsdB, Spa,  
ClfA, ClfB, IsdC, SasF or other surface proteins (see Table 1) or sortase substrates  
from any staphylococcus species and strain are contemplated for use in methods of  
30 the invention.

Deletion variants typically lack one or more residues of the native or wild-type protein. Individual residues can be deleted or a number of contiguous amino acids can be deleted. A stop codon may be introduced (by substitution or insertion) into an

encoding nucleic acid sequence to generate a truncated protein. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. This may include the insertion of one or more residues. Terminal additions, called fusion proteins, may also be generated.

5           Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, with or without the loss of other functions or properties. Substitutions may be conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art  
10 and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to  
15 tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine. Alternatively, substitutions may be non-conservative such that a function or activity of the polypeptide is affected. Non-conservative changes typically involve substituting a residue with one that is chemically dissimilar, such as a polar or  
20 charged amino acid for a nonpolar or uncharged amino acid, and vice versa.

**Table 1. Exemplary surface proteins of *S. aureus* strains.**

SAV #	SA#	Surface	MW2	Mu50	N315	Newman	MRSA252*	MSSA476*
SAV0111	SA0107	Spa	492	450	450		516	492
SAV2503	SA2291	FnBPA	1015	1038	1038		-	1015
SAV2502	SA2290	FnBPB	943	961	961		965	957
SAV0811	SA0742	ClfA	946	935	989	933	1029	928
SAV2630	SA2423	ClfB	907	877	877	913	873	905
Np	np	Can	1183	-	-		1183	1183
SAV0561	SA0519	SdrC	955	953	953	947	906	957
SAV0562	SA0520	SdrD	1347	1385	1385	1315	-	1365
SAV0563	SA0521	SdrE	1141	1141	1141	1166	1137	1141
Np	np	Pls	-	-	-		-	-
SAV2654	SA2447	SasA	2275	2271	2271		1351	2275
SAV2160	SA1964	SasB	686	2481	2481		2222	685
	SA1577	SasC	2186	213	2186		2189	2186
SAV0134	SA0129	SasD	241	241	241		221	241
SAV1130	SA0977	SasE/IsdA	350	350	350		354	350
SAV2646	SA2439	SasF	635	635	635		627	635
SAV2496		SasG	1371	525	927		-	1371
SAV0023	SA0022	SasH	772	-	772		786	786
SAV1731	SA1552	SasI	895	891	891		534	895
SAV1129	SA0976	SasJ/IsdB	645	645	645		652	645
	SA2381	SasK	198	211	211		-	197
	np	SasL	-	232	-		-	-
SAV1131	SA0978	IsdC	227	227	227		227	227

Proteins of the invention may be recombinant, or synthesized *in vitro*. Alternatively, a non-recombinant or recombinant protein may be isolated from bacteria. It is also contemplated that a bacteria containing such a variant may be implemented in compositions and methods of the invention. Consequently, a protein need not be isolated.

The term “functionally equivalent codon” is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 2, below).

**Table 2 Codon Table**

Amino Acids			Codons
<b>Alanine</b>	<b>Ala</b>	<b>A</b>	<b>GCA GCC GCG GCU</b>
<b>Cysteine</b>	<b>Cys</b>	<b>C</b>	<b>UGC UGU</b>
<b>Aspartic acid</b>	<b>Asp</b>	<b>D</b>	<b>GAC GAU</b>
<b>Glutamic acid</b>	<b>Glu</b>	<b>E</b>	<b>GAA GAG</b>
<b>Phenylalanine</b>	<b>Phe</b>	<b>F</b>	<b>UUC UUU</b>
<b>Glycine</b>	<b>Gly</b>	<b>G</b>	<b>GGA GGC GGG GGU</b>
<b>Histidine</b>	<b>His</b>	<b>H</b>	<b>CAC CAU</b>
<b>Isoleucine</b>	<b>Ile</b>	<b>I</b>	<b>AUA AUC AUU</b>
<b>Lysine</b>	<b>Lys</b>	<b>K</b>	<b>AAA AAG</b>
<b>Leucine</b>	<b>Leu</b>	<b>L</b>	<b>UUA UUG CUA CUC CUG CUU</b>
<b>Methionine</b>	<b>Met</b>	<b>M</b>	<b>AUG</b>
<b>Asparagine</b>	<b>Asn</b>	<b>N</b>	<b>AAC AAU</b>
<b>Proline</b>	<b>Pro</b>	<b>P</b>	<b>CCA CCC CCG CCU</b>
<b>Glutamine</b>	<b>Gln</b>	<b>Q</b>	<b>CAA CAG</b>
<b>Arginine</b>	<b>Arg</b>	<b>R</b>	<b>AGA AGG CGA CGC CGG CGU</b>
<b>Serine</b>	<b>Ser</b>	<b>S</b>	<b>AGC AGU UCA UCC UCG UCU</b>
<b>Threonine</b>	<b>Thr</b>	<b>T</b>	<b>ACA ACC ACG ACU</b>
<b>Valine</b>	<b>Val</b>	<b>V</b>	<b>GUA GUC GUG GUU</b>
<b>Tryptophan</b>	<b>Trp</b>	<b>W</b>	<b>UGG</b>
<b>Tyrosine</b>	<b>Tyr</b>	<b>Y</b>	<b>UAC UAU</b>

It also will be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids, or 5' or 3' sequences, respectively, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region.

The following is a discussion based upon changing of the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA coding sequence, and nevertheless produce a protein with like properties. It is thus contemplated by the



inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring  
5 interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

10 It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. It is understood that an amino acid can be  
15 substituted for another having a similar hydrophilicity value and still produce a biologically equivalent and immunologically equivalent protein.

As outlined above, amino acid substitutions generally are based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions  
20 that take into consideration the various foregoing characteristics are well known and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

It is contemplated that in compositions of the invention, there is between about 0.001 mg and about 10 mg of total protein per ml. Thus, the concentration of protein  
25 in a composition can be about, at least about or at most about 0.001, 0.010, 0.050, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 mg/ml or more (or any range derivable therein). Of this, about, at least about, or at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,  
14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36,  
30 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82,

83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100% may be EsxA protein, EsxB protein, or another protein transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or other sortase substrates.

The present invention contemplates the administration of EsxA or EsxB  
5 polypeptides or peptides, as well as any other protein transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or other sortase substrates, to effect a preventative therapy against the development of a disease or condition associated with infection by a staphylococcus pathogen.

In addition, U.S. Patent 4,554,101 (Hopp), which is incorporated herein by  
10 reference, teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in Hopp, one of skill in the art would be able to identify potential epitopes from within an amino acid sequence and confirm their immunogenicity. Numerous scientific  
15 publications have also been devoted to the prediction of secondary structure and to the identification of epitopes, from analyses of amino acid sequences (Chou & Fasman, 1974a,b; 1978a,b, 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

#### **A. Other antigens**

Compositions of the invention can include one or more additional active  
20 agents. Such agents include, but are not limited to one or more (a) staphylococcal antigens, (b) non-staphylococcal antigens, (c) nucleic acid molecules encoding (a) or (b), and antibodies which specifically bind to (a) or (b).

##### **1. Staphylococcal antigens**

Staphylococcal antigens which can be included in compositions of the  
25 invention include *S. aureus* type 5 and 8 capsular polysaccharides optionally conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A, such as StaphVAX®, antigens derived from extracellular polysaccharides (lipoteichoic acid, cell wall teichoic acid, poly-N-acetylglucosamine (PNAG) exopolysacchride) or antigens derived from surface proteins, invasins (leukocidin, kinases, hyaluronidase),  
30 surface factors that inhibit phagocytic engulfment (capsule, Protein A), carotenoids, catalase production, Protein A, coagulase, clotting factor, and/or membrane-damaging

toxins (optionally detoxified) that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin).

## 2. Non-staphylococcal antigens

Compositions of the invention may be administered in conjunction with one or more antigens for use in therapeutic, prophylactic, or diagnostic methods of the present invention. Compositions of the invention optionally can comprise one or more additional polypeptide antigens which are not derived from staphylococcal proteins. Preferred antigens include those listed below. Additionally, the compositions of the present invention may be used to treat or prevent infections caused by any of the below-listed pathogens. In addition to combination with the antigens described below, the compositions of the invention may also be combined with an adjuvant as described herein.

Antigens for use with the invention include, but are not limited to, one or more of the following antigens set forth below, or antigens derived from one or more of the pathogens set forth below:

### a. Bacterial Antigens

Bacterial antigens suitable for use in the invention include proteins, polysaccharides, lipopolysaccharides, and outer membrane vesicles which may be isolated, purified or derived from a bacteria. In addition, bacterial antigens may include bacterial lysates and inactivated bacteria formulations. Bacteria antigens may be produced by recombinant expression. Bacterial antigens preferably include epitopes which are exposed on the surface of the bacteria during at least one stage of its life cycle. Bacterial antigens are preferably conserved across multiple serotypes. Bacterial antigens include antigens derived from one or more of the bacteria set forth below as well as the specific antigens examples identified below.

*Neisseria meningitides*: Meningitides antigens may include proteins, saccharides (including a polysaccharide, oligosaccharide or lipopolysaccharide), or outer-membrane vesicles purified or derived from any *N. meningitides* serogroups. Meningitides protein antigens may be selected from adhesions, autotransporters, toxins, Fe acquisition proteins, and membrane associated proteins (preferably integral outer membrane protein).

*Streptococcus pneumoniae*: *Streptococcus pneumoniae* antigens may include a saccharide (including a polysaccharide or an oligosaccharide) and/or protein from *Streptococcus pneumoniae*. Saccharide antigens may be selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, bA, hA, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 5 and 33F. Protein antigens may be selected from a protein identified in WO 98/18931, WO 98/18930, US Patent No. 6,699,703, US Patent No. 6,800,744, WO 97/43303, and WO 97/37026. *Streptococcus pneumoniae* proteins may be selected from the Poly Histidine Triad family (PhtX), the Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric 10 proteins, pneumolysin (Ply), PspA, PsaA, Sp128, SplO1, Sp130, Sp125 or Sp133.

*Streptococcus pyogenes* (Group A Streptococcus): GAS antigens may include a protein identified in WO 02/34771 or WO 2005/032582 (including GBS 40), fusions of fragments of GBS M proteins (including those described in WO 02/094851, and Dale, Vaccine (1999) 17:193-200, and Dale, Vaccine 14(10): 944- 15 948), fibronectin binding protein (STh 1), Streptococcal heme-associated protein (Shp), and Streptolysin S (SagA).

*Streptococcus agalactiae* (Group B Streptococcus): Group B Streptococcus antigens include a protein or saccharide antigen identified in WO 02/34771, WO 03/093306, WO 04/041157, or WO 2005/0026 19 (including proteins GBS 80, GBS 20 104, GBS 276 and GBS 322, and including saccharide antigens derived from serotypes Ia, Tb, Ia/c, II, III, IV, V, VI, VII and VIII).

*Moraxella catarrhalis*: Moraxella antigens include antigens identified in WO 02/18595 and WO 99/58562, outer membrane protein antigens (HMW-OMP), C-antigen, and/or LPS.

25 *Bordetella pertussis*: Pertussis antigens include pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B. pertussis*, optionally also combination with pertactin and/or agglutinogens 2 and 3 antigen.

*Clostridium tetani* (Tetanus): Tetanus antigens include tetanus toxoid (TT), preferably used as a carrier protein in conjunction/conjugated with the compositions 30 of the present invention.

*Cornynebacterium diphtheriae* (Diphtheria): Diphtheria antigens include diphtheria toxin, preferably detoxified, such as CRM197. Additionally antigens capable of modulating, inhibiting or associated with ADP ribosylation are contemplated for combination/co-administration/conjugation with the compositions of  
5 the present invention. The diphtheria toxoids may be used as carrier proteins.

*Haemophilus influenzae* B (Hib): Hib antigens include a Hib saccharide antigen.

*Pseudomonas aeruginosa*: Pseudomonas antigens include endotoxin A, Wzz protein, *P. aeruginosa* LPS, more particularly LPS isolated from PAO1 (05 serotype),  
10 and/or Outer Membrane Proteins, including Outer Membrane Proteins F (OprF) (Infect Immun. 2001 May; 69(5) : 3510-3515).

*Legionella pneumophila*: Bacterial antigens may be derived from *Legionella pneumophila*.

*Neisseria gonorrhoeae*: *Gonorrhoeae* antigens include Por (or porin) protein, such as PorB (see Zhu et al., Vaccine (2004) 22:660 - 669), a transferring binding protein, such as TbpA and TbpB (See Price et al., Infection and Immunity (2004) 71(1):277 - 283), a opacity protein (such as Opa), a reduction-modifiable protein (Rmp), and outer membrane vesicle (OMV) preparations (see Plante et al., J Infectious Disease (2000) 182:848 - 855), also see e.g. WO99/24578, WO99/36544,  
20 WO99/57280.

*Chlamydia trachomatis*: *Chlamydia trachomatis* antigens include antigens derived from serotypes A, B, Ba and C (agents of trachoma, a cause of blindness), serotypes Li, L2 & L3 (associated with Lymphogranuloma venereum), and serotypes, D-K. *Chlamydia trachomas* antigens may also include an antigen identified in WO  
25 00/37494, WO 03/049762, WO 03/068811, or WO 05/0026 19, including PepA (CT045), LcrE (CT089), ArtJ (CT381), DnaK (CT396), CT398, OmpH-like (CT242), L7/L12 (CT316), OmcA (CT444), AtosS (CT467), CT547, Eno (CT587), HrtA (CT823), and MurG (CT761).

*Treponema pallidum* (Syphilis): Syphilis antigens include TmpA antigen.

*Haemophilus ducreyi* (causing chancroid): Ducreyi antigens include outer membrane protein (DsrA).

*Enterococcus faecalis* or *Enterococcus faecium*: Antigens include a trisaccharide repeat or other Enterococcus derived antigens provided in US Patent No. 5 6,756,361.

*Helicobacter pylori*: *H. pylori* antigens include Cag, Vac, Nap, HopX, HopY and/or urease antigen.

*Yersinia enterocolitica* : *Yersinia enterocolitica* antigens include LPS (Infect Immun. 2002 August; 70(8): 4414).

10 *E. coli*: *E. coli* antigens may be derived from enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAaggEC), diffusely adhering *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), and/or enterohemorrhagic *E. coli* (EHEC).

*Bacillus anthracis* (anthrax): *B. anthracis* antigens are optionally detoxified and may be selected from A-components (lethal factor (LF) and edema factor (EF)), 15 both of which can share a common B-component known as protective antigen (PA).

*Yersinia pestis* (plague): Plague antigens include F 1 capsular antigen (Infect Immun. 2003 Jan; 71(1): 374-383), LPS (Infect Immun. 1999 Oct; 67(10): 5395), *Yersinia pestis* V antigen (Infect Immun. 1997 Nov; 65(11): 4476-4482).

*Mycobacterium tuberculosis*: Tuberculosis antigens include lipoproteins, LPS, 20 BCG antigens, a fusion protein of antigen 85B (Ag85B) and/or ESAT-6 optionally formulated in cationic lipid vesicles (Infect Immun. 2004 October; 72(10): 6148), *Mycobacterium tuberculosis* (Mtb) isocitrate dehydrogenase associated antigens (Proc Natl Acad Sci U S A. 2004 Aug 24; 101(34): 12652), and/or MPT51 antigens (Infect Immun. 2004 July; 72(7): 3829).

25 *Rickettsia*: Antigens include outer membrane proteins, including the outer membrane protein A and/or B (OmpB) (Biochim Biophys Acta. 2004 Nov 1;1702(2):145), LPS, and surface protein antigen (SPA) (J Autoimmun. 1989 Jun;2 Suppl:81).

*Listeria monocytogenes*: Bacterial antigens may be derived from *Listeria monocytogenes*.

*Chlamydia pneumoniae*: Antigens include those identified in WO 02/02606.

5 *Vibrio cholera*: Antigens include proteinase antigens, LPS, particularly lipopolysaccharides of *Vibrio cholera*, 0-specific polysaccharides, *V. cholera* 0139, antigens of IEM108 vaccine (Infect Immun. 2003 Oct;71(10):5498-504), and/or *Zonula occludens* toxin (Zot).

*Salmonella typhi* (typhoid fever): Antigens include capsular polysaccharides preferably conjugates (Vi, i.e. vax-TyVi).

10 *Borrelia burgdorferi* (Lyme disease): Antigens include lipoproteins (such as OspA, OspB, Osp C and Osp D), other surface proteins such as OspE-related proteins (Erps), decorin-binding proteins (such as DbpA), and antigenically variable VI proteins, such as antigens associated with P39 and P13 (an integral membrane protein, Infect Immun. 2001 May; 69(5): 3323-3334), VlsE Antigenic Variation Protein (J  
15 Clin Microbiol. 1999 Dec; 37(12): 3997).

*Porphyromonas gingivalis*: Antigens include *P. gingivalis* outer membrane protein (OMP).

*Kiebsiella*: Antigens include outer membrane proteins, including OmpA, or a polysaccharide optionally conjugated to tetanus toxoid.

20 Further bacterial antigens of the invention may be capsular antigens, polysaccharide antigens or protein antigens of any of the above. Further bacterial antigens may also include an outer membrane vesicle (OMV) preparation. Additionally, antigens include live, attenuated, and/or purified versions of any of the aforementioned bacteria. The antigens of the present invention may be derived from  
25 gram-negative or gram-positive bacteria. The antigens of the present invention may be derived from aerobic or anaerobic bacteria.

Additionally, any of the above bacterial-derived saccharides (polysaccharides, LPS, LOS or oligosaccharides) can be conjugated to another agent or antigen, such as a carrier protein (for example CRM197). Such conjugation may be direct conjugation

effected by reductive amination of carbonyl moieties on the saccharide to amino groups on the protein, as provided in US Patent No. 5,360,897 and Can J Biochem Cell Biol. 1984 May;62(5):270-5. Alternatively, the saccharides can be conjugated through a linker, such as, with succinamide or other linkages.

5 **b. Viral Antigens**

Viral antigens suitable for use in the invention include inactivated (or killed) virus, attenuated virus, split virus formulations, purified subunit formulations, viral proteins which may be isolated, purified or derived from a virus, and Virus Like Particles (VLPs). Viral antigens may be derived from viruses propagated on cell  
10 culture or other substrate. Alternatively, viral antigens may be expressed recombinantly. Viral antigens preferably include epitopes which are exposed on the surface of the virus during at least one stage of its life cycle. Viral antigens are preferably conserved across multiple serotypes or isolates. Viral antigens include antigens derived from one or more of the viruses set forth below as well as the  
15 specific antigens examples identified below.

Orthomyxovirus: Viral antigens may be derived from an Orthomyxovirus, such as Influenza A, B and C. Orthomyxovirus antigens may be selected from one or more of the viral proteins, including haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M1), membrane protein (M2), one or more of the  
20 transcriptase components (PB 1, PB2 and PA). Preferred antigens include HA and NA. Influenza antigens may be derived from interpandemic (annual) flu strains. Alternatively influenza antigens may be derived from strains with the potential to cause a pandemic outbreak (i.e., influenza strains with new haemagglutinin compared to the haemagglutinin in currently circulating strains, or influenza strains which are  
25 pathogenic in avian subjects and have the potential to be transmitted horizontally in the human population, or influenza strains which are pathogenic to humans).

Paramyxoviridae viruses: Viral antigens may be derived from Paramyxoviridae viruses, such as Pneumoviruses (RSV), Paramyxoviruses (PIV) and Morbilliviruses (Measles).

30 Pneumovirus: Viral antigens may be derived from a Pneumovirus, such as Respiratory syncytial virus (RSV), Bovine respiratory syncytial virus, Pneumonia



virus of mice, and Turkey rhinotracheitis virus. Preferably, the Pneumovirus is RSV. Pneumovirus antigens may be selected from one or more of the following proteins, including surface proteins Fusion (F), Glycoprotein (G) and Small Hydrophobic protein (SH), matrix proteins M and M2, nucleocapsid proteins N, P and L and  
5 nonstructural proteins NS 1 and NS2. Preferred Pneumovirus antigens include F, G and M. Pneumovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV.

Paramyxovirus: Viral antigens may be derived from a Paramyxovirus, such as  
10 Parainfluenza virus types 1 - 4 (PIV), Mumps, Sendai viruses, Simian virus 5, Bovine parainfluenza virus and Newcastle disease virus. Preferably, the Paramyxovirus is PIV or Mumps. Paramyxovirus antigens may be selected from one or more of the following proteins: Hemagglutinin - Neuraminidase (HN), Fusion proteins F1 and F2, Nucleoprotein (NP), Phosphoprotein (P), Large protein (L), and Matrix protein (M).  
15 Preferred Paramyxovirus proteins include HN, F1 and F2. Paramyxovirus antigens may also be formulated in or derived from chimeric viruses. For example, chimeric RSV/PIV viruses may comprise components of both RSV and PIV. Commercially available mumps vaccines include live attenuated mumps virus, in either a monovalent form or in combination with measles and rubella vaccines (MMR).

20 Morbillivirus: Viral antigens may be derived from a Morbillivirus, such as Measles. Morbillivirus antigens may be selected from one or more of the following proteins: hemagglutinin (H), Glycoprotein (G), Fusion factor (F), Large protein (L), Nucleoprotein (NP), Polymerase phosphoprotein (P), and Matrix (M). Commercially available measles vaccines include live attenuated measles virus, typically in  
25 combination with mumps and rubella (MMR).

Picornavirus: Viral antigens may be derived from Picornaviruses, such as Enteroviruses, Rhinoviruses, Heparnavirus, Cardioviruses and Aphthoviruses. Antigens derived from Enteroviruses, such as Poliovirus are preferred.

Enterovirus: Viral antigens may be derived from an Enterovirus, such as  
30 Poliovirus types 1, 2 or 3, Coxsackie A virus types 1 to 22 and 24, Coxsackie B virus types 1 to 6, Echovirus (ECHO) virus) types 1 to 9, 11 to 27 and 29 to 34 and

Enterovirus 68 to 71. Preferably, the Enterovirus is poliovirus. Enterovirus antigens are preferably selected from one or more of the following Capsid proteins VP1, VP2, VP3 and VP4. Commercially available polio vaccines include Inactivated Polio Vaccine (IPV) and Oral poliovirus vaccine (OPV).

5           Heparnavirus: Viral antigens may be derived from an Heparnavirus, such as Hepatitis A virus (HAV). Commercially available HAV vaccines include inactivated HAV vaccine.

          Togavirus: Viral antigens may be derived from a Togavirus, such as a Rubivirus, an Alphavirus, or an Arterivirus. Antigens derived from Rubivirus, such as  
10 Rubella virus, are preferred. Togavirus antigens may be selected from E1, E2, E3, C, NSP-1, NSPO-2, NSP- 3 or NSP-4. Togavirus antigens are preferably selected from E1, E2 or E3. Commercially available Rubella vaccines include a live cold-adapted virus, typically in combination with mumps and measles vaccines (MMR).

          Flavivirus: Viral antigens may be derived from a Flavivirus, such as Tick-  
15 borne encephalitis (TBE), Dengue (types 1, 2, 3 or 4), Yellow Fever, Japanese encephalitis, West Nile encephalitis, St. Louis encephalitis, Russian spring-summer encephalitis, Powassan encephalitis. Flavivirus antigens may be selected from PrM, M, C, E, NS-1, NS-2a, NS2b, N53, N54a, NS4b, and NS5. Flavivirus antigens are preferably selected from PrM, M and E. Commercially available TBE vaccine include  
20 inactivated virus vaccines.

          Pestivirus: Viral antigens may be derived from a Pestivirus, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).

          Hepadnavirus: Viral antigens may be derived from a Hepadnavirus, such as Hepatitis B virus. Hepadnavirus antigens may be selected from surface antigens (L, M  
25 and S), core antigens (HBc, HBe). Commercially available HBV vaccines include subunit vaccines comprising the surface antigen S protein.

          Hepatitis C virus: Viral antigens may be derived from a Hepatitis C virus (HCV). HCV antigens may be selected from one or more of E1, E2, E1/E2, NS345 polyprotein, NS 345-core polyprotein, core, and/or peptides from the nonstructural  
30 regions (Houghton et al., Hepatology (1991) 14:381).

Rhabdovirus: Viral antigens may be derived from a Rhabdovirus, such as a Lyssavirus (Rabies virus) and Vesiculovirus (VSV). Rhabdovirus antigens may be selected from glycoprotein (G), nucleoprotein (N), large protein (L), nonstructural proteins (NS). Commercially available Rabies virus vaccine comprise killed virus  
5 grown on human diploid cells or fetal rhesus lung cells.

Calciviridae: Viral antigens may be derived from Calciviridae, such as Norwalk virus, and Norwalk-like Viruses, such as Hawaii Virus and Snow Mountain Virus.

Coronavirus: Viral antigens may be derived from a Coronavirus, SARS,  
10 Human respiratory coronavirus, Avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible Gastroenteritis virus (TGEV). Coronavirus antigens may be selected from spike (S), envelope (E), matrix (M), nucleocapsid (N), and Hemagglutinin-esterase glycoprotein (HE). Preferably, the Coronavirus antigen is derived from a SARS virus.

15 Retrovirus: Viral antigens may be derived from a Retrovirus, such as an Oncovirus, a Lentivirus or a Spumavirus. Oncovirus antigens may be derived from HTLV-1, HTLV-2 or HTLV-5. Lentivirus antigens may be derived from HIV-1 or HIV-2. Retrovirus antigens may be selected from gag, pol, env, tax, tat, rex, rev, nef, vif, vpr, and vpu. HIV antigens may be selected from gag (p24gag and p55gag), env  
20 (gp160 and gp41), pol, tat, nef, rev vpr, miniproteins. HIV antigens may be derived from one or more of the following strains: HIV111b, HIVSF2, HIVLAV, HIVLAI, HIVMN, HIV-1CM235, HIV-1US4.

Reovirus: Viral antigens may be derived from a Reovirus, such as an Orthoreovirus, a Rotavirus, an Orbivirus, or a Coltivirus. Reovirus antigens may be  
25 selected from structural proteins or nonstructural proteins. Preferred Reovirus antigens may be derived from a Rotavirus. Rotavirus antigens may be selected from VP1, VP2, VP3, VP4 (or the cleaved product VP5 and -40 - VP8), NSP 1, VP6, NSP3, NSP2, VP7, NSP4, or NSP5. Preferred Rotavirus antigens include VP4 (or the cleaved product VP5 and VP8), and VP7.

Parvovirus: Viral antigens may be derived from a Parvovirus, such as Parvovirus B 19. Parvovirus antigens may be selected from VP-1, VP-2, VP-3, NS-1 and NS-2. Preferably, the Parvovirus antigen is capsid protein VP-2.

Delta hepatitis virus (HDV): Viral antigens may be derived HDV, particularly s-antigen from HDV (see, e.g., U.S. Patent 5,378,814).

Hepatitis E virus (HEV): Viral antigens may be derived from HEV.

Hepatitis G virus (HGV): Viral antigens may be derived from HGV.

Human Herpesvirus: Viral antigens may be derived from a Human Herpesvirus, such as Herpes Simplex Viruses (HSV), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8). Human Herpesvirus antigens may be selected from immediate early proteins (a), early proteins (J3), and late proteins (y). HSV antigens may be derived from HSV-1 or HSV-2 strains. HSV antigens may be selected from glycoproteins gB, gC, gD and gH, fusion protein (gB), or immune escape proteins (gC, gE, or gI). VZV antigens may be selected from core, nucleocapsid, tegument, or envelope proteins. A live attenuated VZV vaccine is commercially available. EBV antigens may be selected from early antigen (BA) proteins, viral capsid antigen (VCA), and glycoproteins of the membrane antigen (MA). CMV antigens may be selected from capsid proteins, envelope glycoproteins (such as gB and gH), and tegument proteins.

Papovaviruses: Antigens may be derived from Papovaviruses, such as Papillomaviruses and Polyomaviruses. Papillomaviruses include HPV serotypes 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 and 65. Preferably, HPV antigens are derived from serotypes 6, 11, 16 or 18. HPV antigens may be selected from capsid proteins (L1) and (L2), or E1 - E7, or fusions thereof. HPV antigens are preferably formulated into virus-like particles (VLPs). Polyomyavirus viruses include BK virus and JK virus. Polyomavirus antigens may be selected from VP1, VP2 or VP3.

### c. Fungal Antigens

Fungal antigens for use in the invention may be derived from one or more of the fungi set forth below.

Fungal antigens may be derived from Dermatophytes, including:  
 5 *Epidermophyton floccusum*, *Microsporum audouini*, *Microsporum canis*,  
*Microsporum distortum*, *Microsporum equinum*, *Microsporum gypsum*, *Microsporum*  
*nanum*, *Trichophyton concentricum*, *Trichophyton equinum*, *Trichophyton gallinae*,  
*Trichophyton gypseum*, *Trichophyton megnini*, *Trichophyton mentagrophytes*,  
*Trichophyton quinckeanum*, *Trichophyton rubrum*, *Trichophyton schoenleini*,  
 10 *Trichophyton tonsurans*, *Trichophyton verrucosum*, *T. verrucosum* var. *album*, var.  
*discoides*, var. *ochraceum*, *Trichophyton violaceum*, and/or *Trichophyton faviforme*.

Fungal pathogens may be derived from *Aspergillus fumigatus*, *Aspergillus*  
*flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus*  
*sydowi*, *Aspergillus flavatus*, *Aspergillus glaucus*, *Blastoschizomyces capitatus*,  
 15 *Candida albicans*, *Candida enolase*, *Candida tropicalis*, *Candida glabrata*, *Candida*  
*krusei*, *Candida parapsilosis*, *Candida stellatoidea*, *Candida kusei*, *Candida*  
*parakwsei*, *Candida lusitanae*, *Candida pseudotropicalis*, *Candida guilliermondi*,  
*Cladosporium carrionii*, *Coccidioides immitis*, *Blastomyces dermatidis*, *Cryptococcus*  
*neoformans*, *Geotrichum clavatum*, *Histoplasma capsulatum*, *Klebsiella pneumoniae*,  
 20 *Paracoccidioides brasiliensis*, *Pneumocystis carinii*, *Pythium insidiosum*,  
*Pityrosporum ovale*, *Sacharomyces cerevisiae*, *Saccharomyces boulardii*,  
*Saccharomyces pombe*, *Scedosporium apiosperum*, *Sporothrix schenckii*,  
*Trichosporon beigeli*, *Toxoplasma gondii*, *Penicillium marneffe*, *Malassezia* spp.,  
*Fonsecaea* spp., *Wangiella* spp., *Sporothrix* spp., *Basidiobolus* spp., *Conidiobolus*  
 25 spp., *Rhizopus* spp., *Mucor* spp., *Absidia* spp., *Mortierella* spp., *Cunninghamella*  
spp., *Saksenaea* spp., *Alternaria* spp., *Curvularia* spp., *Helminthosporium* spp.,  
*Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Monolinia* spp., *Rhizoctonia* spp.,  
*Paecilomyces* spp., *Pithomyces* spp., and *Cladosporium* spp.

Processes for producing fungal antigens are well known in the art (see US  
 30 Patent No. 6,333,164). In a preferred method a solubilized fraction extracted and  
separated from an insoluble fraction obtainable from fungal cells of which cell wall  
has been substantially removed or at least partially removed, characterized in that the

process comprises the steps of: obtaining living fungal cells; obtaining fungal cells of which cell wall has been substantially removed or at least partially removed; bursting the fungal cells of which cell wall has been substantially removed or at least partially removed; obtaining an insoluble fraction; and extracting and separating a solubilized  
5 fraction from the insoluble fraction.

#### **d. STD Antigens**

The compositions of the invention may include one or more antigens derived from a sexually transmitted disease (STD). Such antigens may provide for prophylaxis or therapy for STD's such as chlamydia, genital herpes, hepatitis (such as  
10 HCV), genital warts, gonorrhea, syphilis and/or chancroid (See, W000/15255). Antigens may be derived from one or more viral or bacterial STD's. Viral STD antigens for use in the invention may be derived from, for example, HIV, herpes simplex virus (HSV-1 and HSV-2), human papillomavirus (HPV), and hepatitis (HCV). Bacterial STD antigens for use in the invention may be derived from, for  
15 example, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, *Haemophilus ducreyi*, *E. coli*, and *Streptococcus agalactiae*. Examples of specific antigens derived from these pathogens are described above.

#### **e. Respiratory Antigens**

The compositions of the invention may include one or more antigens derived  
20 from a pathogen which causes respiratory disease. For example, respiratory antigens may be derived from a respiratory virus such as Orthomyxoviruses (influenza), Pneumovirus (RSV), Paramyxovirus (Ply), Morbillivirus (measles), Togavirus (Rubella), VZV, and Coronavirus (SARS). Respiratory antigens may be derived from a bacterium which causes respiratory disease, such as *Streptococcus pneumoniae*,  
25 *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Bacillus anthracis*, and *Moraxella catarrhalis*. Examples of specific antigens derived from these pathogens are described above.

#### **f. Pediatric Vaccine Antigens.**

30 The compositions of the invention may include one or more antigens suitable for use in pediatric subjects. Pediatric subjects are typically less than about 3 years old

or less than about 2 years old or less than about 1 year old. Pediatric antigens may be administered multiple times over the course of 6 months, 1, 2 or 3 years. Pediatric antigens may be derived from a virus which may target pediatric populations and/or a virus from which pediatric populations are susceptible to infection. Pediatric viral antigens include antigens derived from one or more of Orthomyxovirus (influenza), Pneumovirus (RSV), Paramyxovirus (PIY and Mumps), Morbillivirus (measles), Togavirus (Rubella), Enterovirus (polio), HBV, Coronavirus (SARS), and Varicella-zoster virus (VZV), Epstein Barr virus (EBV). Pediatric bacterial antigens include antigens derived from one or more of Streptococcus pneumoniae, Neisseria meningitides, Streptococcus pyogenes (Group A Streptococcus), Moraxella catarrhalis, Bordetella pertussis, Clostridium tetani (Tetanus), Corynebacterium diphtheriae (Diphtheria), Haemophilus influenzae B (Hib), Pseudomonas aeruginosa, Streptococcus agalactiae (Group B Streptococcus), and E. coli. Examples of specific antigens derived from these pathogens are described above.

15 **g. Antigens suitable for use in Elderly or Immunocompromised Individuals.**

The compositions of the invention may include one or more antigens suitable for use in elderly or immunocompromised individuals. Such individuals may need to be vaccinated more frequently, with higher doses or with adjuvanted formulations to improve their immune response to the targeted antigens. Antigens which may be targeted for use in Elderly or Immunocompromised individuals include antigens derived from one or more of the following pathogens: Neisseria meningitides, Streptococcus pneumoniae, Streptococcus pyogenes (Group A Streptococcus), Moraxella catarrhalis, Bordetella pertussis, Staphylococcus epidermis, Clostridium tetani (Tetanus), Corynebacterium diphtheriae (Diphtheria), Haemophilus influenzae B (Hib), Pseudomonas aeruginosa, Legionella pneumophila, Streptococcus agalactiae (Group B Streptococcus), Enterococcus faecalis, Helicobacter pylori, Chlamydia pneumoniae, Orthomyxovirus (influenza), Pneumovirus (RSV), Paramyxovirus (PIV and Mumps), Morbillivirus (measles), Togavirus (Rubella), Enterovirus (polio), HBV, Coronavirus (SARS), Varicella-zoster virus (VZV), Epstein Barr virus (EBV), Cytomegalovirus (CMV). Examples of specific antigens derived from these pathogens are described above.

#### **h. Antigens suitable for use in Adolescent Vaccines**

The compositions of the invention may include one or more antigens suitable for use in adolescent subjects. Adolescents may be in need of a boost of a previously administered pediatric antigen. Pediatric antigens which may be suitable for use in adolescents are described above. In addition, adolescents may be targeted to receive antigens derived from an STD pathogen in order to ensure protective or therapeutic immunity before the beginning of sexual activity. STD antigens which may be suitable for use in adolescents are described above.

## **II. PROTEIN PRODUCTION**

### **A. Synthetic Proteins**

The present invention describes polypeptides, peptides, and proteins for use in various embodiments of the present invention. For example, specific polypeptides are assayed for their abilities to elicit an immune response. In specific embodiments, all or part of the proteins of the invention can also be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, (1984); Tam *et al.*, (1983); Merrifield, (1986); and Barany and Merrifield (1979), each incorporated herein by reference. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

#### **1. *In Vitro* Protein Production**

One embodiment of the invention includes the use of gene transfer to cells, including microorganisms, for the production and/or presentation of proteins. The gene for the protein of interest may be transferred into appropriate host cells followed by culture of cells under the appropriate conditions. A nucleic acid encoding virtually any polypeptide may be employed. The generation of recombinant expression vectors, and the elements included therein, are discussed herein. Alternatively, the



protein to be produced may be an endogenous protein normally synthesized by the cell used for protein production.

Another embodiment of the present invention uses autologous B lymphocyte cell lines, which are transfected with a viral vector that expresses an immunogen product, and more specifically, a protein having immunogenic activity. Other examples of mammalian host cell lines include, but are not limited to Vero and HeLa cells, other B- and T- cell lines, such as CEM, 721.221, H9, Jurkat, Raji, as well as cell lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, 3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or that modifies and processes the gene product in the manner desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

A number of selection systems may be used including, but not limited to HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes, in tk-, hgprt- or aprt- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection: for dhfr, which confers resistance to trimethoprim and methotrexate; gpt, which confers resistance to mycophenolic acid; neo, which confers resistance to the aminoglycoside G418; and hygromycin, which confers resistance to hygromycin.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage-dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

### III. NUCLEIC ACIDS

The present invention concerns recombinant polynucleotides encoding the proteins, polypeptides, peptides of the invention. The nucleic acid sequences for wild-type EsxA, EsxB, or any other polypeptide transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or other surface proteins or sortase substrates, are included, all of which are incorporated by reference, and can be used to prepare an EsxA, EsxB, or any other polypeptide transported by the Ess pathway, and/or SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, IsdC, SasF or other sortase substrates. Similarly, nucleic acid sequences encoding any other wild-type protein that is transported by the Ess pathway or are substrates for sortase mechanism are included.

As used in this application, the term “polynucleotide” refers to a nucleic acid molecule that either is recombinant or has been isolated free of total genomic nucleic acid. Included within the term “polynucleotide” are oligonucleotides (nucleic acids 100 residues or less in length), recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like. Polynucleotides include, in certain aspects, regulatory sequences, isolated substantially away from their naturally occurring genes or protein encoding sequences. Polynucleotides may be RNA, DNA, analogs thereof, or a combination thereof.

In this respect, the term “gene,” “polynucleotide” or “nucleic acid” is used for to refer to a nucleic acid that encodes a protein, polypeptide, or peptide (including any sequences required for proper transcription, post-translational modification, or localization). As will be understood by those in the art, this term encompasses genomic sequences, expression cassettes, cDNA sequences, and smaller engineered nucleic acid segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a polypeptide may contain a contiguous nucleic acid sequence encoding all or a portion of such a polypeptide of the following lengths: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880,

890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040, 1050, 1060, 1070, 1080, 1090, 1095, 1100, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 9000, 10000, or more nucleotides, nucleosides, or base pairs. It also is contemplated that a particular polypeptide from a  
5 given species may be encoded by nucleic acids containing natural variations that having slightly different nucleic acid sequences but, nonetheless, encode the same or substantially similar protein (see Table 2 above).

In particular embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode  
10 an EsxA, EsxB, or any other protein transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or other sortase substrates. Thus, an isolated nucleic acid segment or vector containing a nucleic acid segment may encode, for example, an EsxA, EsxB, or other Ess pathway protein, and/or SdrD, SdrE, IsdA, IsdB, or other sortase substrates that is immunogenic. The term "recombinant" may be used in conjunction  
15 with a polypeptide or the name of a specific polypeptide, and this generally refers to a polypeptide produced from a nucleic acid molecule that has been manipulated *in vitro* or that is a replication product of such a molecule.

In other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating nucleic acid sequences that encode a EsxA,  
20 EsxB, or other Ess transported polypeptide or peptide, and/or SdrD, SdrE, IsdA, IsdB, or other sortase substrate polypeptide or peptide that can be used to generate an immune response in a subject. In various embodiments the nucleic acids of the invention may be used in genetic vaccines.

The nucleic acid segments used in the present invention, regardless of the  
25 length of the coding sequence itself, may be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably  
30 being limited by the ease of preparation and use in the intended recombinant nucleic acid protocol. In some cases, a nucleic acid sequence may encode a polypeptide sequence with additional heterologous coding sequences, for example to allow for

purification of the polypeptide, transport, secretion, post-translational modification, or for therapeutic benefits such as targeting or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a polypeptide that is not the same as the  
5 modified polypeptide.

The nucleic acid used in the present invention encode EsxA, EsxB, or any other peptide or protein from a polypeptide transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or any other peptides or protein processed by the sortase mechanism. Such sequences may arise as a consequence of codon redundancy and  
10 functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes  
15 designed by human may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein.

In certain other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors that include within their sequence a contiguous  
20 nucleic acid sequence from SEQ ID NO:1 (EsxA), SEQ ID NO:3 (EsxB), SEQ ID NO:5 (SdrD), SEQ ID NO:7 (SdrE), SEQ ID NO:9 (IsdA), SEQ ID NO:11 (IsdB), SEQ ID NO:13 (Spa), SEQ ID NO:15 (ClfB), SEQ ID NO:17 (IsdC), SEQ ID NO:19 (SasF), SEQ ID NO:21 (SdrC), SEQ ID NO:23 (ClfA) or any other nucleic acid sequences encoding secreted virulence factors and/or surface proteins including  
25 proteins transported by the Ess pathway, processed by sortase, or proteins incorporated herein by reference.

#### **A. Vectors**

Polypeptides of the invention may be encoded by a nucleic acid molecule comprised in a vector. The term "vector" is used to refer to a carrier nucleic acid  
30 molecule into which a heterologous nucleic acid sequence can be inserted for introduction into a cell where it can be replicated and expressed. A nucleic acid

sequence can be “heterologous,” which means that it is in a context foreign to the cell in which the vector is being introduced, which includes a sequence homologous to a sequence in the cell but in a position within the host cell where it is ordinarily not found. Vectors include DNAs, RNAs, plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (for example Sambrook *et al.*, 2001; Ausubel *et al.*, 1996, both incorporated herein by reference). In addition to encoding an EsxA, EsxB, or other Ess transported polypeptide, and/or SdrD, SdrE, IsdA, IsdB, or any other peptides or protein processed by sortase, a vector may encode polypeptide sequences such as a tag or immunogenicity enhancing peptide. Useful vectors encoding such fusion proteins include pIN vectors (Inouye *et al.*, 1985), vectors encoding a stretch of histidines, and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage.

Vectors of the invention may be used in a host cell to produce an EsxA, EsxB, or other Ess transported polypeptide, and/or a SdrD, SdrE, IsdA, IsdB, or any other peptides or protein processed by the sortase mechanism that may subsequently be purified for administration to a subject or the vector may be purified for direct administration to a subject for expression of the protein in the subject.

The term “expression vector” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

### 1. Promoters and Enhancers

A “promoter” is a control sequence. The promoter is typically a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It

may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon. Such a promoter can be referred to as “endogenous.” Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural state. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not “naturally occurring,” *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR<sup>TM</sup>, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference).

Naturally, it may be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression (see Sambrook *et al.*, 2001, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, or inducible and in certain

embodiments may direct high level expression of the introduced DNA segment under specified conditions, such as large-scale production of recombinant proteins or peptides.

Various elements/promoters that may be employed in the context of the present invention to regulate the expression of a gene. Examples of such inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus, include but are not limited to Immunoglobulin Heavy Chain (Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Atchinson *et al.*, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990), Immunoglobulin Light Chain (Queen *et al.*, 1983; Picard *et al.*, 1984), T Cell Receptor (Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990), HLA DQ  $\alpha$  and/or DQ  $\beta$  (Sullivan *et al.*, 1987),  $\beta$  Interferon (Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988), Interleukin-2 (Greene *et al.*, 1989), Interleukin-2 Receptor (Greene *et al.*, 1989; Lin *et al.*, 1990), MHC Class II 5 (Koch *et al.*, 1989), MHC Class II HLA-DR $\alpha$  (Sherman *et al.*, 1989),  $\beta$ -Actin (Kawamoto *et al.*, 1988; Ng *et al.*; 1989), Muscle Creatine Kinase (MCK) (Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989), Prealbumin (Transthyretin) (Costa *et al.*, 1988), Elastase I (Omitz *et al.*, 1987), Metallothionein (MTII) (Karin *et al.*, 1987; Culotta *et al.*, 1989), Collagenase (Pinkert *et al.*, 1987; Angel *et al.*, 1987), Albumin (Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990),  $\alpha$ -Fetoprotein (Godbout *et al.*, 1988; Campere *et al.*, 1989),  $\gamma$ -Globin (Bodine *et al.*, 1987; Perez-Stable *et al.*, 1990),  $\beta$ -Globin (Trudel *et al.*, 1987), c-fos (Cohen *et al.*, 1987), c-HA-ras (Triesman, 1986; Deschamps *et al.*, 1985), Insulin (Edlund *et al.*, 1985), Neural Cell Adhesion Molecule (NCAM) (Hirsh *et al.*, 1990),  $\alpha$ 1-Antitrypsin (Latimer *et al.*, 1990), H2B (TH2B) Histone (Hwang *et al.*, 1990), Mouse and/or Type I Collagen (Ripe *et al.*, 1989), Glucose-Regulated Proteins (GRP94 and GRP78) (Chang *et al.*, 1989), Rat Growth Hormone (Larsen *et al.*, 1986), Human Serum Amyloid A (SAA) (Edbrooke *et al.*, 1989), Troponin I (TN I) (Yutzey *et al.*, 1989), Platelet-Derived Growth Factor (PDGF) (Pech *et al.*, 1989), Duchenne Muscular Dystrophy (Klamut *et al.*, 1990), SV40 (Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleight *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988), Polyoma (Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981;

Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell *et al.*, 1988), Retroviruses (Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman *et al.*, 1989), Papilloma Virus (Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987), Hepatitis B Virus (Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988), Human Immunodeficiency Virus (Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989), Cytomegalovirus (CMV) IE (Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986), Gibbon Ape Leukemia Virus (Holbrook *et al.*, 1987; Quinn *et al.*, 1989).

15 Inducible Elements include, but are not limited to MT II - Phorbol Ester (TFA)/Heavy metals (Palmiter *et al.*, 1982; Haslinger *et al.*, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989); MMTV (mouse mammary tumor virus) – Glucocorticoids (Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988);  $\beta$ -Interferon - poly(rI)x/poly(rc) (Tavernier *et al.*, 1983); Adenovirus 5 E2 – E1A (Imperiale *et al.*, 1984); Collagenase - Phorbol Ester (TPA) (Angel *et al.*, 1987a); Stromelysin - Phorbol Ester (TPA) (Angel *et al.*, 1987b); SV40 - Phorbol Ester (TPA) (Angel *et al.*, 1987b); Murine MX Gene - Interferon, Newcastle Disease Virus (Hug *et al.*, 1988); GRP78 Gene - A23187 (Resendez *et al.*, 1988);  $\alpha$ -2-Macroglobulin - IL-6 (Kunz *et al.*, 1989); Vimentin – Serum (Rittling *et al.*, 1989); MHC Class I Gene H-2kb – Interferon (Blanar *et al.*, 1989); HSP70 – E1A/SV40 Large T Antigen (Taylor *et al.*, 1989, 1990a, 1990b); Proliferin - Phorbol Ester/TPA (Mordacq *et al.*, 1989); Tumor Necrosis Factor – PMA (Hensel *et al.*, 1989); and Thyroid Stimulating Hormone  $\alpha$  Gene - Thyroid Hormone (Chatterjee *et al.*, 1989).

Also contemplated as useful in the present invention are the dectin-1 and dectin-2 promoters. Additionally any promoter/enhancer combination (as per the



Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes, protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest.

The particular promoter that is employed to control the expression of peptide  
5 or protein encoding polynucleotide of the invention is not believed to be critical, so long as it is capable of expressing the polynucleotide in a targeted cell, preferably a bacterial cell. Where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter  
10 might include either a bacterial, human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, and the Rous sarcoma virus long terminal repeat can be used to obtain high level expression of an EsxA-, EsxB-, or other Ess-related polynucleotide, and/or SdrD, SdrE, IsdA, IsdB, or any other sortase substrate  
15 related polynucleotide. The use of other viral or mammalian cellular or bacterial phage promoters, which are well known in the art, to achieve expression of polynucleotides is contemplated as well.

In embodiments in which a vector is administered to a subject for expression of the protein, it is contemplated that a desirable promoter for use with the vector is  
20 one that is not down-regulated by cytokines or one that is strong enough that even if down-regulated, it produces an effective amount of an EsxA, EsxB, or other Ess transported protein, and/or SdrD, SdrE, IsdA, IsdB, or any other peptides or protein processed by sortase in a subject to elicit an immune response. Non-limiting examples of these are CMV IE and RSV LTR. In other embodiments, a promoter that  
25 is up-regulated in the presence of cytokines is employed. The MHC I promoter increases expression in the presence of IFN- $\gamma$ .

Tissue specific promoters can be used, particularly if expression is in cells in which expression of an antigen is desirable, such as dendritic cells or macrophages. The mammalian MHC I and MHC II promoters are examples of such tissue-specific  
30 promoters.

## 2. Initiation Signals and Internal Ribosome Binding Sites (IRES)

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent  
5 sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational  
10 control signals and initiation codons can be either natural or synthetic and may be operable in bacteria or mammalian cells. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES  
15 elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements  
20 can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patents 5,925,565 and  
25 5,935,819, herein incorporated by reference).

## 3. Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See  
30 Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) Frequently, a vector is linearized or fragmented using a restriction

enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

#### 4. Splicing Sites

5 Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. (See Chandler *et al.*, 1997, incorporated herein by reference.)

#### 10 5. Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A “termination signal” or “terminator” is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the  
15 production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a  
20 stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message.

25 Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the bovine growth hormone terminator or viral termination sequences, such as the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as  
30 due to a sequence truncation.

## 6. Polyadenylation Signals

In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and/or any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

## 7. Origins of Replication

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

## 8. Selectable and Screenable Markers

In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by encoding a screenable or selectable marker in the expression vector. When transcribed and translated, a marker confers an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, markers that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin or histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP for colorimetric analysis.

Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers that can be used in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a protein of the invention. Further examples of selectable and screenable markers are well known to one of skill in the art.

## B. Host Cells

As used herein, the terms “cell,” “cell line,” and “cell culture” may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors or viruses. A host cell may be “transfected” or “transformed,” which refers to a process by which exogenous nucleic acid, such as a recombinant protein-encoding sequence, is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

Host cells may be derived from prokaryotes or eukaryotes, including bacteria, yeast cells, insect cells, and mammalian cells for replication of the vector or expression of part or all of the nucleic acid sequence(s). Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials ([www.atcc.org](http://www.atcc.org)). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors or expression of encoded proteins. Bacterial cells used as host cells for vector replication and/or expression include Staphylococcus strains, DH5 $\alpha$ , JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE<sup>®</sup> Competent Cells and SOLOPACK<sup>™</sup> Gold Cells (STRATAGENE<sup>®</sup>, La Jolla, CA). Alternatively, bacterial

cells such as *E. coli* LE392 could be used as host cells for phage viruses. Appropriate yeast cells include *Saccharomyces cerevisiae*, *Saccharomyces pombe*, and *Pichia pastoris*.

5 Examples of eukaryotic host cells for replication and/or expression of a vector include HeLa, NIH3T3, Jurkat, 293, Cos, CHO, Saos, and PC12. Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either a eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector.

10 Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale  
15 production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

### C. Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be  
20 employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents  
25 5,871,986, 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MAXBAC® 2.0 from INVITROGEN® and BACPACK™ BACULOVIRUS EXPRESSION SYSTEM FROM CLONTECH®.

In addition to the disclosed expression systems of the invention, other examples of expression systems include STRATAGENE®'s COMPLETE  
30 CONTROL™ Inducible Mammalian Expression System, which involves a synthetic

ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN® also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

#### 10           **D.       Amplification of Nucleic Acids**

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 2001). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

The term “primer,” as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to sequences of genes identified herein are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids containing one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis.

Multiple rounds of amplification, also referred to as “cycles,” are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the  
5 detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals (Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known  
10 amplification methods is the polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988, each of which is incorporated herein by reference in their entirety.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents  
15 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

#### **E. Methods of Gene Transfer**

Suitable methods for nucleic acid delivery to effect expression of  
20 compositions of the present invention are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA, including viral and nonviral vectors) can be introduced into a cell, a tissue or an organism, as described herein or as would be known to one of ordinary skill in the art. Such methods include, but are not limited  
25 to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated herein by reference), including microinjection (Harland and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent No. 5,384,253, incorporated herein by reference); by  
30 calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE dextran followed by polyethylene glycol



(Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaepler *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); by Agrobacterium mediated transformation (U.S. Patents 5,591,616 and 5,563,055, each incorporated herein by reference); or by PEG mediated transformation of protoplasts (Omirulleh *et al.*, 1993; U.S. Patents 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition mediated DNA uptake (Potrykus *et al.*, 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

#### 15 IV. IMMUNE RESPONSE AND ASSAYS

As discussed above, the invention concerns evoking an immune response in a subject against a secreted virulence factor or surface protein, including EsxA, EsxB, or other polypeptide transported by the Ess pathway, and/or SdrC, SdrD, SdrE, IsdA, IsdB, Spa, ClfA, ClfB, SasF, IsdC or any other peptide or protein processed by sortase. In one embodiment, the immune response can protect against or treat a subject having, suspected of having, or at risk of developing an infection or related disease, particularly those related to staphylococci.

##### A. Immunoassays

The present invention includes the implementation of serological assays to evaluate whether and to what extent an immune response is induced or evoked by EsxA, EsxB, or any other polypeptide transported by the Ess pathway, and/or SdrD, SdrE, IsdA, IsdB, or any other sortase process peptide or protein. There are many types of immunoassays that can be implemented. Immunoassays encompassed by the present invention include, but are not limited to, those described in U.S. Patent 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent 4,452,901

(western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

Immunoassays generally are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and  
5 radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful.

In one exemplary ELISA, the antibodies or antigens are immobilized on a selected surface, such as a well in a polystyrene microtiter plate, dipstick, or column support. Then, a test composition suspected of containing the desired antigen or  
10 antibody, such as a clinical sample, is added to the wells. After binding and washing to remove non specifically bound immune complexes, the bound antigen or antibody may be detected. Detection is generally achieved by the addition of another antibody, specific for the desired antigen or antibody, that is linked to a detectable label. This type of ELISA is known as a "sandwich ELISA". Detection also may be achieved by  
15 the addition of a second antibody specific for the desired antigen, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

Variations on ELISA techniques are known to those of skill in the art. In one such variation, the samples suspected of containing a target antigen or antibody are  
20 immobilized onto the well surface and then contacted with the antibodies or antigens of the invention. After binding and appropriate washing, the bound immune complexes are detected. Where the initial antigen specific antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the  
25 first antigen specific antibody, with the second antibody being linked to a detectable label.

Competition ELISAs are also possible in which test samples compete for binding with known amounts of labeled antigens or antibodies. The amount of reactive species in the unknown sample is determined by mixing the sample with the  
30 known labeled species before or during incubation with coated wells. The presence of

reactive species in the sample acts to reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non specifically  
5 bound species, and detecting the bound immune complexes.

Antigen or antibodies may also be linked to a solid support, such as in the form of plate, beads, dipstick, membrane, or column matrix, and the sample to be analyzed is applied to the immobilized antigen or antibody. In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a  
10 solution of the antigen or antibody, either overnight or for a specified period. The wells of the plate will then be washed to remove incompletely-adsorbed material. Any remaining available surfaces of the wells are then “coated” with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein, and solutions of milk powder. The coating  
15 allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of the antigen or antibody to the  
20 well, coating with a non reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or  
25 antibody in conjunction with a labeled tertiary antibody or third binding ligand.

“Under conditions effective to allow immune complex (antigen/antibody) formation” means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction  
30 of nonspecific background.

The suitable conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

5           After all incubation steps in an ELISA are followed, the contacted surface is washed so as to remove non complexed material. Washing often includes washing with a solution of PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes  
10   may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or  
15   second immune complex with a urease, glucose oxidase, alkaline phosphatase, or hydrogen peroxidase conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation, *e.g.*, incubation for 2 hours at room temperature in a PBS containing solution such as PBS Tween.

After incubation with the labeled antibody, and subsequent to washing to  
20   remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2' azino-di(3-ethyl benzthiazoline-6-sulfonic acid [ABTS] and H<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer. Alternatively, the label  
25   may be a chemiluminescent label (see, U.S. Patents 5,310,687, 5,238,808 and 5,221,605).

## **B.       Diagnosis of Bacterial Infection**

In addition to the use of proteins, polypeptides, and/or peptides, as well as antibodies binding these polypeptides, proteins, and/or peptides to treat or prevent  
30   infection as described above, the present invention contemplates the use of these polypeptides, proteins, peptides, and/or antibodies in a variety of ways, including the

detection of the presence of Staphylococci to diagnose an infection, whether in a patient or on medical equipment which may also become infected. In accordance with the invention, a preferred method of detecting the presence of infections involves the steps of obtaining a sample suspected of being infected by one or more staphylococcal bacteria species or strains, such as a sample taken from an individual, for example, from one's blood, saliva, tissues, bone, muscle, cartilage, or skin. Following isolation of the sample, diagnostic assays utilizing the polypeptides, proteins, peptides, and/or antibodies of the present invention may be carried out to detect the presence of staphylococci, and such assay techniques for determining such presence in a sample are well known to those skilled in the art and include methods such as radioimmunoassay, western blot analysis and ELISA assays. In general, in accordance with the invention, a method of diagnosing an infection is contemplated wherein a sample suspected of being infected with staphylococci has added to it the polypeptide, protein, peptide, antibody, or monoclonal antibody in accordance with the present invention, and staphylococci are indicated by antibody binding to the polypeptides, proteins, and/or peptides, or polypeptides, proteins, and/or peptides binding to the antibodies in the sample.

Accordingly, antibodies in accordance with the invention may be used for the prevention of infection from staphylococcal bacteria, for the treatment of an ongoing infection, or for use as research tools. The term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, such as those fragments which maintain the binding specificity of the antibodies, including the products of an Fab immunoglobulin expression library. Accordingly, the invention contemplates the use of single chains such as the variable heavy and light chains of the antibodies. Generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art. Specific examples of the generation of an antibody to a bacterial protein can be found in U.S. Patent Application Pub. No. 20030153022, which is incorporated herein by reference in its entirety.

Any of the above described polypeptides, proteins, peptides, and/or antibodies may be labeled directly with a detectable label for identification and quantification of staphylococcal bacteria. Labels for use in immunoassays are generally known to

those skilled in the art and include enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances, including colored particles such as colloidal gold or latex beads. Suitable immunoassays include enzyme-linked immunosorbent assays (ELISA).

5           **C.     Protective Immunity**

In some embodiments of the invention, proteinaceous compositions confer protective immunity on a subject. Protective immunity refers to a body's ability to mount a specific immune response that protects the subject from developing a particular disease or condition that involves the agent against which there is an  
10 immune response. An immunogenically effective amount is capable of conferring protective immunity to the subject.

As used herein in the specification and in the claims section that follows, the term polypeptide refers to a stretch of amino acids covalently linked there amongst via peptide bonds. Different polypeptides have different functionalities according to  
15 the present invention. While according to one aspect a polypeptide is derived from an immunogen designed to induce an active immune response in a recipient, according to another aspect of the invention, a polypeptide is derived from an antibody which results following the elicitation of an active immune response, in, for example, an animal, and which can serve to induce a passive immune response in the recipient. In  
20 both cases, however, the polypeptide is encoded by a polynucleotide according to any possible codon usage.

As used herein the phrase "immune response" or its equivalent "immunological response" refers to the development of a humoral (antibody mediated), cellular (mediated by antigen-specific T cells or their secretion products)  
25 or both humoral and cellular response directed against a protein, peptide, or polypeptide of the invention in a recipient patient. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibody, antibody containing material, or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in  
30 association with Class I or Class II MHC molecules, to activate antigen-specific CD4 (+) T helper cells and/or CD8 (+) cytotoxic T cells. The response may also involve

activation of monocytes, macrophages, NK cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity.

As used herein "active immunity" refers to any immunity conferred upon a subject by administration of an antigen.

5 As used herein "passive immunity" refers to any immunity conferred upon a subject without administration of an antigen to the subject. "Passive immunity" therefore includes, but is not limited to, administration of activated immune effectors including cellular mediators or protein mediators (*e.g.*, monoclonal and/or polyclonal antibodies) of an immune response. A monoclonal or polyclonal antibody  
10 composition may be used in passive immunization for the prevention or treatment of infection by organisms that carry the antigen recognized by the antibody. An antibody composition may include antibodies that bind to a variety of antigens that may in turn be associated with various organisms. The antibody component can be a polyclonal antiserum. In certain aspects the antibody or antibodies are affinity  
15 purified from an animal or second subject that has been challenged with an antigen(s). Alternatively, an antibody mixture may be used, which is a mixture of monoclonal and/or polyclonal antibodies to antigens present in the same, related, or different microbes or organisms, such as gram-positive bacteria, gram-negative bacteria, including but not limited to staphylococcus bacteria.

20 Passive immunity may be imparted to a patient or subject by administering to the patient immunoglobulins (Ig) and/or other immune factors obtained from a donor or other non-patient source having a known immunoreactivity. In other aspects, an antigenic composition of the present invention can be administered to a subject who then acts as a source or donor for globulin, produced in response to challenge from the  
25 composition ("hyperimmune globulin"), that contains antibodies directed against Staphylococcus or other organism. A subject thus treated would donate plasma from which hyperimmune globulin would then be obtained, *via* conventional plasma-fractionation methodology, and administered to another subject in order to impart resistance against or to treat staphylococcus infection. Hyperimmune globulins  
30 according to the invention are particularly useful for immune-compromised individuals, for individuals undergoing invasive procedures or where time does not permit the individual to produce his own antibodies in response to vaccination. See

U.S. Patents 6,936,258, 6,770,278, 6,756,361, 5,548,066, 5,512,282, 4,338,298, and 4,748,018, each of which is incorporated herein by reference in its entirety, for exemplary methods and compositions related to passive immunity.

For purposes of this specification and the accompanying claims the terms  
5 "epitope" and "antigenic determinant" are used interchangeably to refer to a site on an antigen to which B and/or T cells respond or recognize. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by  
10 tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, Epitope Mapping Protocols (1996). Antibodies that  
15 recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. T-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by in vitro assays that measure antigen-dependent proliferation, as determined by <sup>3</sup>H-  
20 thymidine incorporation by primed T cells in response to an epitope (Burke *et al.*, 1994), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges *et al.*, 1996) or by cytokine secretion.

The presence of a cell-mediated immunological response can be determined by proliferation assays (CD4 (+) T cells) or CTL (cytotoxic T lymphocyte) assays.  
25 The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating IgG and T-cells from an immunized syngeneic animal and measuring protective or therapeutic effect in a second subject.

As used herein and in the claims, the terms "antibody" or "immunoglobulin"  
30 are used interchangeably and refer to any of several classes of structurally related proteins that function as part of the immune response of an animal or recipient, which proteins include IgG, IgD, IgE, IgA, IgM and related proteins.



Under normal physiological conditions antibodies are found in plasma and other body fluids and in the membrane of certain cells and are produced by lymphocytes of the type denoted B cells or their functional equivalent. Antibodies of the IgG class are made up of four polypeptide chains linked together by disulfide  
5 bonds. The four chains of intact IgG molecules are two identical heavy chains referred to as H-chains and two identical light chains referred to as L-chains.

In order to produce polyclonal antibodies, a host, such as a rabbit or goat, is immunized with the antigen or antigen fragment, generally with an adjuvant and, if necessary, coupled to a carrier. Antibodies to the antigen are subsequently collected  
10 from the sera of the host. The polyclonal antibody can be affinity purified against the antigen rendering it monospecific.

In order to produce monoclonal antibodies, hyperimmunization of an appropriate donor, generally a mouse, with the antigen is undertaken. Isolation of splenic antibody producing cells is then carried out. These cells are fused to a cell  
15 characterized by immortality, such as a myeloma cell, to provide a fused cell hybrid (hybridoma) which can be maintained in culture and which secretes the required monoclonal antibody. The cells are then be cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use. By definition, monoclonal antibodies are specific to a single epitope. Monoclonal antibodies often have lower  
20 affinity constants than polyclonal antibodies raised against similar antigens for this reason.

Monoclonal antibodies may also be produced *ex-vivo* by use of primary cultures of splenic cells or cell lines derived from spleen (Anavi, 1998). In order to produce recombinant antibody (see generally Huston *et al.*, 1991; Johnson *et al.*,  
25 1991; Mernaugh *et al.*, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complementary DNAs (cDNAs). Antibody cDNA, which can be full length or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The  
30 antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone *et al.* (1982). The binding of antibodies to a solid support substrate is also well  
5 known in the art (Harlow *et al.*, 1988; Borrebaeck, 1992).

As used herein and in the claims, the phrase "an immunological portion of an antibody" include a Fab fragment of an antibody, a Fv fragment of an antibody, a heavy chain of an antibody, a light chain of an antibody, an unassociated mixture of a heavy chain and a light chain of an antibody, a heterodimer consisting of a heavy  
10 chain and a light chain of an antibody, a catalytic domain of a heavy chain of an antibody, a catalytic domain of a light chain of an antibody, a variable fragment of a light chain of an antibody, a variable fragment of a heavy chain of an antibody, and a single chain variant of an antibody, which is also known as scFv. In addition, the term includes chimeric immunoglobulins which are the expression products of fused  
15 genes derived from different species, one of the species can be a human, in which case a chimeric immunoglobulin is said to be humanized. Typically, an immunological portion of an antibody competes with the intact antibody from which it was derived for specific binding to an antigen.

Optionally, an antibody or preferably an immunological portion of an  
20 antibody, can be chemically conjugated to, or expressed as, a fusion protein with other proteins. For purposes of this specification and the accompanying claims, all such fused proteins are included in the definition of antibodies or an immunological portion of an antibody.

As used herein the terms "immunogenic agent" or "immunogen" or "antigen"  
25 are used interchangeably to describe a molecule capable of inducing an immunological response against itself on administration to a recipient, either alone, in conjunction with an adjuvant, or presented on a display vehicle.

#### **D. Treatment Methods**

A method of the present invention includes treatment for a disease or  
30 condition caused by a staphylococcus pathogen. An immunogenic polypeptide of the invention can be given to induce an immune response in a person infected with

staphylococcus or suspected of having been exposed to staphylococcus. Methods may be employed with respect to individuals who have tested positive for exposure to staphylococcus or who are deemed to be at risk for infection based on possible exposure.

5 In some embodiments, the treatment is administered in the presence of adjuvants or carriers or other staphylococcal antigens. Furthermore, in some examples, treatment comprises administration of other agents commonly used against bacterial infection, such as one or more antibiotics.

10 The use of peptides for vaccination typically requires conjugation of the peptide to an immunogenic carrier protein, such as hepatitis B surface antigen, keyhole limpet hemocyanin, or bovine serum albumin. Methods for performing this conjugation are well known in the art.

## V. VACCINE AND OTHER PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

### 15 A. Vaccines

The present invention includes methods for preventing or ameliorating staphylococcus infections. As such, the invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly  
20 from immunogenic secreted virulence proteins or surface proteins, including EsxA, EsxB, or any other polypeptide transported by the Ess pathway, and/or SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, Spa, ClfA, ClfB, SasF or any other sortase processed peptide or protein prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or  
25 lyophilized for more ready formulation into a desired vehicle. The invention includes compositions that can be used to induce an immune response against a polypeptide or peptide derived from a member of the Ess pathway, in certain aspects an Esx protein and more specifically an EsxA, EsxB, or any other polypeptide transported by the Ess pathway, and/or a polypeptide or peptide derived from a peptide or protein processed  
30 by the sortase pathway, in certain aspects, SdrD, SdrE, IsdA, IsdB, or any other sortase processed peptide or protein so as to protect against infection by a

staphylococcus and against developing a condition or disease caused by such a pathogen.

Alternatively, other viable and important options for a protein/peptide-based vaccine involve introducing nucleic acids encoding the antigen(s) as DNA vaccines. In this regard, recent reports described construction of recombinant vaccinia viruses expressing either 10 contiguous minimal CTL epitopes (Thomson, 1996) or a combination of B cell, CTL, and TH epitopes from several microbes (An, 1997), and successful use of such constructs to immunize mice for priming protective immune responses. Thus, there is ample evidence in the literature for successful utilization of peptides, peptide-pulsed APCs, and peptide-encoding constructs for efficient *in vivo* priming of protective immune responses. The use of nucleic acid sequences as vaccines is exemplified in U.S. Patents 5,958,895 and 5,620,896.

The preparation of vaccines that contain polypeptide or peptide sequence(s) as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all of which are incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions: solid forms suitable for solution in or suspension in liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants that enhance the effectiveness of the vaccines. In specific embodiments, vaccines are formulated with a combination of substances, as described in U.S. Patents 6,793,923 and 6,733,754, which are incorporated herein by reference.

Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides: such suppositories may be

formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1% to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10% to about 95% of active ingredient, preferably about 25% to about 70%.

The polypeptides and polypeptide-encoding DNA constructs may be formulated into a vaccine as neutral or salt forms. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the peptide) and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Typically, vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including the capacity of the individual's immune system to synthesize antibodies and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection and the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size and health of the subject.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations, and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at two to twelve week intervals, more usually from  
5 three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies against the antigens, as described supra, U.S. Patents 3,791,932; 4,174,384 and 3,949,064, are illustrative of these types of assays.

## 10 **1. Carriers**

A given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as  
15 ovalbumin, mouse serum albumin, or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide, and bis-biazotized benzidine.

## **2. Adjuvants**

20 The immunogenicity of polypeptide or peptide compositions can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable adjuvants include all acceptable immunostimulatory compounds, such as cytokines, toxins, or synthetic compositions.

A number of adjuvants can be used to enhance an antibody response against  
25 an EsxA, EsxB, or any other polypeptide transported by the Ess pathway and/or against a SdrD, SdrE, IsdA, IsdB, or any other sortase processed peptide or protein. Adjuvants can 1) trap the antigen in the body to cause a slow release; 2) attract cells involved in the immune response to the site of administration; 3) induce proliferation or activation of immune system cells; or 4) improve the spread of the antigen  
30 throughout the subject's body.

Adjuvants include, but are not limited to, oil-in-water emulsions, water-in-oil emulsions, mineral salts, polynucleotides, and natural substances. Specific adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12,  $\gamma$ -interferon, GMCSF, BCG, aluminum hydroxide or other aluminum compound, MDP compounds, such as thur-  
5 MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM), and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion. MHC antigens may even be used. Others adjuvants or methods are exemplified in U.S. Patents 6,814,971, 5,084,269, 6,656,462, each of which is  
10 incorporated herein by reference).

Various methods of achieving adjuvant affect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the  
15 protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin-treated (Fab) antibodies to albumin; mixture with bacterial cells (e.g., *C. parvum*), endotoxins or lipopolysaccharide components of Gram-negative bacteria; emulsion in physiologically acceptable oil vehicles (e.g., mannide  
20 mono-oleate (Aracel A)); or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed to produce an adjuvant effect.

Exemplary, often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium*  
25 *tuberculosis*), incomplete Freund's adjuvants, and aluminum hydroxide.

In addition to adjuvants, it may be desirable to co-administer biologic response modifiers (BRM) to enhance immune responses. BRMs have been shown to upregulate T cell immunity or downregulate suppresser cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); or  
30 low-dose Cyclophosphamide (CYP; 300 mg/m<sup>2</sup>) (Johnson/ Mead, NJ) and cytokines such as  $\gamma$ -interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B-7.

## B. Lipid Components and Moieties

In certain embodiments, the present invention concerns compositions comprising one or more lipids associated with a nucleic acid or a polypeptide/peptide. A lipid is a substance that is insoluble in water and extractable with an organic solvent. Compounds other than those specifically described herein are understood by one of skill in the art as lipids, and are encompassed by the compositions and methods of the present invention. A lipid component and a non-lipid may be attached to one another, either covalently or non-covalently.

A lipid may be a naturally occurring lipid or a synthetic lipid. However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glucolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof.

A nucleic acid molecule or a polypeptide/peptide, associated with a lipid may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid or otherwise associated with a lipid. A lipid or lipid-poxvirus-associated composition of the present invention is not limited to any particular structure. For example, they may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape. In another example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. In another non-limiting example, a lipofectamine(Gibco BRL)-poxvirus or Superfect (Qiagen)-poxvirus complex is also contemplated.

In certain embodiments, a composition may comprise about 1%, about 2%, about 3%, about 4% about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about 32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%,



about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%,  
5 about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or any range therebetween, of a particular lipid, lipid type, or non-lipid component such as an adjuvant, antigen, peptide, polypeptide, sugar, nucleic acid or other material disclosed  
10 herein or as would be known to one of skill in the art. In a non-limiting example, a composition may comprise about 10% to about 20% neutral lipids, and about 33% to about 34% of a cerebroside, and about 1% cholesterol. In another non-limiting example, a liposome may comprise about 4% to about 12% terpenes, wherein about 1% of the micelle is specifically lycopene, leaving about 3% to about 11% of the  
15 liposome as comprising other terpenes; and about 10% to about 35% phosphatidyl choline, and about 1% of a non-lipid component. Thus, it is contemplated that compositions of the present invention may comprise any of the lipids, lipid types or other components in any combination or percentage range.

### C. Combination Therapy

20 The compositions and related methods of the present invention, particularly administration of a secreted virulence factor or surface protein, including a polypeptide or peptide of a EsxA, EsxB, or other polypeptide transported by the Ess pathway, and/or a polypeptide or peptide of a SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, Spa, ClfA, ClfB, SasF or any other sortase processed peptide or protein to a  
25 patient/subject, may also be used in combination with the administration of traditional therapies. These include, but are not limited to, the administration of antibiotics such as streptomycin, ciprofloxacin, doxycycline, gentamycin, chloramphenicol, trimethoprim, sulfamethoxazole, ampicillin, tetracycline or various combinations of antibiotics.

30 In one aspect, it is contemplated that a polypeptide vaccine and/or therapy is used in conjunction with antibacterial treatment. Alternatively, the therapy may precede or follow the other agent treatment by intervals ranging from minutes to

weeks. In embodiments where the other agents and/or a proteins or polynucleotides are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and antigenic composition would still be able to exert an advantageously combined effect on the subject. In such instances, it is contemplated that one may administer both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for administration significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, for example antibiotic therapy is "A" and the immunogenic molecule given as part of an immune therapy regime, such as an antigen, is "B":

	A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	A/B/B/B	B/A/B/B
	B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A		
15	B/A/B/A	B/A/A/B	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A		

Administration of the immunogenic compositions of the present invention to a patient/subject will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the EsxA-composition, EsxB-composition, or composition of any other polypeptide transported by the Ess pathway and/or a SdrD-composition, SdrE-composition, IsdA-composition, IsdB-composition, or any other sortase processed peptide or protein. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, such as hydration, may be applied in combination with the described therapy.

#### 25 **D. General Pharmaceutical Compositions**

In some embodiments, pharmaceutical compositions are administered to a subject. Different aspects of the present invention involve administering an effective amount of a composition to a subject. In some embodiments of the present invention, members of the Ess pathway and including polypeptides or peptides of the Esx class,

and/or members of sortase substrates may be administered to the patient to protect against infection by one or more staphylococcus pathogens. Alternatively, an expression vector encoding one or more such polypeptides or peptides may be given to a patient as a preventative treatment. Additionally, such compounds can be administered in combination with an antibiotic. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The phrases “pharmaceutically acceptable” or “pharmacologically acceptable” refer to molecular entities and compositions that do not produce an adverse, allergic, or other untoward reaction when administered to an animal, or human. As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in immunogenic and therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-cancer agents, can also be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including creams, lotions, mouthwashes, inhalants and the like.

The active compounds of the present invention can be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains a compound or compounds that increase the expression of an MHC class I molecule will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for use to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and, the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil, or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that it may be easily injected. It also should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The proteinaceous compositions may be formulated into a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use

in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Administration of the compositions according to the present invention will typically be via any common route. This includes, but is not limited to oral, nasal, or buccal administration. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal, intranasal, or intravenous injection. In certain embodiments, a vaccine composition may be inhaled (*e.g.*, U.S. Patent 6,651,655, which is specifically incorporated by reference). Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. As used herein, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem complications commensurate with a reasonable benefit/risk ratio. The term "pharmaceutically acceptable carrier," means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a chemical agent.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal

administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in isotonic NaCl solution and either added to hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, 5 Remington's Pharmaceutical Sciences, 1990). Some variation in dosage will necessarily occur depending on the condition of the subject. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

An effective amount of therapeutic or prophylactic composition is determined 10 based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined quantity of the composition calculated to produce the desired responses discussed above in association with its administration, *i.e.*, the appropriate route and regimen. The quantity to be administered, both according to number of treatments and unit 15 dose, depends on the protection desired.

Precise amounts of the composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the subject, route of administration, intended goal of treatment (alleviation of symptoms versus cure), and potency, stability, and toxicity of 20 the particular composition.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically or prophylactically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above.

#### 25 **E. *In Vitro*, *Ex Vivo*, or *In Vivo* Administration**

As used herein, the term *in vitro* administration refers to manipulations performed on cells removed from or outside of an animal, including, but not limited to cells in culture. The term *ex vivo* administration refers to cells which have been manipulated *in vitro*, and are subsequently administered to a living animal. The term 30 *in vivo* administration includes all manipulations performed within an animal.

In certain aspects of the present invention, the compositions may be administered either *in vitro*, *ex vivo*, or *in vivo*. In certain *in vitro* embodiments, autologous B-lymphocyte cell lines are incubated with a virus vector of the instant invention for 24 to 48 hours or with EsxA, EsxB, or any other polypeptide transported  
5 by the Ess pathway, and/or a polypeptide or peptide of a SdrC, SdrD, SdrE, IsdA, IsdB, IsdC, Spa, ClfA, ClfB, SasF or any other sortase processed peptide or protein (or any combination thereof) for two hours. The transduced cells can then be used for *in vitro* analysis, or alternatively for *ex vivo* administration.

U.S. Patents 4,690,915 and 5,199,942, both incorporated herein by reference,  
10 disclose methods for *ex vivo* manipulation of blood mononuclear cells and bone marrow cells for use in therapeutic applications.

### EXAMPLES

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any  
15 fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the  
20 invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

#### EXAMPLE 1

##### **Staphylococcus Mutant in *esxA*, *esxB* or *essC* are Defective in the Pathogenesis of 25 Staphylococcal Abscesses**

*Staphylococcus aureus* host infection and dissemination within organ tissues is dependent on staphylococcal synthesis and secretion of a wide variety of virulence factors (Novick, 2003). Because of the astonishing armament of staphylococci with virulence factors, the pathogenesis of *S. aureus* infections is considered multi-factorial  
30 (Novick, 2003). Thus, with the exception of  $\alpha$ -hemolysin (*hla*) mutants (Bhakdi and Trantum-Jensen, 1991), strains carrying mutations that abrogate the expression of individual exoproteins typically do not display significant defects in the pathogenesis

of *S. aureus* infections. It was contemplated that the Ess secretion pathway is involved in the establishment of staphylococcal disease. *S. aureus* Newman variant strains *esxA24*, *essC::erm* and *esxB::erm* do not produce EsxA and EsxB. These strains were chosen for measurement of bacterial virulence. Viable staphylococci of mutant or wild-type Newman strains (approximately  $10^6$  staphylococci) were administered intravenously via retroorbital injection into mice. Four days after infection, the animals were killed. Internal organs were removed, inspected for abscess formation, and then homogenized and spread on agar medium to quantify staphylococcal replication in host tissues via colony formation (Albus *et al.*, 1991). Mutations in *esxA*, *esxB* and *essC* genes caused a significant reduction in the ability of *S. aureus* Newman to establish kidney or spleen abscesses in infected mice (FIG. 2, data not shown). Kidneys of mice infected with Newman carried a mean value of  $3.89 \times 10^7$  colony forming units (CFU)/organ while those of mice infected with strains lacking *esxA*, *esxB* and *essC* harbored a mean value of  $3.00 \times 10^4$  ( $p < 2.09 \times 10^{-5}$ ),  $6.17 \times 10^5$  ( $p < 1.2 \times 10^{-2}$ ) and  $2.51 \times 10^5$  ( $p < 5.53 \times 10^{-3}$ ) CFU/organ, respectively. It should be noted that half of the animals infected with mutant bacteria had cleared most staphylococci from the kidneys after four days, suggesting that complete clearing may occur by day five, post infection with the mutant strains. The formation of liver abscesses in infected animals was even more affected when staphylococci carried transposon insertions in *essC* and *esxB*. Homogenized livers of mice infected with Newman were found to contain a mean value of  $2.09 \times 10^6$  CFU/organ of staphylococci, while the livers of mutants lacking *esxA*, *esxB* and *essC* contained a mean value of  $9 \times 10^1$  ( $p < 3.01 \times 10^{-7}$ ),  $4.17 \times 10^2$  ( $p < 4.33 \times 10^{-6}$ ) and  $1.35 \times 10^3$  ( $p < 1.72 \times 10^{-4}$ ) CFU/organ, respectively.

25

## EXAMPLE 2

### Immune Response of Mice to EsxA and EsxB During Staphylococcal Infection

A hallmark of virulence factors secreted by pathogenic microbes is the development of specific humoral or cellular immune responses. Often such acquired immune responses neutralize the pathogenesis functions of such essential virulence factors and thereby contribute to opsono-phagocytic clearances of microbial infections (Mills *et al.*, 1997). As EsxA or EsxB represent secreted virulence factors, the inventors studied the response of the immune system of animals infected with *S. aureus* relative to these polypeptides as antigens, in particular the generation of



specific humoral immune responses. In addressing this issue, three week old BALB/c mice were infected by intravenous inoculation with  $1 \times 10^7$  cfu of *S. aureus* Newman (a sublethal dose of infection) and compared with mock-infected animals as a control. As reported above, staphylococci invade multiple tissues and seed abscesses in liver and other internal organs, which are resolved when acquired immune responses of BALB/c mice are mounted that eventually clear these infections after 10-14 days. During this time interval, the animals mount a plethora of humoral immune responses, first via the production IgM type antibodies and then via the secretion of mature IgG type antibodies (Janeway *et al.*, 1999). To examine whether animals acutely infected with *S. aureus* generate humoral immune responses to EsxA and EsxB, infected animals were euthanized via terminal bleeding on day 0 and 30 after intravenous inoculation. Serum samples were subjected to quantitative analysis for specific IgG responses using purified EsxA and EsxB in an ELISA assay. Data in FIG. 6 show that animals infected with *S. aureus* developed IgG type antibodies against EsxA and EsxB, whereas mock-infected animals did not. It is contemplated that the observed humoral immune responses confer protection against staphylococcal infection.

Efficacy of purified EsxA antigen in raising protective immunity was studied in a sub-lethal bacterial mouse challenge model (FIG. 7.). On day 0, ten four-week old BALB/c mice were immunized by intra-muscular injection with 50  $\mu$ g of purified antigen (EsxA) in phosphate buffered saline and mixed (50% v/v) with complete freund adjuvant (CFA). Immunized animals were boosted with the same antigen formulation at days 11 and 20. Two groups of ten mice from immunized and control animals (PBS/CFA 50% v/v) were challenged on day 21 after the first immunization. Animals were killed 96 h post-infection, and bacterial counts were performed on their kidneys and livers. The experiment suggests that immunization with purified EsxA antigen confers partial protection against *S. aureus* disease.

### EXAMPLE 3

#### Immunization with Purified EsxA and EsxB

Preliminary studies have shown that *bursa aurealis* insertion in *esxA*, *essC* or *esxB* caused a 3-4 log reduction in the ability of *S. aureus* strain Newman to form liver abscesses following intravenous infection of BALB/c mice (FIG. 5). The ability of mutant staphylococci to seed abscesses in internal organs was also diminished for

spleen and kidneys, indicating that Ess-mediated secretion plays generally an important role during the pathogenesis of *S. aureus* infections. Similarly severe virulence defects have only been observed for accessory gene regulation two component genes (*agr*) (Cheung *et al.* 1994), sortase (Ton-That *et al.*, 2000) or heme iron transport mutants (Skaar *et al.*, 2004), whereas mutations in single surface protein or exoprotein genes cause only small reductions in the ability of staphylococci to establish murine disease (Ni Eidhin *et al.*, 1998). In the system used by the inventors, staphylococci ( $1 \times 10^6$  or  $1 \times 10^7$  cfu of staphylococci grown in tryptic soy broth) are injected intravenously (Albus *et al.*, 1991). In the first hour after infection, staphylococci are slowly cleared from the blood stream, in part via phagocytic killing or by bacterial binding to host organ tissues (Thakker *et al.*, 1998). Staphylococci that escape killing by early innate immune responses (Thomas *et al.*, 2000) begin to replicate in infected tissues and generate pro-inflammatory responses with the release of cytokines from macrophages, neutrophils and other immune cells (Jonsson *et al.*, 2003). The resulting massive invasion of immune cells into the site of infection is accompanied by central liquefaction necrosis and formation of peripheral fibrin walls in an effort to prevent infection spread (Jonsson *et al.*, 1985). Eventually, the central liquefaction necrosis is drained to organ or skin surfaces, while infectious material is being phagocytosed and epithelia repaired by wound healing (Jonsson *et al.*, 1985; Patel *et al.*, 1987). All of these events represent physiological host responses, producing abscesses in variable degrees of development as the macroscopic and microscopic anatomic-pathological substrate of *S. aureus* infections. The experimental data reported in FIG. 5 however account only for the presence of abscesses as well as for the viability of staphylococci within infected organ tissue.

Previous work in the field sought to identify immuno-dominant antigen epitopes reactive with human serum and individual antibodies were selected because of their high titer of opsonic activity in phagocytic killing assays (Etz *et al.*, 2002). *S. aureus* whole genome expression libraries, either used as fusions to *E. coli* surface proteins (Etz *et al.*, 2002) or synthesized *via* the ribosome display method (Weichhart *et al.*, 2003) isolated either 60 (Etz *et al.*, 2002) or 84 immuno-dominant antigens (Weichhart *et al.*, 2003), respectively. EsxA and EsxB were not among the identified immuno-dominant antigens, however considering the limitations of gene expression and bacterial surface display or the limitations of using human sera without disease -

there are many reasons to suspect that these genome scale studies were far from exhaustive (Weichhart *et al.*, 2003). Since animals acutely infected with *S. aureus* mount an immune response to EsxA and EsxB as judged by our ELISA experiment (FIG. 6), the efficacy of purified antigens of raising protective immunity will be tested  
5 in sub-lethal bacterial mouse challenge models. On day 0, mice will be immunized by intra-muscular injection with 100 µg of purified antigen (EsxA or EsxB or both) in phosphate buffered saline and adsorbed to adjuvant by mixing (50% v/v). Immunized animals will then be boosted with the same antigen formulation after one week (day 7). Blood samples will be withdrawn on day 0, 7 and 21, either before or during  
10 immunization for each animal, blood cells, and clotting factors will be removed and the serum examined for immune-reactivity to purified recombinant staphylococcal antigen using an ELISA based assay. The data output of these experiments are the relative levels of IgG or IgM antibodies that bind to each antigen, thereby documenting a successful immunization. Groups of 5 mice from immunized and  
15 control animals will be challenged on day 21 after the first immunization. Animals will be euthanized 96 h post-infection, and bacterial counts will be performed on their kidneys, livers, and peritoneal lavage fluids. These experiments will allow us to measure whether specific EsxA and/or EsxB antibodies induced by active immunization confer protection against *S. aureus* disease.

20

#### EXAMPLE 4

##### EsxA And EsxB Are Secreted By An Esat-6-Like System

###### A. Materials and Methods

**Strains, Media, and Growth Conditions.** *Escherichia coli* and *S. aureus* were grown in Luria–Bertani broth and tryptic soy broth at 37°C, respectively. Ampicillin and erythromycin were used at 100 mg/liter and 10 mg/liter, respectively. *essABC*,  
25 *esaAB*, and *esxB* mutants were obtained from the Phoenix (ΦNE) library (Bae *et al.*, 2004) (Table 2). Each Phoenix isolate is a derivative of the clinical isolate Newman (Kuroda *et al.*, 2001) and carries the transposon *bursa aurealis* at a site on the chromosome that has been determined by DNA sequencing (Bae *et al.*, 2004) and  
30 compared to *S. aureus* Mu50 genomic sequence (Kuroda *et al.*, 2001). All *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman by using bacteriophage φ85 (Mazmanian *et al.*, 2000).

**Culture Fractionation.** Cultures were grown to an optical density of 0.8 at 660 nm (OD<sub>660</sub>), and 1.5 ml of culture was centrifuged at 10,000 x g for 4 min. Proteins in 1 ml of supernatant were precipitated with 7.5% trichloroacetic acid (TCA), using deoxycholic acid (0.2%) as a carrier, and sedimented by centrifugation  
5 10,000 x g for 10 min (medium fraction). Culture (1.5 ml) was incubated in the presence of lysostaphin (100 µg/ml) for 30 min at 37°C. A 1-ml aliquot was precipitated with TCA (total culture). For extraction of proteins that are associated with the cell wall (loosely associated fraction), the cells of 1.5 ml of culture were washed three times with an equal volume of TSM buffer (50 mM Tris-HCl, pH 7.5  
10 / 0.5 M sucrose / 10 mM MgCl<sub>2</sub>).

A 1-ml cell suspension was transferred to a fresh tube and precipitated with TCA. All TCA precipitates were washed with ice-cold acetone, solubilized in 50 µl of 0.5 M Tris-HCl (pH 8.0) / 4% SDS and heated at 90°C for 10 min. Proteins were separated on SDS-PAGE and transferred to poly(vinylidene difluoride) membrane for  
15 immunoblot analysis with appropriate polyclonal antibodies. Immunoreactive signals were revealed by using a secondary antibody coupled to horseradish peroxidase and chemiluminescence.

**Staphylococcal Fractionation.** Cultures were centrifuged as described above and supernatants TCA precipitated in the presence of deoxycholic acid (medium).  
20 Cell pellets were suspended in 1 ml TSM buffer containing 100 µg/ml lysostaphin and incubated at 37°C for 30 min. Protoplasts were collected by centrifugation at 10,000 x g for 10 min, and the supernatant (cell wall fraction) was precipitated with TCA.

The protoplasts were suspended in membrane buffer (0.1 M Tris-HCl, pH 7.5 /  
25 0.1 M NaCl / 10 mM MgCl<sub>2</sub>) and subjected to five rounds of freeze-thaw in a dry ice ethanol bath. Soluble proteins (cytoplasmic fraction) were separated from insoluble materials and membranes (membrane fraction) by centrifugation at 100,000 x g for 30 min. All samples were TCA-precipitated before immunoblotting.

**Murine Abscess Model.** *S. aureus* strains were grown at 37°C overnight in  
30 tryptic soy broth, diluted 100-fold in fresh broth and incubated at 37°C until an OD<sub>660</sub> of 0.4 was reached. Cells were washed and suspended in PBS, and 100 µl of

bacterial suspension was injected intravenously into 10 6- to 8-week-old female BALB-c mice anesthetized by intraperitoneal injection of 100 mg/ml ketamine and 2 mg/ml xylazine. Viable staphylococci were enumerated by colony formation on tryptic soy agar to quantify the infection dose [ $\sim 10^6$  colony forming units (cfu)/ml].

5 Four days after challenge, mice were killed by CO<sub>2</sub> asphyxiation. Spleen, kidneys, and liver were removed, and organs were homogenized in 1 ml of 1% Triton X-100 in PBS. Dilutions of the homogenates were plated on agar for enumeration of viable staphylococci. The statistical analysis of the data were performed with Student's t test using ANALYZE-IT (Analyze-It Software, Leeds, England).

## 10 B. Results

*A Cluster of S. aureus Genes Encoding ESAT-6-Like Proteins.* *S. aureus* EsxA and EsxB correspond to the Mu50 ORFs SAV0282 and SAV0290 (Kuroda *et al.*, 2001) and display 20.8% and 17.8% identity as well as 25% and 35% similarity to *M. tuberculosis* EsxA, respectively (FIG. 1A). The peptide sequences of *S. aureus*

15 EsxA and EsxB encompass the WXG motif, a signature sequence of ESAT-6-like proteins (Pallen *et al.*, 2002) (FIG. 1A). EsxA and EsxB are encoded within a cluster comprised of eight predicted ORFs (*esxA*, *esaA*, *essa*, *esaB*, *essB*, *essC*, *esaC*, and *esxB*) as shown in FIG. 1B (*esa*, ESAT-6 secretion accessory gene). Beside *esxA* and *esxB*, the other genes in this cluster appear to be unique in the chromosome. The

20 cluster is referred to as the Ess cluster. Genes located immediately downstream of *esxB* are not depicted in FIG. 1B. However, their conservation in other related clusters from low G+C organisms suggest that they may also be functionally associated with the Ess cluster. The deduced peptide sequence of one of these genes, *essC*, displays similarity with FSD proteins of the *M. tuberculosis* RD1 cluster (FIG.

25 1B). Because of a sequencing error, *essC* entry in the Mu50 genome appears as two ORFs (SAV0287–SAV0288) when it is, in fact, a single combined ORF (data not shown). FIG. 1C shows the membrane topology of four proteins, EsaA, EssA, EssB, and EssC, as predicted by the TMHMM algorithm ([www.cbs.dtu.dk/services/TMHMM-2.0](http://www.cbs.dtu.dk/services/TMHMM-2.0)) and PSORT-B (Gardy *et al.*, 2003). Four

30 other factors (EsxA, EsxB, EsaB, and EsaC) are predicted by the same computational methods to be soluble, and not membrane associated. A summary of the relevant features of proteins encoded by the *ess* locus is presented in Table 3.

**Table 3. *S. aureus* strains used in this study**

Strain	Genotype	<i>bursa aurealis</i> insertion/mutation			Phenotype	
		nt position	AA position	Orientation	EsxA secretion	EsxB secretion
Newman	Clinical isolate				Yes	Yes
ΦNE187-01	<i>esxA24</i>	24	8	NA	No	No
ΦNE 09349	<i>esaA::erm</i>	1110	370	(+)	Yes	Yes
ΦNE 03742	<i>essA::erm</i>	121	41	(-)	No	No
ΦNE 13152	<i>esaB::erm</i>	43	15	(+)	Yes	Yes
ΦNE 02411	<i>essB::erm</i>	199	67	(+)	No	No
ΦNE 04464	<i>essC::erm</i>	792	264	(-)	No	No
ΦNE 02191	<i>essC::erm</i>	1854	618	(-)	No	No
ΦNE 02832	<i>essC::erm</i>	2161	721	(+)	Low	Low
ΦNE 13038	<i>essC::erm</i>	3835	1,279	(+)	Yes	Yes
ΦNE 07912	<i>esxB::erm</i>	2	1	(+)	No	No

All Phoenix strains (ΦNE) are isogenic to *S. aureus* Newman (Duthie and Lorenz, 1952) and are part of inventor's laboratory collection (Bae *et al.*, 2004). In strain *esxA24*, a stop codon replaces the coding codon at position 8. AA, amino acid; *erm*, erythromycin resistance gene; nt, nucleotide.

*EsxA and EsxB Are Secreted into the Culture Medium of S. aureus.* To study the location of EsxA and EsxB, cultures of *S. aureus* strain Newman were centrifuged and bacterial cells in the sediment were separated from the supernatant containing conditioned culture medium. Staphylococci were suspended in buffer and the cell wall was digested with lysostaphin, an endopeptidase that cleaves staphylococcal peptidoglycan (Schindler and Schuhardt, 1964). Proteins in the total lysate of staphylococci (T) or the culture medium (MD) were precipitated with TCA before separation on SDS-PAGE and immunoblotting. EsxA and EsxB were detected with specific rabbit antisera and were found in the total lysate and in the culture medium, indicating that *S. aureus* strain Newman secretes both polypeptides across the bacterial envelope (FIG. 2A).

As a control, *S. aureus* IsdG, a cytoplasmic protein, and IsdE, a membrane lipoprotein, were found in total lysates but not in the bacterial culture medium (FIG. 2A) (Jonsson *et al.*, 2003). As a control for polypeptides that are covalently anchored to the cell wall, staphylococcal protein A was examined (Schneewind *et al.*, 1995).  
5 Cell wall degradation products, *i.e.*, peptidoglycan fragments with linked polypeptide, are being released from the staphylococcal surface, and some protein A can therefore be found in both the total lysate and culture medium (Navarre and Schneewind, 1999) (FIG. 2A). Staphylococci were harvested from cultures by centrifugation and were boiled in hot SDS to release polypeptides that are only loosely associated with the cell  
10 wall (L). Boiling in hot SDS released small amounts of protein A, but not IsdE and IsdG. This is an expected result as the cell wall of staphylococci cannot be disintegrated by boiling in ionic detergents (Schneewind *et al.*, 1992). EsxA and EsxB were not found to be associated with the staphylococcal surface after boiling bacteria in hot SDS, suggesting that staphylococci ESAT-6-like proteins are soluble in  
15 the extracellular milieu.

***Subcellular Localization of EsxA and EsxB.*** To examine the subcellular localization of EsxA and EsxB in staphylococci, cultures of *S. aureus* strain Newman were separated into cytoplasm, membrane, cell wall, and medium fractions (FIG. 2B). Proteins in all fractions were revealed by immunoblotting with specific antibodies.  
20 EsxA and EsxB were found exclusively in the culture medium. As a control, protein A was detected in the cell wall fraction, whereas IsdG and IsdE resided in the cytoplasm and the plasma membrane or cell wall of staphylococci, respectively (FIG. 2B). Together, these results demonstrate that *S. aureus* strain Newman secretes EsxA and EsxB across the bacterial envelope into the culture medium.

***Factors Affecting the Production and Secretion of EsxA and EsxB.***  
25 Previous work from the inventors' laboratory generated the Phoenix ( $\Phi$ NE) library, a collection of *bursa aurealis* insertion mutants in *S. aureus* strain Newman that have been mapped by inverted PCR and DNA sequencing (Bae *et al.*, 2004). *S. aureus* strain Newman variants  $\Phi$ NE09349,  $\Phi$ NE03742,  $\Phi$ NE13152,  $\Phi$ NE02411,  
30  $\Phi$ NE04464, and  $\Phi$ NE07912 carry *bursa aurealis* insertions in *esaA*, *essA*, *esaB*, *essB*, *essC*, or *esxB*, respectively (Table 3 and FIG. 3A). *S. aureus* Newman variant  $\Phi$ NE187-01 carries a stop codon at position 8 of *esxA* coding sequence. The ability

of these mutant strains to synthesize and secrete EsxA and EsxB was examined by immunoblotting of culture medium and total lysostaphin lysate samples as described in the legend of FIG. 2A.

*Bursa aurealis* insertions at nucleotide positions 1,110 (codon 370) of *esaA*, or  
5 43 (codon 15) of *esaB* did not affect the production or secretion of EsxA and EsxB  
when examined by immunoblot analysis (FIG. 3B). However, *bursa aurealis*  
insertions at nucleotide positions 121 (codon 41) of *essA*, 328 (codon 110) of *essB*, or  
792 (codon 264) of *essC* abolished production and thus secretion of both EsxA and  
EsxB polypeptides as immunoreactive species could not be detected in either the  
10 medium or total culture lysate of strains  $\Phi$ NE03742,  $\Phi$ NE02411, and  $\Phi$ NE04464,  
respectively (FIG. 3B). One *bursa aurealis* insertion in the Phoenix library mapped  
to nucleotide 2 (codon 1) of *esxB* (mutant  $\Phi$ NE07912), a mutation that abolished the  
expression of all EsxB immunoreactive species (FIG. 3B). The inability of strain  
 $\Phi$ NE07912 (*esxB::erm*) to produce EsxB abolished all EsxA production and,  
15 therefore, secretion. Conversely, the inability of strain  $\Phi$ NE187-01 (*esxA24*) to  
produce EsxA abolished all EsxB production as well (FIG. 3B). None of the mutants  
used here abolished the transcription of either *esxA* or *esxB* as judged by RT-PCR,  
suggesting that the phenotypes observed were not caused by polar effects of mutations  
or to transcriptional down-regulation.

20 EsaA is a 1,009-aa polytopic membrane protein with six predicted  
transmembrane helices that is conserved in staphylococcal and listerial species but  
absent from mycobacteria (FIG. 1B). *essA* and *esaB* specify 152- and 80-aa long  
protein products, respectively. Bioinformatic prediction of subcellular localization  
suggests that EssA contains one transmembrane domain, whereas EsaB may be  
25 soluble as it lacks a canonical signal peptide. EssB and EsaB share sequence  
homology with listerial genes located in a similar *ess* cluster, and with *B. subtilis*  
YukC and YukD, respectively (Oudega *et al.*, 1997). The function of YukC and  
YukD is unknown, but their corresponding genes map to a chromosomal locus with  
*yukA*, *yukB*, and *yukE* (Oudega *et al.*, 1997) (FIG. 1B). YukE bears the WXG motif  
30 typical of ESAT-6-like proteins.

EssC is homologous to two genes of *M. tuberculosis*, Rv3870 (*snm1*) and  
Rv3871 (*snm2*), and to *B. subtilis* YukA–YukB. Recent sequence analysis revealed



that *yukA-yukB* represents a single ORF (Sao-Jose *et al.*, 2004). EssC, Rv3870, Rv3871, and YukA sequences encompass one or more FSDs (Pallen *et al.*, 2002). Gene SA0276 (*essC*, in the N315 genome) is annotated as “conserved hypothetical protein, similar to diarrheal toxin.” The annotation derives from similarity to the  
5 *Bacillus cereus* gene *bceT* and appears to be erroneous (Hansen *et al.*, 2003). Bioinformatic analysis predicted two transmembrane helices at the N terminus of the 1,479-aa EssC polypeptide (FIG. 1C). The N and C termini of EssC are presumed to reside in the staphylococcal cytoplasm based on the assumption that the FSDs hydrolyze ATP in this compartment.

10 ***Phenotypic Analysis of Various Insertion Mutants in essC.*** The Phoenix library contains several strains carrying *bursa aurealis* insertions in the *essC* gene (Table 3 and FIG. 4A). Four *essC* mutants were used to analyze the effect of EssC truncations on the secretion of EsxA and EsxB (FIG. 4B). strain  $\Phi$ NE04464 carries a transposon insertion at nucleotide 792 (codon 264, mutant 1), whereas strains  
15  $\Phi$ NE02191,  $\Phi$ NE02832, and  $\Phi$ NE13038 carry *bursa aurealis* insertions at nucleotide positions 1854 (codon 618, mutant 2), 2161 (codon 721, mutant 3), and 3835 (codon 1,279, mutant 4), respectively (FIG. 4A). None of these transposon insertions affected the transcription of the two genes downstream (data not shown). Transposon insertion at codons 264 and 618 of *essC* abolished all production and secretion of  
20 EsxA and EsxB. In contrast, the synthesis and secretion of EsxB or EsxA occurred when *bursa aurealis* inserted at codons 721 and 1279 (FIG. 4B). These results imply that a single FSD domain is sufficient for EssC activity, and suggest that EsxA and EsxB secretion may be fueled by EssC mediated ATP hydrolysis.

25 ***esxA, esxB, and essC Mutants Are Defective in the Pathogenesis of Staphylococcal Abscesses.*** *S. aureus* host infection and dissemination within organ tissues depends on staphylococcal synthesis and secretion of a wide variety of virulence factors (Novick, 2003). Because of the astonishing armament of staphylococci with virulence factors, the pathogenesis of *S. aureus* infections is considered multifactorial (Novick, 2003). Thus, with the exception of  $\alpha$ -hemolysin  
30 (*hla*) mutants (Bhakdi and Tranum-Jensen, 1991), strains carrying mutations that abrogate the expression of individual exoproteins typically do not display significant defects in the pathogenesis of *S. aureus* infections (O'Reilly *et al.*, 1986; Jonsson *et*

*al.*, 1985; Patel *et al.*, 1987). *S. aureus* Newman variants  $\Phi$ NE187-01 (*esxA24*),  $\Phi$ NE07912 (*esxB::erm*), and  $\Phi$ NE04464 (*essC::erm*) do not synthesize EsxA and EsxB. These strains were chosen for experimental measurement of bacterial virulence. Viable staphylococci of mutant or wild-type Newman strains ( $\sim 10^6$  staphylococci) were administered intravenously via retro-orbital injection into mice. The animals were killed 4 days after infection. Internal organs were removed, inspected for abscess formation, and then homogenized and spread on agar medium to quantify staphylococcal replication in host tissues by colony formation (Albus *et al.*, 1991). Mutations in *esxA*, *esxB*, and *essC* genes caused a significant reduction in the ability of *S. aureus* Newman to establish kidney or liver abscesses in infected mice (FIG. 5 and data not shown). Kidneys of mice infected with Newman carried a mean value of  $3.89 \times 10^7$  cfu per organ, whereas those of mice infected with strains lacking *esxA*, *esxB*, or *essC* harbored a mean value of  $3.00 \times 10^4$  cfu per organ ( $P < 2.09 \times 10^{-5}$ ),  $6.17 \times 10^5$  cfu per organ ( $P < 1.2 \times 10^{-2}$ ), and  $2.51 \times 10^5$  cfu per organ ( $P < 5.53 \times 10^{-3}$ ), respectively. It should be noted that half of the animals infected with mutant bacteria had cleared most staphylococci from the kidneys after 4 days, suggesting that complete clearing may occur by day 5 after infection with the mutant strains. The formation of liver abscesses in infected animals was even more affected when staphylococci did not produce EsxA and EsxB. Homogenized livers of mice infected with Newman were found to contain a mean value of  $2.09 \times 10^6$  cfu per organ of staphylococci, whereas the livers of mutants lacking *esxA*, *esxB*, or *essC* contained a mean value of  $9 \times 10^1$  cfu per organ ( $P < 3.01 \times 10^{-7}$ ),  $4.17 \times 10^2$  cfu per organ ( $P < 4.33 \times 10^{-6}$ ), and  $1.35 \times 10^3$  cfu per organ ( $P < 1.72 \times 10^{-4}$ ), respectively.

## EXAMPLE 5

### 25 *Staphylococcus aureus* Vaccine Assembled From Surface Protein Antigens

#### A. Materials and Methods

**Cloning, Expression and Purification** -- Surface antigens were amplified via PCR from *S. aureus* strain N315. Primers were designed excluding the predicted signal peptide sequence and sortase recognition motif. PCR products were cloned into either pET-15b (Novagen) or pGEX-2T to yield recombinant proteins with either a 6-His or glutathione-S-transferase (GST) tag, respectively.

**Animal Immunization and Challenge.** Groups of 25-day-old female BALB/c mice (nine or ten mice per group, Charles River Labs, MA) were immunized by intramuscular injection into the hind leg with purified protein. These proteins (100 µg of each) were administered to mice on days 0 (adsorbed to Complete Freund's adjuvant, CFA) and 11 (adsorbed to Incomplete Freund's adjuvant, IFA). Blood samples were withdrawn via retro-orbital bleeding on days 0, 11 and 20. Following blood clotting, sera were retrieved and used for measurements of antibody production. Immunized animals were challenged on day 21 by retro-orbital injection of 100 µl bacterial suspension ( $\sim 10^6$  CFU). For these experiments, *S. aureus* strain Newman was grown overnight in tryptic soy broth (TSB) at 37°C, diluted 1:100 in fresh TSB, and grown for 3 h at 37°C. The staphylococci were washed twice and diluted in sterile PBS to yield an OD<sub>600</sub> of 0.4. The infection inoculum was verified by a plate count. Mice were anaesthetized for this procedure using an intraperitoneal injection of 80-120 mg/kg ketamine and 3-6 mg/kg xylazine. On day 25, mice were euthanized by a compressed CO<sub>2</sub> overdose and their kidneys were excised. The organs were homogenized in 1% Triton X-100 and diluted and plated in triplicate for determination of CFU. Serum immunoglobulin G (IgG) levels with specific antigen binding activity were determined by enzyme-linked immunosorbent assay (ELISA) at the GLRCE Immunology Core, University of Chicago. All animal experiments were performed in accordance with institutional guidelines following experimental protocol review and approval by the Institutional Animal Care and Use Committee.

## **B. Results**

The pathogenesis of staphylococcal infections relies on many different virulence factors such as secreted exotoxins, exopolysaccharides, and surface adhesins. However, deletion of single genes encoding such factors causes either no defect or results in only modest reduction of virulence. The development of staphylococcal vaccines is hindered by the multifaceted nature of staphylococcal invasion strategies. Previous work demonstrated that immunization of mice with capsular polysaccharide, poly-N-acetylglucosamine (PNAG) exopolysacchride or the surface protein clumping factor A (ClfA) afforded partial protection against staphylococcal replication and disease in murine models of infection. Staphylococci rely on surface protein mediated-adhesion to host cells or invasion of tissues as a strategy for escape from immune defenses. Furthermore, *S. aureus* utilize surface

proteins to sequester iron from the host during infection. The majority of surface proteins involved in staphylococcal pathogenesis carry C-terminal sorting signals, *i.e.*, they are covalently linked to the cell wall envelope by sortase. Further, staphylococcal strains lacking the genes required for surface protein anchoring, *i.e.*,  
5 sortase A and B, display a dramatic defect in the virulence of several different mouse models of disease. Thus, surface protein antigens represent a validated vaccine target as the corresponding genes are essential for the development of staphylococcal disease.

Using cell wall sorting signals as queries for BLAST searches and previous  
10 genome analysis, 22 surface protein genes were identified in six staphylococcal strains. Large portions of N-terminal, surface exposed amino acid sequences of 20 different surface proteins were expressed in *Escherichia coli* and recombinant proteins purified by affinity chromatography. Antigen, 100 µg purified, recombinant protein emulsified with complete Freund's adjuvant, was injected into muscle tissue  
15 of the hind leg of 25-day-old female BALB/c mice on day 0. Immune responses of animals were boosted by immunization with 100 µg antigen emulsified in incomplete Freund's adjuvant on day 11. Blood samples were withdrawn via retro-orbital bleeding on days 0, 11 and 20 following immunization and serum antibody titers to individual antigens determined by enzyme linked immuno-sorbent assay (ELISA).  
20 Animals were challenged on day 21 with intra-venous injection of  $10^6$  colony forming units (cfu) of *S. aureus* strain Newman, a human clinical isolate. Four days following infection, animals were euthanized and organ tissues removed. The ability of staphylococci to seed abscesses in kidneys was assessed by macroscopic inspection and by plating tissue homogenate on agar media followed by colony formation and  
25 enumeration.

The results show that animals immunized with a buffer control were infected by staphylococci, harboring abscesses with approximately  $1 \times 10^7$  cfu of staphylococci in their kidneys (FIG. 8). None of the twenty recombinant surface proteins used as vaccine antigens generated immune responses that afforded complete protection  
30 against staphylococcal disease. However, the bacterial load in the kidneys of animals immunized with SdrD, SdrE, IsdA, and IsdB was reduced by three to four logs of cfu, respectively. Immunization with Spa, ClfA, ClfB, SdrC, IsdC or SasF afforded a two

to three log reduction in the bacterial burden of kidney tissues, whereas immunization with other antigens generated very little or no vaccine protection.

## EXAMPLE 6

### Vaccine Assembly from Combinatins of Surface Proteins Of Staphylococcus

#### 5 A. Methods

*Bioinformatics.* Genome sequences of *S. aureus* strains were analyzed for surface proteins using sorting signals as queries in BLAST searches. Signal peptide cleavage sites were predicted with the SignalP 3.0 algorithm (Bendtsen *et al.*, 2004).

*Bacterial Strains and Growth.* Staphylococci were cultured on tryptic soy  
10 agar or broth at 37°C. *S. aureus* NRS70 (N315), NRS123 (USA400/MW2), NRS248,  
NRS252 and NRS382 (USA100) were obtained through the Network on  
Antimicrobial Resistance in *Staphylococcus aureus* (NARSA, NIAID Contract No.  
N01-AI-95359). *S. aureus* SEJ2 is a  $\Delta$ (*spa*) variant of strain Newman carrying a  
deletion of the protein A gene. *Escherichia coli* strains DH5 $\alpha$  and BL21(DE3) were  
15 cultured on Luria agar or broth at 37°C. Carbenicillin (50  $\mu$ g/mL) and erythromycin  
(10  $\mu$ g/mL) were used for plasmid selection.

*Cloning and Purification.* Coding sequences for surface proteins excluding  
the signal peptide and sorting signal were PCR amplified using *S. aureus* N315  
template DNA (see Table 7 for a listing of primer sequences). PCR products were  
20 cloned into either pET-15b or pGEX-2T to express recombinant proteins with N-  
terminal six histidyl tag or glutathione-S-transferase (GST) fusion, respectively.  
Following bacterial disintegration, proteins were affinity purified from cleared lysates  
using Ni-NTA or glutathione sepharose. Protein eluate was subjected to endotoxin  
removal by Triton X-114 phase separation, surface proteins were purified further by  
25 gel filtration and protein concentration determined.

*Immunization.* BALB/c mice (24-day-old female, eight to ten mice per group,  
Charles River Labs, MA) were immunized by intramuscular injection into the hind  
leg with purified protein. Proteins (100  $\mu$ g of each) were administered on days 0  
(emulsified 1:1 with CFA) and 11 (emulsified 1:1 with IFA). Pilot studies compared  
30 aluminum hydroxide, CFA, or IFA as possible adjuvants for surface protein  
immunization and eliminated aluminum hydroxide because of immune responses that

interfere with murine abscess formation. Blood samples were drawn via retro-orbital bleeding on days 0, 11, and 20. Sera were examined by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) titers with specific antigen binding activity. Animal experiments were performed in accordance with  
5 institutional guidelines following experimental protocol review and approval by the Institutional Animal Care and Use Committee.

*Renal Abscess.* Immunized animals were challenged on day 21 by retro-orbital injection of 100  $\mu$ L bacterial suspension ( $3-5 \times 10^6$  CFU). Overnight cultures of *S. aureus* Newman were diluted 1:100 into fresh TSB and grown for 3 hours at  
10 37°C. Staphylococci were centrifuged, washed twice and diluted in PBS to yield an OD<sub>600</sub> of 0.4 ( $3-5 \times 10^7$  CFU/mL). Further dilutions were needed for the desired inoculum, which was experimentally verified by agar plating and colony formation. Mice were anesthetized by intra-peritoneal injection of 80-120 mg/kg ketamine and 3-6 mg/kg xylazine. On day 25, mice were euthanized by compressed CO<sub>2</sub> inhalation.  
15 Kidneys were removed and homogenized in 1% Triton X-100, aliquots diluted and plated on agar media for triplicate determination of CFU. For histology, kidney tissue was incubated at room temperature in 10% formalin for 24 hours. Tissues were embedded in paraffin, thin sectioned, hematoxylin/eosin stained and examined by microscopy.

20 *Lethal Challenge.* Immunized animals were challenged on day 21 by intra-peritoneal injection with  $2 \times 10^{10}$  CFU of *S. aureus* Newman or  $3-10 \times 10^9$  CFU of clinical *S. aureus* isolates. Animals were monitored for seven days and lethal disease recorded.

*Opsonophagocytic Killing.* Polymorphonuclear leukocytes (PMNs) were  
25 isolated from healthy human volunteers. Cells were counted, examined for viability (trypan blue exclusion) and diluted to  $2-5 \times 10^6$  PMN/mL. To remove antibodies that react with the bacterial target, infant rabbit serum was pre-adsorbed with *S. aureus* SEJ2 suspensions by mixing at 4°C for 30 minutes. Serum was then centrifuged, filter sterilized and used as a source of complement. *S. aureus* SEJ2 was adjusted to  
30  $2-5 \times 10^5$  CFU/mL. Rabbit antibodies to IsdA, IsdB, SdrD and SdrE were normalized to the same IgG titer. Equal volumes (100  $\mu$ L) of PMNs, complement, bacteria, and diluted antibodies were mixed and incubated at 37°C for 90 minutes prior to dilution,

agar plating and bacterial enumeration. The percent amount of bacterial killing was calculated as  $(1 - [\text{the number of CFU recovered in the presence of PMNs} / \text{the number of CFU recovered in the absence of PMNs}]) \times 100$ .

*Statistical Analysis.* One-tailed Student's t tests were performed to analyze statistical significance of renal abscess data. Fisher's exact test was used to analyze statistical significance of the lethal challenge data.

## B. Results

*S. aureus*, a gram-positive bacterial commensal of human skin and nares, is the leading cause of bloodstream, lower respiratory tract and skin/soft tissue infections (Diekema *et al.*, 2001). The broad spectrum of important staphylococcal diseases also includes endocarditis, septic arthritis, toxic shock syndrome, scalded skin syndrome and food poisoning (Archer and Climo, 2001). *S. aureus* strains exhibiting multiple antibiotic resistances are isolated in about 60% of community and up to 80% of hospital infections (Kaplan *et al.*, 2005). For example, *S. aureus* strains with intermediate (VISA) or full resistance (VRSA) to vancomycin, which is considered the therapy of last resort for methicillin-resistant *S. aureus* (MRSA), have recently emerged (Weigel *et al.*, 2003).

Generation of protective immunity against invasive *S. aureus* disease has been a goal since the discovery of this microbe. Whole-cell live or killed vaccines largely fail to generate protective immune responses (Rogers and Melly, 1965). Purified capsular polysaccharides (CP), types 5 and 8 (which represent more than 80% of all capsular types found in clinical isolates (Arbeit *et al.*, 1984)), shows promise when used as a conjugate vaccine in experimental animals or in patients with end-stage renal disease (Fattom *et al.*, 1990; Fattom *et al.*, 2004). Immunization with PNAG, a *S. aureus* surface carbohydrate synthesized by *icaABC* products (Heilmann *et al.*, 1996), also protects mice against staphylococcal disease (McKenney *et al.*, 1999; Maira-Litran *et al.*, 2005). Subunit vaccines composed of individual surface proteins, for example clumping factor A (ClfA) (Josefsson *et al.*, 2001), clumping factor B (ClfB) (Schaffer *et al.*, 2006), iron-regulated surface determinant B (IsdB) (Kuklin *et al.*, 2006) or fibronectin-binding protein (FnBP) (Zhou *et al.*, 2006) generate immune responses that afford partial protection against *S. aureus* challenge of experimental animals. However, in order for a *S. aureus* vaccine to be successful, genetic

determinants for specific antigens must be essential for staphylococcal virulence (Maira-Litran *et al.*, 2004). This has not been observed for mutants lacking the genetic determinants of CP (Albus *et al.*, 1991) or of individual surface proteins (Mazmanian *et al.*, 2001).

5 Rappuoli and colleagues exploited information encrypted in bacterial genome sequences to distinguish pan-genomes, *i.e.*, genes in all strains of a pathogen, from conserved genes found in all members of its species (Medini *et al.*, 2005). Rational vaccine design was achieved by interrogating conserved antigens (secreted or surface displayed) for protective immunity, which led to the identification of multiple surface  
10 proteins of group B streptococci (GBS) as candidates (Maione *et al.*, 2005). By combining multiple antigens into a single vaccine, broad spectrum protective immunity against many different clinical isolates was achieved (Maione *et al.*, 2005).

The inventors contemplate a broad spectrum *S. aureus* vaccine derived from staphylococcal surface proteins for protective immunity. *S. aureus* sortase A (srtA)  
15 mutants, which cannot display surface proteins, are unable to establish infections in experimental models, indicating that the sum of all twenty-three surface proteins is essential for pathogenesis (Mazmanian *et al.*, 2000). Sortase A cleaves sorting signals of surface proteins and anchors polypeptides to the cell wall envelope (Mazmanian *et al.*, 2001). Eight staphylococcal genome sequences (Kuroda *et al.*, 2001; Holden *et al.*, 2004; Diep *et al.*, 2006) were examined for the presence of sortase substrate genes  
20 using sorting signals as queries in BLAST searches and nineteen conserved surface protein genes were identified (Table 4). These genes were expressed in *E. coli* as soluble His-tagged or glutathione S-transferase fusions and recombinant proteins purified by affinity chromatography. Groups of mice were immunized by intra-  
25 muscular injection with 100 µg purified protein emulsified in complete Freund's adjuvant (CFA) and eleven days later with protein emulsified in incomplete Freund's adjuvant (IFA). Blood samples were drawn before, during and after immunization and specific serum IgG levels determined by ELISA, demonstrating that surface proteins generated humoral immune responses to immunization (Table 5). Mice were  
30 challenged 21 days after the primary immunization with 3-5 x 10<sup>6</sup> colony forming units (CFU) of *S. aureus* Newman (Duthie and Lorenz, 1952) via retro-orbital injection. Four days after infection, mice were killed, kidneys removed and the



bacterial load in homogenized tissues measured (Albus *et al.*, 1991). When compared to mock immunized animals, some recombinant surface proteins generated specific immune responses that afforded partial protection against staphylococcal disease. Bacterial load in kidneys of animals immunized with ClfA, SdrD, SdrE, IsdA or IsdB  
5 was reduced by three to four logs, while immunization with Spa, ClfB, IsdC, SdrC, SasD or SasF afforded a two to three log reduction in staphylococcal burden. FnBPA, SasG or IsdH immunization generated even smaller reductions in bacterial load, while immunization with FnBPB, SasA, SasB, SasC or SasK did not result in significant protection (Table 5).

**Table 4. Sortase-anchored surface proteins of *S. aureus***

Surface Protein	AA	Ligand	MRSA 252	MSSA 476	Mu50	MW2	N315	NCTC 8325	Newman	USA 300
Spa	450	IgG,vWF	+	+	+	+	+	+	+	+
FnBPA	1038	Fibronectin		+	+	+	+	+	+	+
FnBP	961	Fibronectin	+	+	+	+	+	+	+	+
CifA	989	Fibrinogen	+	+	+	+	+	+	+	+
CifB	877	Fibrinogen	+	+	+	+	+	+	+	+
SdrC	953	unknown	+	+	+	+	+	+	+	+
SdrD	1347	unknown	+	+	+	+	+		+	+
SdrE	1141	unknown	+	+	+	+	+	+	+	+
Can	1183	Collagen	+	+		+				
Pls	1166	unknown								
SasA	2271	unknown	+	+	+	+	+	+	+	+
SasB	2481	unknown	+	+	+	+	+	+	+	+
SasC	2186	unknown	+	+	+	+	+	+	+	+
SasD	241	unknown	+	+	+	+	+		+	+
SasE/IsdA	350	Heme	+	+	+	+	+	+	+	+
SasF	635	unknown	+	+	+	+	+	+	+	+
SasG/Aap	525	unknown		+	+	+	+	+		+
SasH	772	unknown	+	+		+	+	+	+	+
SasI/HarA/IsdH	891		+	+	+	+	+	+	+	+
SasJ/IsdB	645	Hemoglobin	+	+	+	+	+	+	+	+
SasK	211	unknown		+	+	+	+			
SasL	232	unknown			+					
IsdC	227	Heme	+	+	+	+	+	+	+	+

Surface proteins were identified as genes harboring a C-terminal sorting signal.

AA, protein length in amino acids.

Motif, consensus motif recognized by sortase and present in C-terminal cell wall sorting signal.

Ligand, molecular component(s) recognized or bound by surface proteins.

Table 5 Immune protection against *S. aureus* abscess formation conferred by immunization of mice with surface protein

Surface Protein	IgG Titer	<i>S. aureus</i> in Kidneys (log <sub>10</sub> CFU/mL)	Reduction of Staphylococci (log <sub>10</sub> CFU/mL)	Significance
Spa	ND	3.911 ± 0.978	2.951	<i>P</i> = 0.00541
FnBPA	18,000 ± 6,771	4.891 ± 0.922	1.971	<i>P</i> = 0.02316
FnBPB	24,300 ± 3,600	6.172 ± 0.437	0.690	<i>P</i> = 0.07434
ClfA	64,800 ± 9,920	3.521 ± 0.922	3.341	<i>P</i> = 0.00361
ClfB	36,600 ± 12,247	4.308 ± 0.797	2.554	<i>P</i> = 0.01012
SdrC	12,125 ± 3,770	4.026 ± 0.979	2.836	<i>P</i> = 0.01012
SdrD	30,000 ± 12,920	3.477 ± 1.039	3.385	<i>P</i> = 0.00613
SdrE	29,000 ± 6,878	2.565 ± 0.913	4.297	<i>P</i> = 0.00068
SasA	8,500 ± 1,936	5.207 ± 1.048	1.655	<i>P</i> = 0.06821
SasB	20,250 ± 6,037	5.709 ± 0.780	1.153	<i>P</i> = 0.09094
SasC	ND	5.649 ± 0.781	1.213	<i>P</i> = 0.09642
SasD	24,500 ± 1,342	4.675 ± 1.082	2.187	<i>P</i> = 0.03782
IsdA/SasE	45,900 ± 9,156	2.518 ± 0.897	4.344	<i>P</i> = 0.00057
SasF	16,900 ± 3,932	3.916 ± 0.781	2.946	<i>P</i> = 0.00187
SasG/Aap	21,875 ± 2,900	5.384 ± 0.715	1.478	<i>P</i> = 0.03597
HarA/IsdH/SasI	30,375 ± 15,093	4.918 ± 0.761	1.944	<i>P</i> = 0.02239
IsdB/SasJ	36,300 ± 2,741	3.394 ± 0.982	3.468	<i>P</i> = 0.00318
SasK	32,800 ± 12,659	5.898 ± 0.746	0.964	<i>P</i> = 0.11032
IsdC	ND	3.779 ± 1.084	3.083	<i>P</i> = 0.00996
PBS mock	-	6.862 ± 0.098	-	-

IgG titers [mean serum titers ± standard error of mean (SEM)] in response to immunization with surface proteins were determined by ELISA (n=5 animals).

Immunized mice challenged with *S. aureus* Newman (n=8-10 animals) and staphylococci in kidney tissues were enumerated [log<sub>10</sub>(CFU)/mL ± SEM].

Reduction of the number of staphylococci in kidney tissues (log<sub>10</sub>(CFU)/mL) as a measure for protective immunity and compared to control mice immunized with phosphate buffered saline (PBS)(n=8-10 animals).

Statistical significance of reduction in staphylococcal burden was assessed with the one-tailed student's *t* test and *P* values recorded.

ND=not determined

Four surface protein vaccine candidates (IsdA, IsdB, SdrD and SdrE) generated the highest levels of protection against staphylococcal renal infection. To determine the nature of protection, antibodies against IsdA, IsdB, SdrD and SdrE were raised in rabbits and analyzed for their ability to induce opsonophagocytic  
5 killing of staphylococci in the presence of white blood cells, an important immunological correlate of protective immunity against *S. aureus* (Fattom *et al.*, 2004; Maira-Litran *et al.*, 2005). Freshly isolated human polymorphonuclear leukocytes (PMNs) were incubated with staphylococci in the presence of complement and specific antibodies. Following incubation, bacterial killing was monitored by  
10 spreading sample aliquots on agar media followed by colony formation and enumeration (FIG. 12). Antibodies against all four surface protein antigens induced opsonophagocytic killing of *S. aureus* (FIG. 12).

IsdA, IsdB, SdrD and SdrE, were assembled into a combined vaccine. To determine whether antibodies are generated against each of the four antigens in a  
15 combined vaccine, serum IgG titers of immunized mice were determined (Table 6). Antibody levels generated via the combined vaccine were similar to those achieved by immunization with individual surface protein antigens. Mice were challenged with  $3 \times 10^6$  CFU of *S. aureus* strain Newman. Four days following infection, animals were killed and kidneys removed. In contrast to immunization with individual  
20 antigens, the combined vaccine afforded complete protection against staphylococcal challenge [a reduction in bacterial load of  $5.062 \log_{10}$  CFU/mL ( $P=0.00074$ ) to a level below detection]. Histological analysis of kidney tissues failed to detect staphylococcal abscesses in mice that had been immunized with the combined vaccine (FIG. 9). In contrast, organs removed from mock-immunized animals harbored  
25 bacterial abscesses with central concentrations of staphylococci that were surrounded by a large cuff of white blood cells (FIG. 9). Kidney tissue of mice immunized with the combined vaccine revealed the physiological architecture of renal tubules as well as small infiltrates of PMNs, likely the anatomical substrate of opsonophagocytic clearance of staphylococci.

Table 6. IgG titers for individual surface proteins and the combined vaccine (IsdA, IsdB, SdrD &amp; SdrE)

Surface Protein	Individual Immunization	Combined Vaccine	Significance
IsdA/SasE	27,000 ± 12,000	41,000 ± 14,000	0.26964
IsdB/SasJ	11,000 ± 2,449	28,000 ± 12,104	0.14330
SdrD	7,000 ± 2,000	9,000 ± 2,449	0.18695
SdrE	15,000 ± 3,162	17,000 ± 3,742	0.35200

IgG titers (mean serum titers ± standard error of mean) in response to immunization of mice as determined by ELISA (n=5 animals).

Specific antibody titers raised against surface proteins were not significantly different when immunizing antigens were administered individually or in combination.

Table 7. Surface protein primers for cloning

Gene	Sequence (SEQ ID NO.)	Term	R.E.	Vector
Spa	gctgcaCATATGgcgcaacacgatgaagctcaac (SEQ ID NO:25.)	N	NdeI	pET15b
Spa	agtGGATCCttatgcttgagctttgtagcatctgc (SEQ ID NO.26)	C	BamHI	pET15b
FnBPA	aaagaaCATATGgcatcagaacaaaagacaactacag (SEQ ID NO.27)	N	NdeI	pET15b
FnBPA	tgaGGATCCttaggactcagtgatcctccaacg (SEQ ID NO28.)	C	BamHI	pET15b
FnBPB	gaaCTCGAGgcatcggaacaaaacaataactacag (SEQ ID NO.29)	N	XhoI	pET15b
FnBPB	aatGGATCCttaatcatttggtttatctttaccatcg (SEQ ID NO.30)	C	BamHI	pET15b
ClfA	gaagcaCATATGagtgaaaatagtgttacgcaatc (SEQ ID NO.31)	N	NdeI	pET15b
ClfA	agaGGATCCttagttattaaatgctacttcgttgtc (SEQ ID NO.32)	C	BamHI	pET15b

ClfB	gcaCTCGAGtcagaacaatcgaacgatacaacg (SEQ ID NO.33)	N	XhoI	pET15b
ClfB	tggGGATCCttaatttactgctgaatcaccatcag (SEQ ID NO.34)	C	BamHI	pET15b
SdrC	gaagctCATATGgcagaaacatacgaatggagaattaaat c (SEQ ID NO.35)	N	NdeI	pET15b
SdrC	ttcGGATCCttaattatcaagtgtgaaatcatcatgatc (SEQ ID NO.36)	C	BamHI	pET15b
SdrD	gcaCTCGAGgcagaaagtactaataaagaattgaacg (SEQ ID NO.37)	N	XhoI	pET15b
SdrD	ttcGGATCCttatattataagttccattttctaatacc (SEQ ID NO.38)	C	BamHI	pET15b
SdrE	gaagctCATATGgctgaaaacactagtagcagaaaatg (SEQ ID NO.39)	N	NdeI	pET15b
SdrE	ttcGGATCCttagttatcaagtgtgaaatcatcatg (SEQ ID NO.40)	C	BamHI	pET15b
SasA	caagctCATATGgcttctgatgcaccattaacttct (SEQ ID NO.41)	N	NdeI	pET15b
SasA	atcGGATCCgctatttcttggttacttcatatttaaagt (SEQ ID NO.42)	C	BamHI	pET15b
SasB	gcgAGATCTgcagagcaaaatcagcctgcac (SEQ ID NO.43)	N	BglII	pGEX-2T
SasB	atcGAATTCttaagttgtggcagcttgcacttga (SEQ ID NO.44)	C	EcoRI	pGEX-2T
SasC	gcaGGATCCttaactacggataataatgtacaaag (SEQ ID NO.45)	N	BamHI	pGEX-2T
SasC	tatGAATTCttaagctttatcatttacagcattgcg (SEQ ID NO.46)	C	EcoRI-9	pGEX-2T
SasD	gcgCTCGAGgacacgacttcaatgaatgtgc	N	XhoI	pET15b

	(SEQ ID NO.47)				
SasD	AgcGGATCCttaaacttttttgttactttggttcatttg	C	BamHI	pET15b	
	(SEQ ID NO.48)				
SasF	GccGGATCCgcatctgaaaaggatactgaaatttc	N	BamHI	pGEX-2T	
	(SEQ ID NO.49)				
SasF	tttGAATTCttatacattaccatcagcatttaataatc	C	EcoRI	pGEX-2T	
	(SEQ ID NO.50)				
SasG/Aap	gaagcaCATATGgctgaaaacaatattgagaatccaac	N	NdeI	pET15b	
	(SEQ ID NO.51)				
SasG/Aap	tgtGGATCCttatttttgtccttctcttgttactttttc	C	BamHI	pET15b	
	(SEQ ID NO.52)				
HarA/IsdH/SasI	gcgCTCGAGgcagaaaatacaaaacttcagataaaatc	N	XhoI	pET15b	
	(SEQ ID NO.53)				
HarA/IsdH/SasI	agtGAATTCttacatcttagattgactaagtttgttttc	C	EcoRI	pET15b	
	(SEQ ID NO.54)				
SasK	aatgcgCATATGgaaaataacaaacctgaaggtgtg	N	NdeI	pET15b	
	(SEQ ID NO.55)				
SasK	tgtGGATCCttattctttcaattggtgcttatgtactg	C	BamHI	pET15b	
	(SEQ ID NO.56)				

IsdA/SasE, IsdB/SasJ and IsdC have been previously described (Mazmanian *et al.*, 2003). Restriction enzyme (R.E.) sites are capitalized.

To study the protection afforded by a combined vaccine against lethal infections, mice were immunized with the combined vaccine or with individual purified surface antigens (IsdA, IsdB, SdrD and SdrE). Challenges of  $2 \times 10^{10}$  CFU of *S. aureus* Newman were administered intra-peritoneally and mice were monitored for 7 days. FIG. 10 shows that immunization with individual surface proteins had either no effect on survival or afforded only very modest protection (FIG. 10), as already reported for purified IsdB (Kuklin *et al.*, 2006). In contrast to individual protein antigens, immunization with the combined vaccine afforded complete protection against a lethal challenge with one LD<sub>50</sub> unit of *S. aureus* Newman ( $P < 0.03$ ) (FIG. 10). These results suggest that immunization with the combined vaccine

can generate increased protection against lethal challenge with a strain expressing all four surface proteins.

In order for staphylococcal vaccines to be effective, protection must be achieved against a wide variety of different strains. By first eliminating surface proteins that are not conserved amongst staphylococcal strains, the inventors aim at developing a vaccine that may be effective against a wide range of *S. aureus* isolates. Five *S. aureus* strains were selected for assessment. NRS252 is a methicillin-sensitive *S. aureus* (MSSA) strain associated with toxic shock syndrome following burn injury, whereas N315, NRS248, USA100 and USA400 are MRSA strains (Kuroda *et al.*, 2001; McDougal *et al.*, 2003; Baba *et al.*, 2002). NRS248 is the causative agent of necrotizing pneumonia. USA400 carries the Panton-Valentine leukocidin genes associated with lethal lung infections (Gillet *et al.*, 2002). USA100 is the most frequent cause of healthcare-associated infections in the United States and protection against this strain is of crucial importance for vaccine efforts (McDougal *et al.*, 2003). Mice were immunized with the combined vaccine or mock immunized and then challenged by intra-peritoneal injection of *S. aureus* suspensions harboring  $3-10 \times 10^9$  CFU. Survival analysis revealed that the combined vaccine afforded significant protection against lethal challenge with any one of the five clinical *S. aureus* isolates (FIG. 11).

The data indicate that a combined vaccine of *S. aureus* surface antigens derived from conserved genome sequences can elicit immune responses that achieve greater protective immunity than immunization with its individual components. Immunization with four surface proteins, IsdA, IsdB, SdrD and SdrE, generated the highest level of protection in mice as compared to fifteen other antigens. Three of the four antigens in the combined vaccine are already known to be immunogenic in humans, as antibodies against IsdB, SdrD and SdrE can be found in healthy individuals or in patients with *S. aureus* disease (Dryla *et al.*, 2005).

## EXAMPLE 7

### Staphylococcal Infection of Mouse Animal Model

Groups of five 3 week-old mice were infected with various staphylococcal isolates ( $10^6$  cfu/ml suspended in PBS and administered IV in a 0.1 ml volume) or PBS (as a control for uninfected animals). Twenty-one days following infection, the



animals were bled and killed. The presence of anti-EsxA (SAV0282), anti-EsxB (SAV0290), anti-EsaC (SAV0289) IgGs in the sera was tested in an Elisa assay (FIG. 13).

The results of immunization studies using recombinant EsxA and EsxB are summarized in Table 8. For these experiments, mice (groups of 10; 3 week-old) were immunized with 100  $\mu$ l of recombinant antigen EsxA or EsxB mixed with complete Freund adjuvant (day 0), or incomplete Freund adjuvant (day 11). Mice were bled on day 0, 11 and 21. IgG titers are shown for day 21 only (Table 8). On day 21, the animals were challenged with  $5 \times 10^6$  cfu of strain Newman (suspended in PBS and administered IV in a 0.1 ml volume). On day 25, animals were killed and colony forming units in organs were measured by plating ground organs on agar plates and counting colonies after overnight growth at 37°C. The Average CFU recovered for each kidney is shown in Table 8. The protection conferred upon immunization is measured as *Reduction* in CFU and calculated as the ratio of CFU for animals immunized with PBS (no protein) over CFU for animals immunized with recombinant antigen.

Patients infected with staphylococci generate antibodies against EsxA and EsxB. Fig. 14 illustrates the serum IgG titers of patients infected with *S. aureus*. Sera of two non infected individuals were used as a control. Various dilutions of sera were analyzed for EsxA and EsxB specific IgG by custom ELISA.

Table 8. Immunization with recombinant EsxA and EsxB

Protein	Avg log <sub>10</sub> CFU/ml	Reduction	Pvalue	IgG Titer
-----	6.21	0.00	-----	-----
EsxA	3.85	2.36	2.8 x 10 <sup>-4</sup>	50000
-----	6.51	0.00	-----	-----
EsxB	4.27	2.24	2.2 x 10 <sup>-3</sup>	75000
EsxAB	5.35	1.16	1.9 x 10 <sup>-3</sup>	10000(EsxA) 75000 (EsxB)
-----	6.36	0.00	-----	-----
EsxA	4.31	2.05	2.9 x 10 <sup>-3</sup>	40500
EsxB	3.77	2.59	1.0 x 10 <sup>-3</sup>	81000
EsxAB	4.93	1.43	5.1 x 10 <sup>-3</sup>	18000 (EsxA) 81000 (EsxB)

5 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 fusion proteins, compositions, 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  
 10 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 appended claims.

15

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### CLAIMS

1. A method for eliciting an immune response against a staphylococcus bacterium in a subject comprising providing to the subject an effective amount of an EsxA and/or EsxB protein.
2. The method of claim 1, further comprising providing to the subject a SdrD, SdrE, IsdA, and/or IsdB protein.
3. The method of claim 1, wherein the subject is provided with an effective amount of an EsxA or EsxB protein by administering to the subject a composition comprising
  - i) an isolated EsxA and/or EsxB protein, or
  - ii) at least one isolated recombinant nucleic acid molecule encoding an EsxA or EsxB protein,wherein the composition is not a staphylococcus bacterium.
4. The method of claim 2, wherein the subject is provided with an SdrD, SdrE, IsdA, and/or IsdB protein by administering to the subject a composition comprising
  - i) an isolated SdrD, SdrE, IsdA, and/or IsdB protein, or
  - ii) at least one isolated recombinant nucleic acid molecule encoding a SdrD, SdrE, IsdA, or IsdB protein.
5. The method of claim 4, wherein the subject is provided with more than one of the following: SdrD, SdrE, IsdA, or IsdB protein.
6. The method of claim 3, wherein the composition comprises isolated EsxA and/or EsxB protein.
7. The method of claim 6, wherein the isolated EsxA and/or EsxB proteins are dimerized.
8. The method of claim 1, where the subject is also administered an adjuvant.



9. The method of claim 6, wherein the composition comprises an adjuvant.
10. The method of claim 6, wherein the composition further comprises an adjuvant linked to the protein.
11. The method of claim 10, wherein the adjuvant is chemically conjugated to the protein.
12. The method of claim 1, wherein the EsxA protein is at least 70% identical to SEQ ID NO:2.
13. The method of claim 1, wherein the EsxA protein is at least 80% identical to SEQ ID NO:2.
14. The method of claim 1, wherein the EsxA protein is at least 90% identical to SEQ ID NO:2.
15. The method of claim 1, wherein the EsxB protein is at least 70% identical to SEQ ID NO:4.
16. The method of claim 1, wherein the EsxB protein is at least 80% identical to SEQ ID NO:4.
17. The method of claim 1, wherein the EsxB protein is at least 90% identical to SEQ ID NO:4.
18. The method of claim 6, wherein the composition is formulated in a pharmaceutically acceptable composition.
19. The method of claim 1, wherein the staphylococcus bacterium is an *Staphylococcus aureus* bacterium.
20. The method of claim 1, further comprising administering the composition more than one time to the subject.

21. The method of claim 1, wherein the composition is administered orally.
22. The method of claim 1, wherein the composition is administered parenterally.
23. The method of claim 22, wherein the composition is administered subcutaneously, intramuscularly, or intravenously.
24. The method of claim 1, wherein the method further comprises administering to the subject a composition comprising a non-EsxA or non-EsxB Ess protein.
25. The method of claim 24, wherein the Ess protein is in the same composition as EsxA protein or EsxB protein.
26. The method of claim 6, wherein the composition comprises a recombinant nucleic acid molecule encoding an EsxA protein or a recombinant nucleic acid molecule encoding an EsxB protein.
27. The method of claim 26, wherein the composition comprises a nucleic acid molecule encoding an EsxA protein and a nucleic acid molecule encoding an EsxB protein.
28. The method of claim 27, wherein a nucleic acid molecule encodes both an EsxA protein and an EsxB protein.
29. The method of claim 26, wherein the recombinant nucleic acid molecule encoding an EsxA or EsxB protein contains a heterologous promoter.
30. The method of claim 26, wherein the recombinant nucleic acid molecule is a vector.
31. The method of claim 30, wherein the vector is a plasmid.
32. The method of claim 30, wherein the vector is a viral vector.

33. The method of claim 26, wherein the composition further comprises a nucleic acid encoding another Ess protein.
34. The method of claim 26, wherein the composition includes a recombinant, non-staphylococcus bacterium containing the EsxA or EsxB protein.
35. The method of claim 34, wherein the recombinant non- staphylococcus bacterium is Salmonella.
36. The method of claim 1, wherein the subject is human.
37. The method of claim 1, wherein the immune response is a protective immune response.
38. A method for stimulating in a subject an immune response against a staphylococcus bacterium comprising providing to the subject an effective amount of a combination of Staphylococcus virulence factors, wherein the combination includes more than one of the following Staphylococcus cell surface proteins: SdrD, SdrE, IsdA, or IsdB.
39. The method of claim 38, wherein the combination further includes SdrC, Spa, IsdC, ClfA, ClfB, SasF, or combinations thereof.
40. The method of claim 38, wherein the combination further includes EsxA and/or EsxB.
41. The method of claim 38, wherein the subject is provided with an effective amount of a combination of Staphylococcus virulence factors by administering to the subject an effective amount of a composition including i) an isolated SdrD, SdrE, IsdA, and/or IsdB protein; or, ii) an isolated nucleic acid molecule encoding a SdrD, SdrE, IsdA, and/or IsdB protein.

42. The method of claim 40, wherein the subject is provided with a combination that includes EsxA and/or EsxB by administering to the subject a composition that includes i) an isolated EsxA or EsxB protein; or, ii) an isolated nucleic acid molecule encoding an EsxA and/or EsxB protein, wherein the composition is not a *Staphylococcus* bacteria.
43. The method of claim 38, wherein the subject is a human.
44. The method of claim 38, wherein the composition is formulated in a pharmaceutically acceptable formulation.
45. The method of claim 38, wherein the *Staphylococcus* bacteria is *Staphylococcus aureus*.
46. The method of claim 38, wherein the patient is not provided a *Staphylococcus* bacterium containing the combination of virulence factors.
47. A vaccine comprising a pharmaceutically acceptable composition having an isolated EsxA and/or EsxB protein, wherein the composition is capable of stimulating an immune response against a *Staphylococcus* bacteria.
48. The vaccine of claim 47, further comprising an isolated SdrD, SdrE, IsdA, and/or IsdB protein.
49. The vaccine of claim 47, comprising an isolated EsxA and an isolated EsxB protein.
50. The vaccine of claim 47, wherein the isolated EsxA and isolated EsxB proteins are dimerized.
51. The vaccine of claim 48 or 49, wherein the composition is contaminated by less than 1% with other *Staphylococcus* bacterial proteins.

52. The vaccine of claim 47, further comprising a non-EsxA or EsxB isolated Ess protein.
53. The vaccine of claim 47, further comprising an adjuvant.
54. The vaccine of claim 53, wherein the protein is linked to the adjuvant.
55. The vaccine of claim 54, wherein the adjuvant is chemically conjugated to the protein.
56. A vaccine comprising a pharmaceutically acceptable composition having an isolated recombinant nucleic acid encoding an EsxA or EsxB protein, wherein the composition is capable of stimulating an immune response against a Staphylococcus bacteria.
57. The vaccine of claim 56, further comprising a recombinant nucleic acid encoding a SdrD, SdrE, IsdA or IsdB protein.
58. The vaccine of claim 56, wherein the composition comprises a recombinant nucleic acid encoding an EsxA protein.
59. The vaccine of claim 56, wherein the composition comprises a recombinant nucleic acid encoding an EsxB protein.
60. The vaccine of claim 56, wherein the composition comprises a recombinant nucleic acid encoding an EsxA protein and a recombinant nucleic acid encoding an EsxB protein.
61. The vaccine of claim 60, further comprising an isolated recombinant nucleic acid encoding a SdrD, SdrE, IsdA and/or IsdB protein
62. The vaccine of claim 60, wherein the composition comprises a recombinant nucleic acid encoding both EsxA and EsxB proteins.

63. The vaccine of claim 56, wherein the recombinant nucleic acid contains a heterologous promoter.
64. The vaccine of claim 56, wherein the recombinant nucleic acid is a vector.
65. The vaccine of claim 64, wherein the vector is a plasmid.
66. The vaccine of claim 64, wherein the vector is a viral vector.
67. The vaccine of claim 56, wherein the composition further comprises a nucleic acid encoding another Ess protein.
68. The vaccine of claim 56, wherein the composition includes a recombinant, non-Staphylococcus bacteria containing the nucleic acid.
69. The vaccine of claim 68, wherein the recombinant non-Staphylococcus bacteria is Salmonella.
70. The vaccine of claim 56, wherein the composition further comprises an adjuvant.
71. A method for eliciting an immune response against a staphylococcus bacterium in a subject comprising providing to the subject an effective amount of a sortase substrate and a second protein or polypeptide.
72. The method of claim 71, wherein the second protein or polypeptide is a second sortase substrate.
73. The method of claim 71, wherein the sortase substrate is SdrD, SdrE, IsdA, or IsdB protein.
74. The method of claim 73, wherein the subject is provided with an effective amount of SdrD, SdrE, IsdA, or IsdB protein by administering to the subject a composition comprising

- i) an isolated SdrD, SdrE, IsdA, or IsdB protein, or
- ii) an isolated recombinant nucleic acid molecule encoding a SdrD, SdrE, IsdA, or IsdB protein,

wherein the composition is not a staphylococcus bacterium.

75. The method of claim 74, wherein three or more of SdrD, SdrE, IsdA, or IsdB proteins are administered to the subject.

76. The method of claim 75, wherein SdrD, SdrE, IsdA, and IsdB proteins are administered to the subject.

77. The method of claim 71, wherein the second polypeptide is a secreted virulence factor.

78. The method of claim 77, wherein the secreted virulence factor is provided to the subject by administering a composition comprising:

- i) an isolated EsxA, and/or EsxB protein, or
- ii) an isolated recombinant nucleic acid molecule encoding a EsxA, and/or EsxB protein.

79. The method of claim 74, wherein the composition comprises isolated SdrD, SdrE, IsdA, or IsdB protein.

80. The method of claim 71, where the subject is also administered an adjuvant.

81. The method of claim 79, wherein the composition comprises an adjuvant.

82. The method of claim 79, wherein the composition further comprises an adjuvant linked to the protein.

83. The method of claim 82, wherein the adjuvant is chemically conjugated to the protein.

84. The method of claim 73, wherein the SdrD protein is at least 70% identical to SEQ ID NO:6.
85. The method of claim 73, wherein the SdrD protein is at least 80% identical to SEQ ID NO:6.
86. The method of claim 73, wherein the SdrD protein is at least 90% identical to SEQ ID NO:6.
87. The method of claim 73, wherein the SdrE protein is at least 70% identical to SEQ ID NO:8.
88. The method of claim 73, wherein the SdrE protein is at least 80% identical to SEQ ID NO:8.
89. The method of claim 73, wherein the SdrE protein is at least 90% identical to SEQ ID NO:8.
90. The method of claim 73, wherein the IsdA protein is at least 70% identical to SEQ ID NO:10.
91. The method of claim 73, wherein the IsdA protein is at least 80% identical to SEQ ID NO:10.
92. The method of claim 73, wherein the IsdA protein is at least 90% identical to SEQ ID NO:10.
93. The method of claim 73, wherein the IsdB protein is at least 70% identical to SEQ ID NO:12.
94. The method of claim 73, wherein the IsdB protein is at least 80% identical to SEQ ID NO:12.



95. The method of claim 73, wherein the IsdB protein is at least 90% identical to SEQ ID NO:12.
96. The method of claim 79, wherein the composition is formulated in a pharmaceutically acceptable composition.
97. The method of claim 71, wherein the staphylococcus bacterium is an *S. aureus* bacterium.
98. The method of claim 71, further comprising administering the composition more than one time to the subject.
99. The method of claim 71, wherein the composition is administered orally.
100. The method of claim 71, wherein the composition is administered parenterally.
101. The method of claim 100, wherein the composition is administered subcutaneously, intramuscularly, or intravenously.
102. The method of claim 74, wherein the composition comprises a recombinant nucleic acid molecule encoding an SdrD protein, a recombinant nucleic acid molecule encoding an SdrE protein, a recombinant nucleic acid molecule encoding an IsdA protein, or a recombinant nucleic acid molecule encoding an IsdB protein.
103. The method of claim 102, wherein the composition comprises a recombinant nucleic acid molecule encoding an SdrD protein, a recombinant nucleic acid molecule encoding an SdrE protein, a recombinant nucleic acid molecule encoding an IsdA protein, and a recombinant nucleic acid molecule encoding an IsdB protein.
104. The method of claim 103, wherein a nucleic acid molecule encodes two or more of a SdrD protein, a SdrE protein, an IsdA protein, or an IsdB protein.
105. The method of claim 102, wherein the recombinant nucleic acid molecule encoding a SdrD, SdrE, IsdA or IsdB protein contains a heterologous promoter.

106. The method of claim 102, wherein the recombinant nucleic acid molecule is a vector.
107. The method of claim 106, wherein the vector is a plasmid.
108. The method of claim 106, wherein the vector is a viral vector.
109. The method of claim 71, wherein the composition includes a recombinant, non-staphylococcus bacterium containing the SdrD, SdrE, IsdA or IsdB protein.
110. The method of claim 109, wherein the recombinant non-staphylococcus bacterium is Salmonella.
111. The method of claim 71, wherein the subject is human.
112. The method of claim 71, wherein the immune response is a protective immune response.
113. A method for stimulating in a subject a protective immune response against a staphylococcus bacterium comprising administering to the subject an effective amount of a composition including i) two or more of SdrD, SdrE, IsdA, or IsdB proteins; or, ii) one or more nucleic acid molecules encoding two or more SdrD, SdrE, IsdA, or IsdB proteins, wherein the composition is not a Staphylococcus bacteria.
114. The method of claim 113, wherein the subject is a human.
115. The method of claim 113, wherein the composition is formulated in a pharmaceutically acceptable formulation.
116. The method of claim 113, wherein the Staphylococcus bacteria is Staphylococcus aureus.

117. A vaccine comprising a pharmaceutically acceptable composition having two or more isolated SdrD, SdrE, IsdA, or IsdB proteins, wherein the composition is capable of stimulating an immune response against a Staphylococcus bacteria.

118. The vaccine of claim 117, further comprising an effective amount of EsxA, EsxB, or EsxA and EsxB.

119. The vaccine of claim 117, comprising an isolated SdrD, an isolated SdrE protein, an isolated IsdA, and an isolated IsdB protein.

120. The vaccine of claim 119, wherein the composition is contaminated by less than 1% with other Staphylococcus bacterial proteins.

121. The vaccine of claim 117, further comprising an adjuvant.

122. The vaccine of claim 121, wherein the protein is linked to the adjuvant.

123. The vaccine of claim 122, wherein the adjuvant is chemically conjugated to the protein.

124. A vaccine comprising a pharmaceutically acceptable composition having a recombinant nucleic acid encoding two or more of SdrD, SdrE, IsdA, or IsdB proteins, wherein the composition is capable of stimulating an immune response against a Staphylococcus bacteria..

125. The vaccine of claim 124, further comprising a recombinant nucleic acid encoding EsxA, EsxB, or EsxA and EsxB.

126. The vaccine of claim 124, wherein the composition comprises a recombinant nucleic acid encoding a SdrD protein, a recombinant nucleic acid encoding a SdrE protein, a recombinant nucleic acid encoding an IsdA protein, or a recombinant nucleic acid encoding an IsdB protein.

127. The vaccine of claim 124, wherein the recombinant nucleic acid contains a heterologous promoter.
128. The vaccine of claim 124, wherein the recombinant nucleic acid is a vector.
129. The vaccine of claim 128, wherein the vector is a plasmid.
130. The vaccine of claim 128, wherein the vector is a viral vector.
131. The vaccine of claim 124, wherein the composition includes a recombinant, non-Staphylococcus bacteria containing the nucleic acid.
132. The vaccine of claim 131, wherein the recombinant non-Staphylococcus bacteria is Salmonella.
133. The vaccine of claim 124, wherein the composition further comprises an adjuvant.

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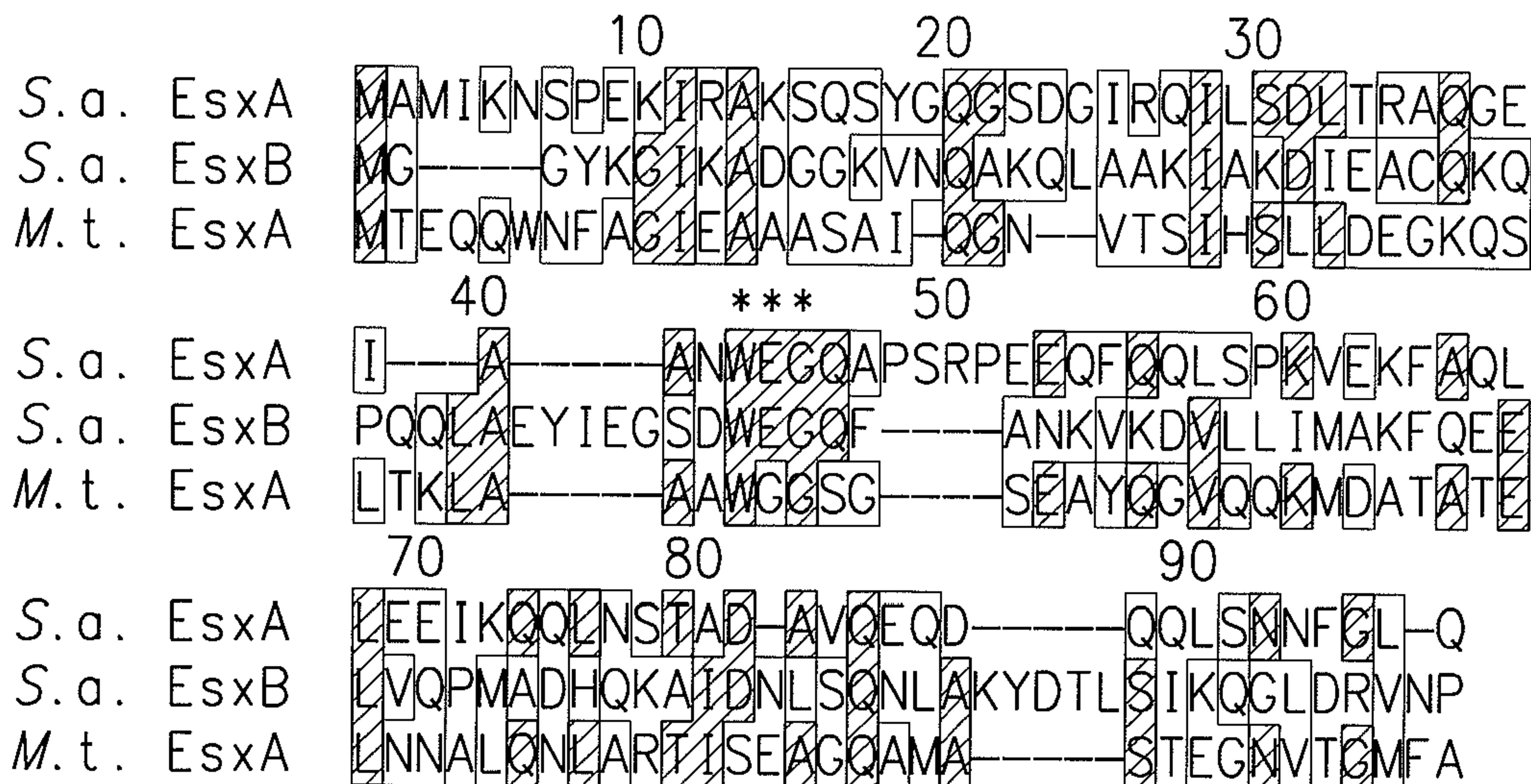


FIG. 1A

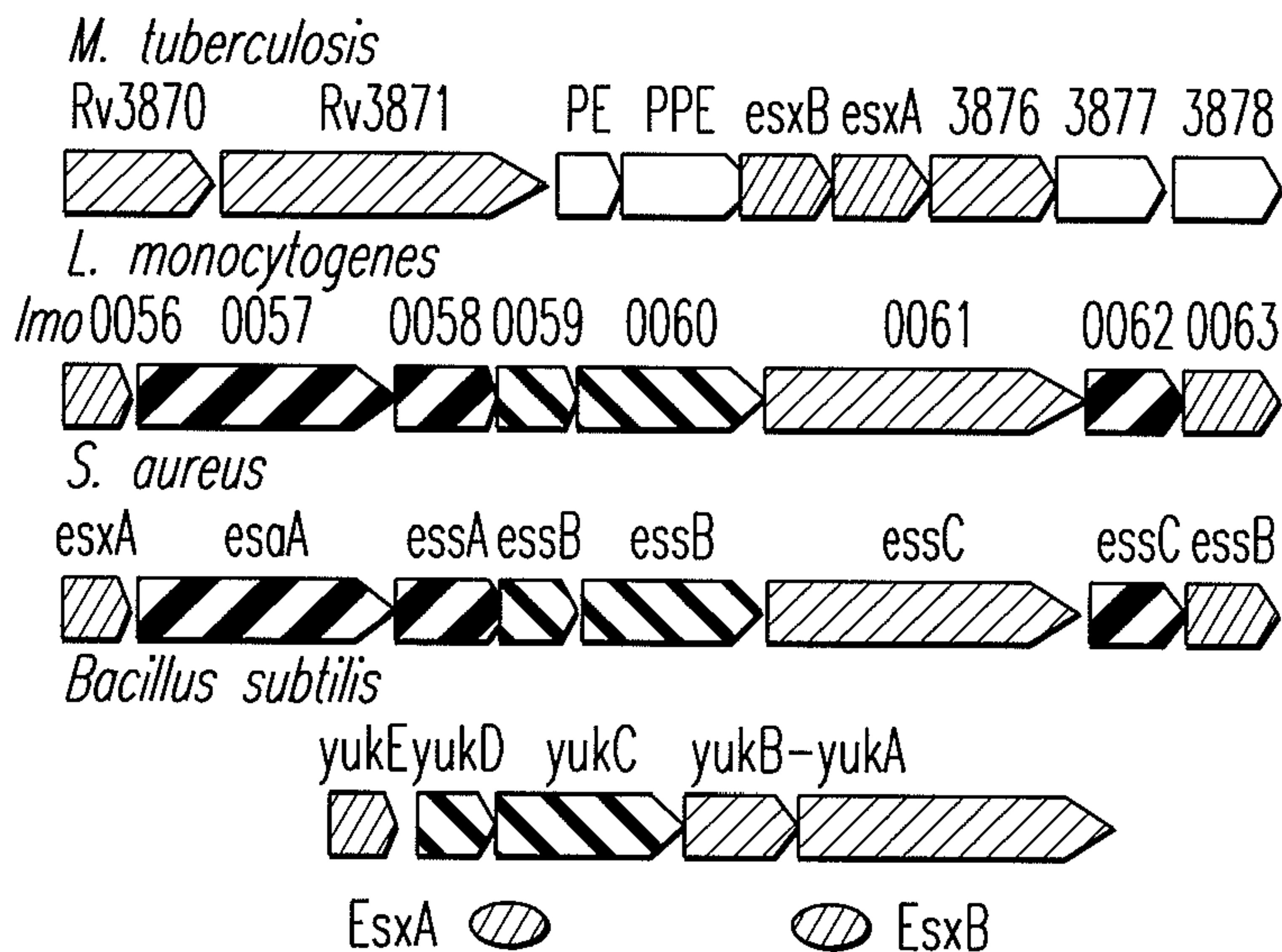


FIG. 1B

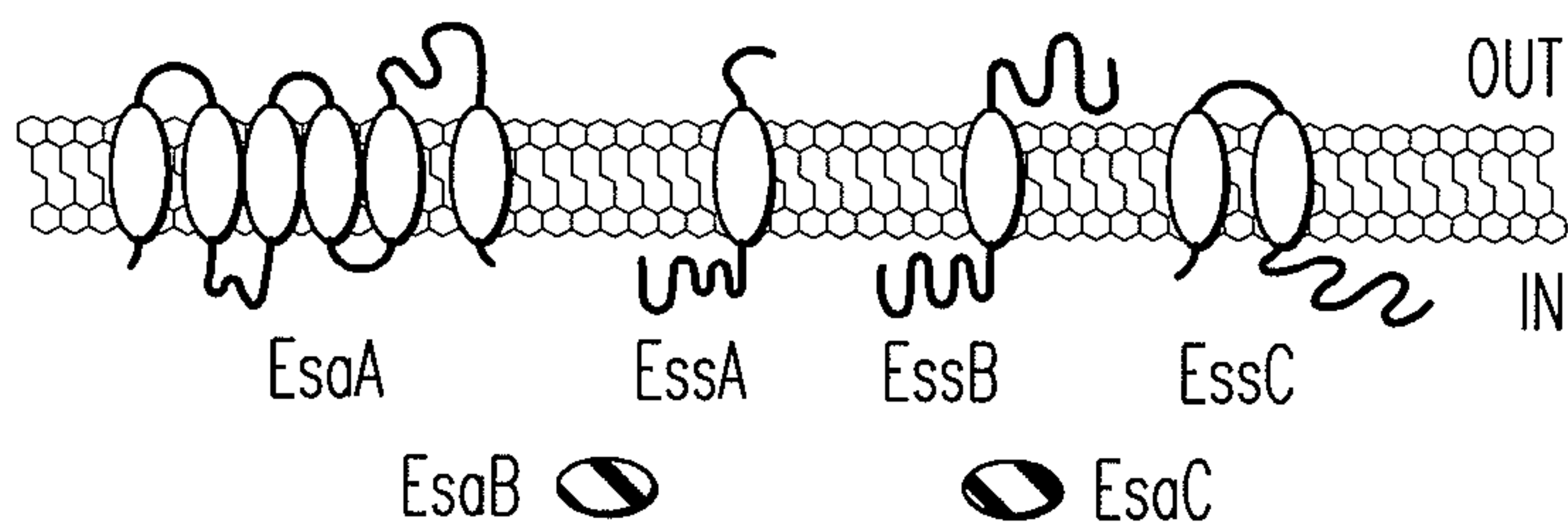


FIG. 1C



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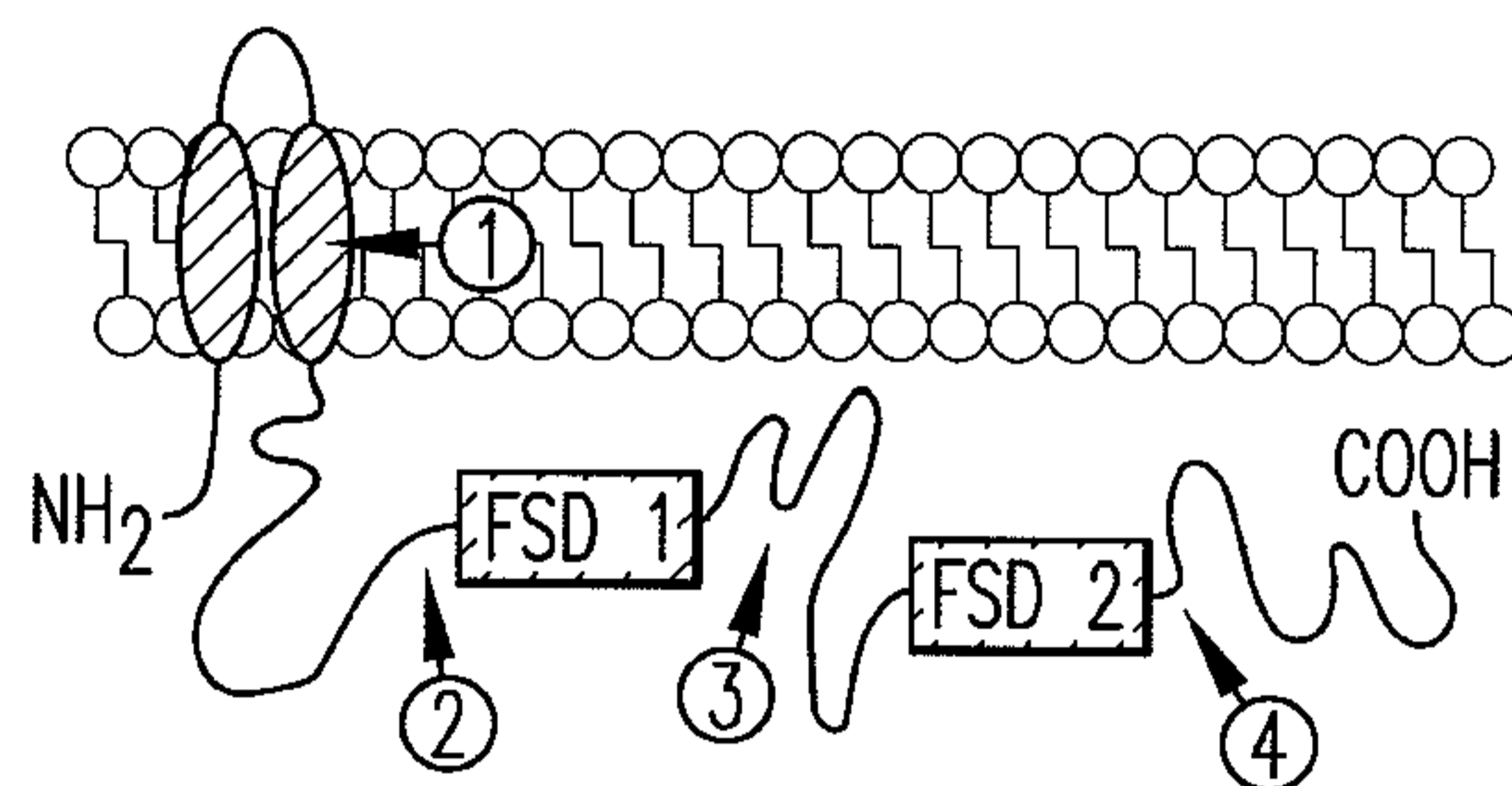


FIG.4A

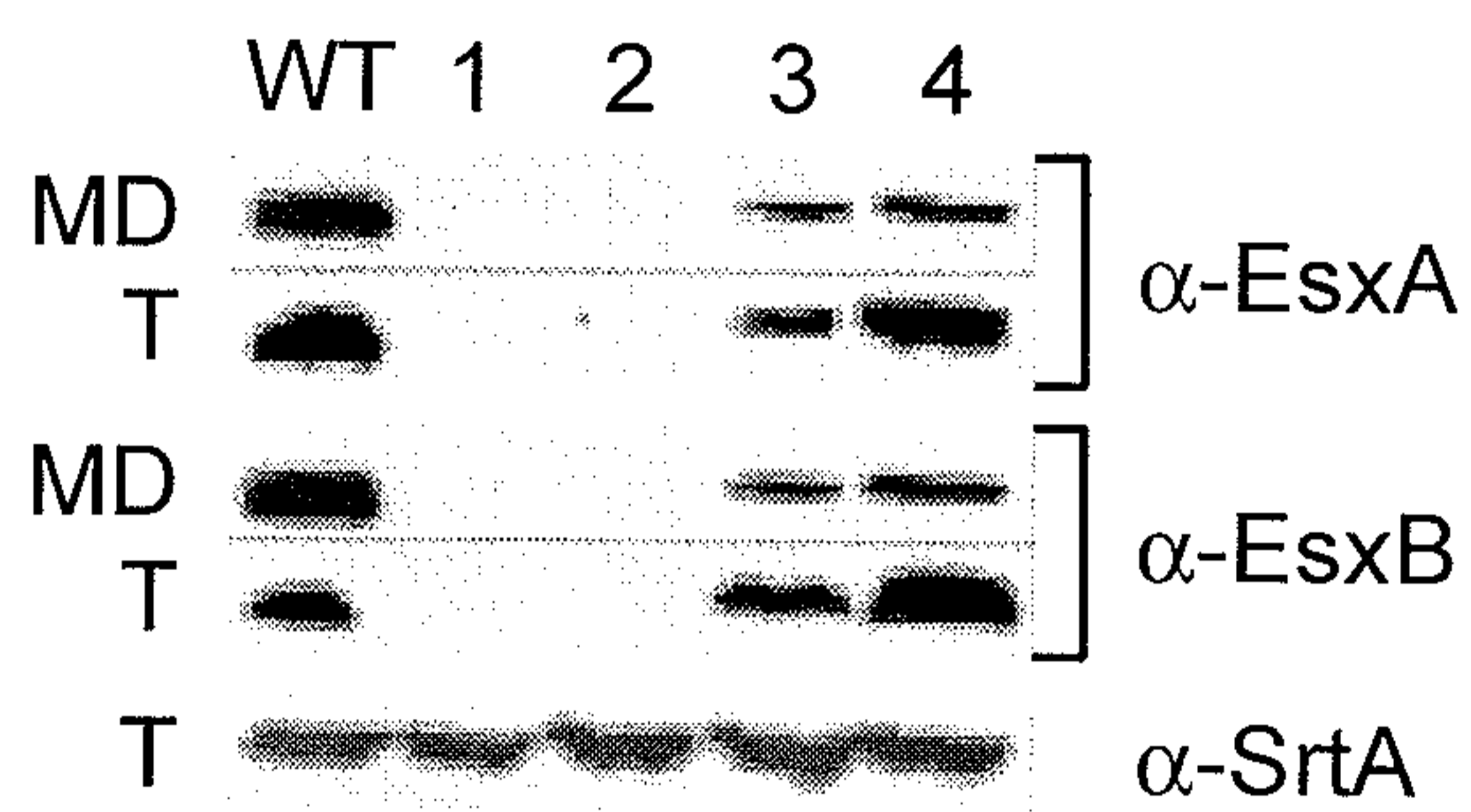


FIG.4B

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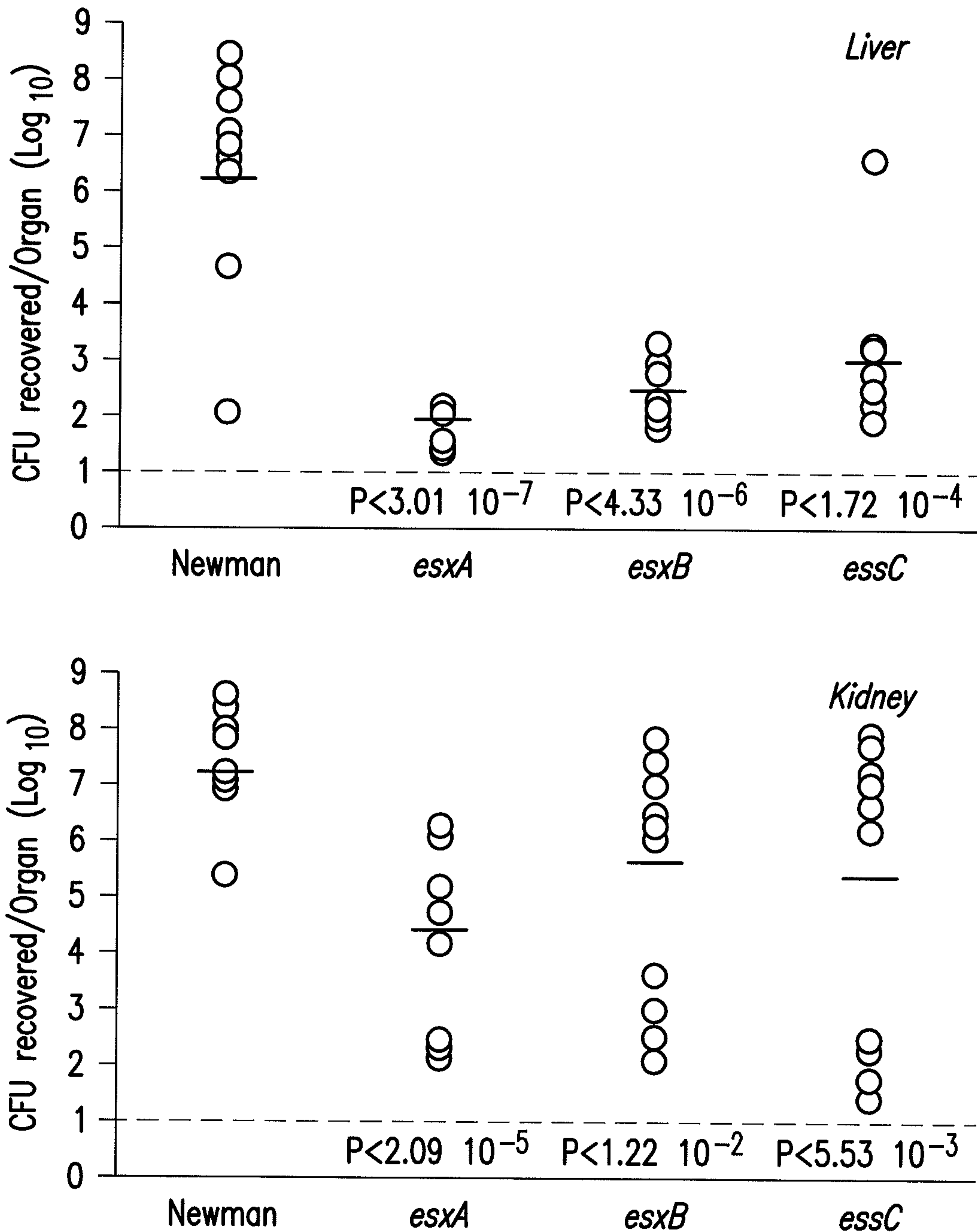


FIG. 5



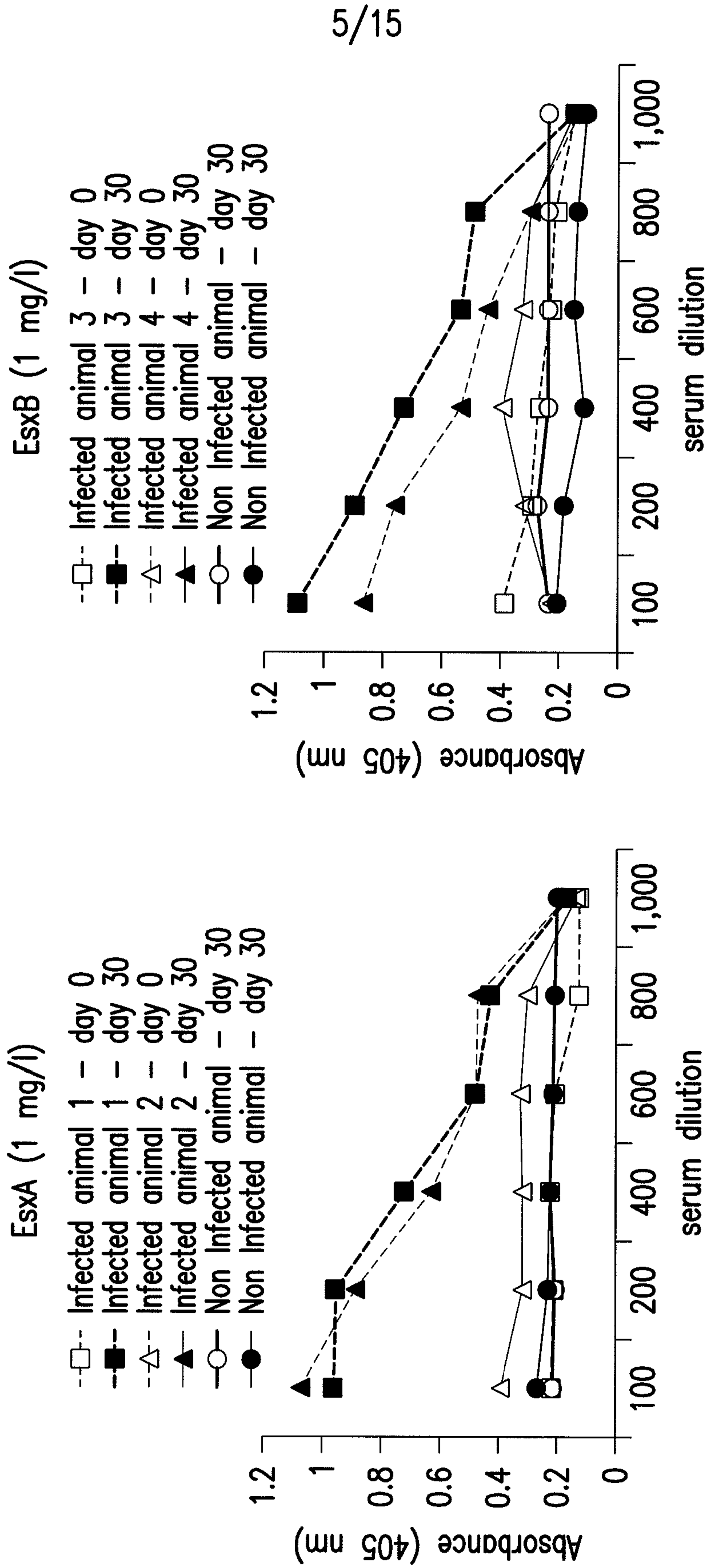


FIG.6

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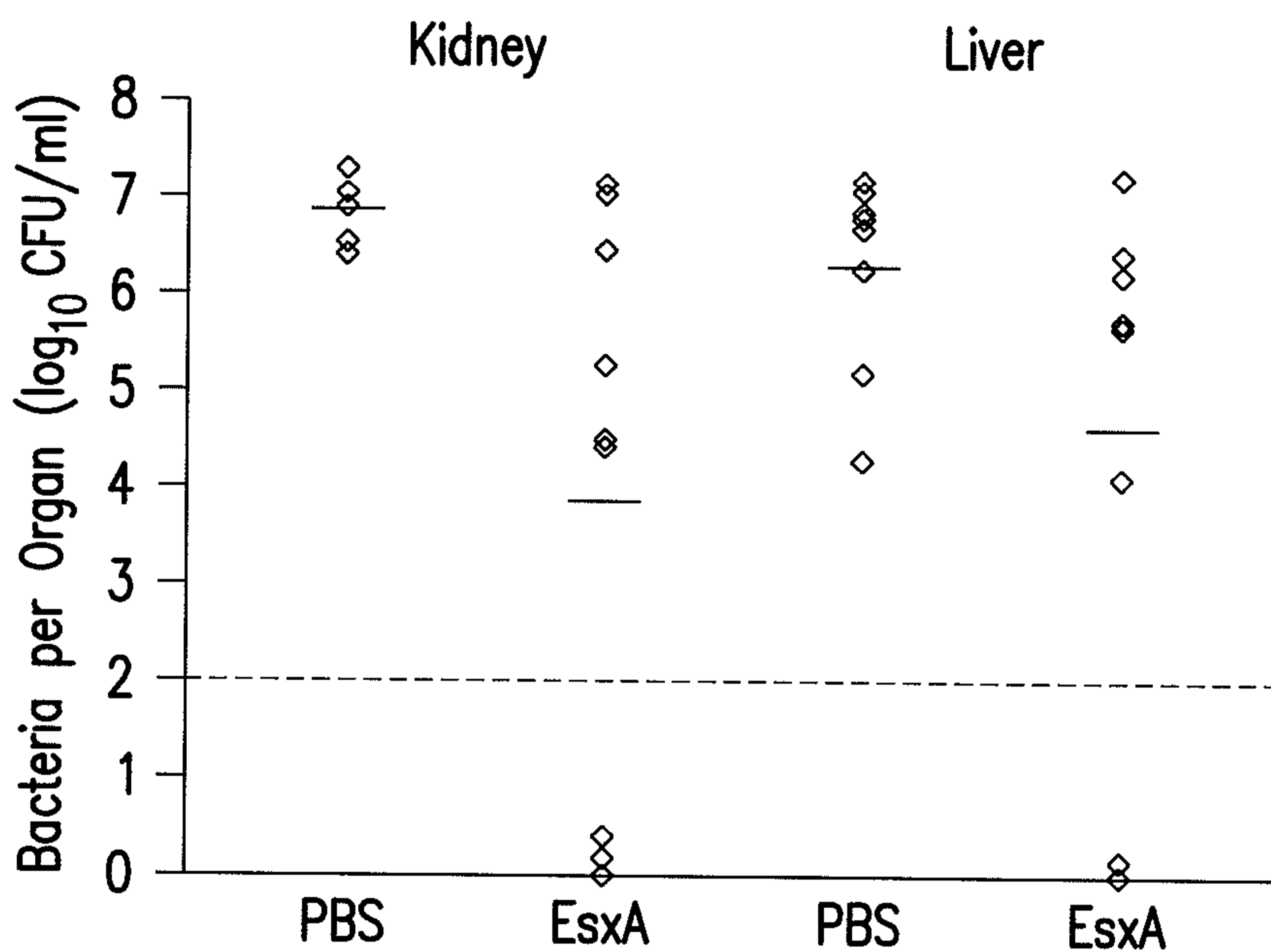
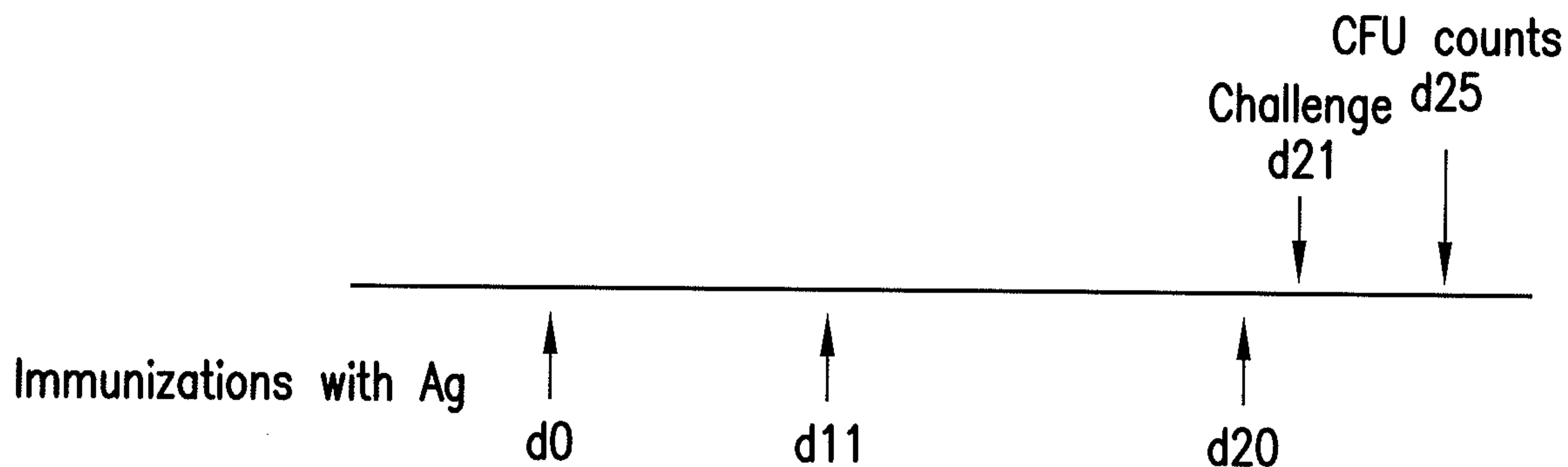


FIG. 7

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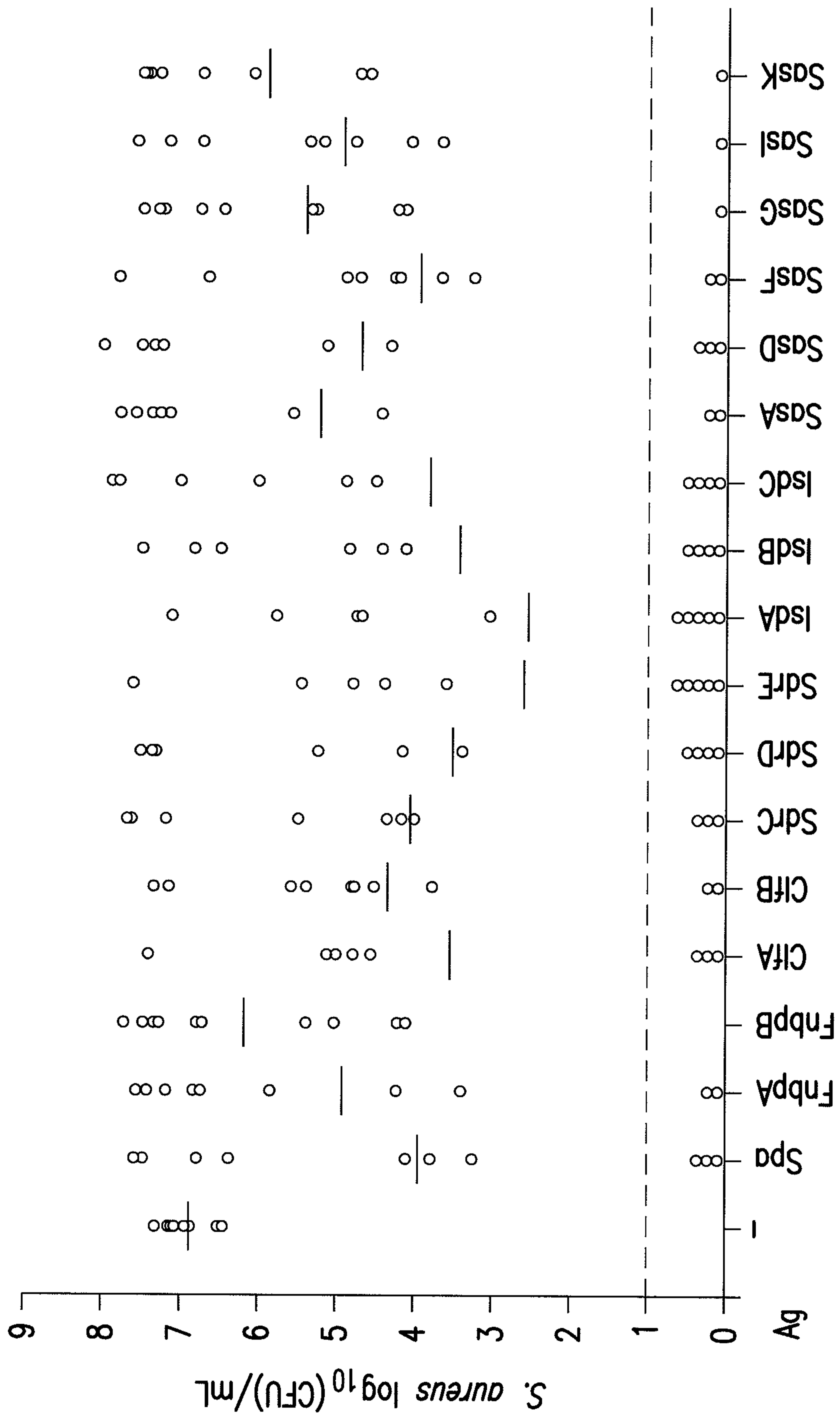


FIG. 8

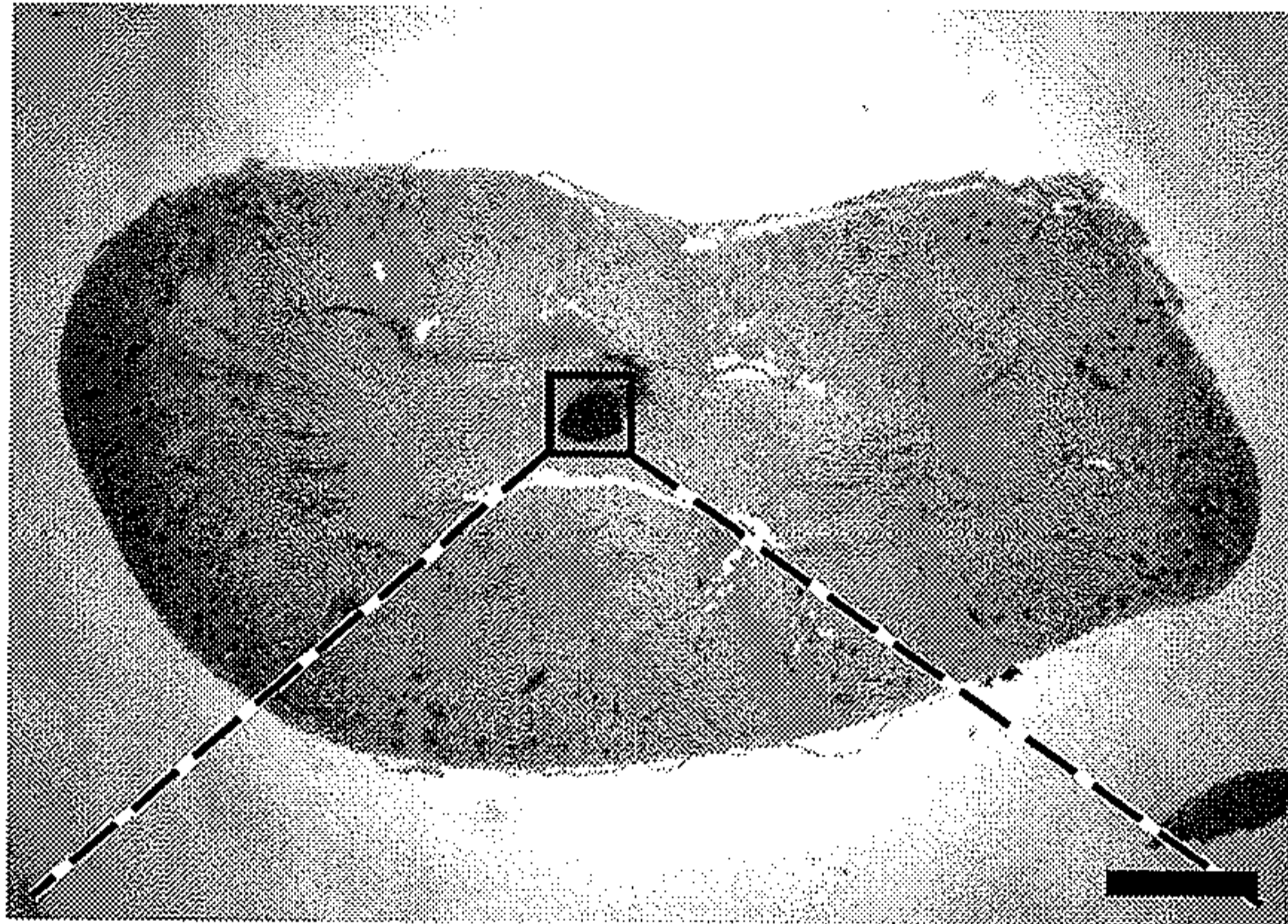


FIG.9A

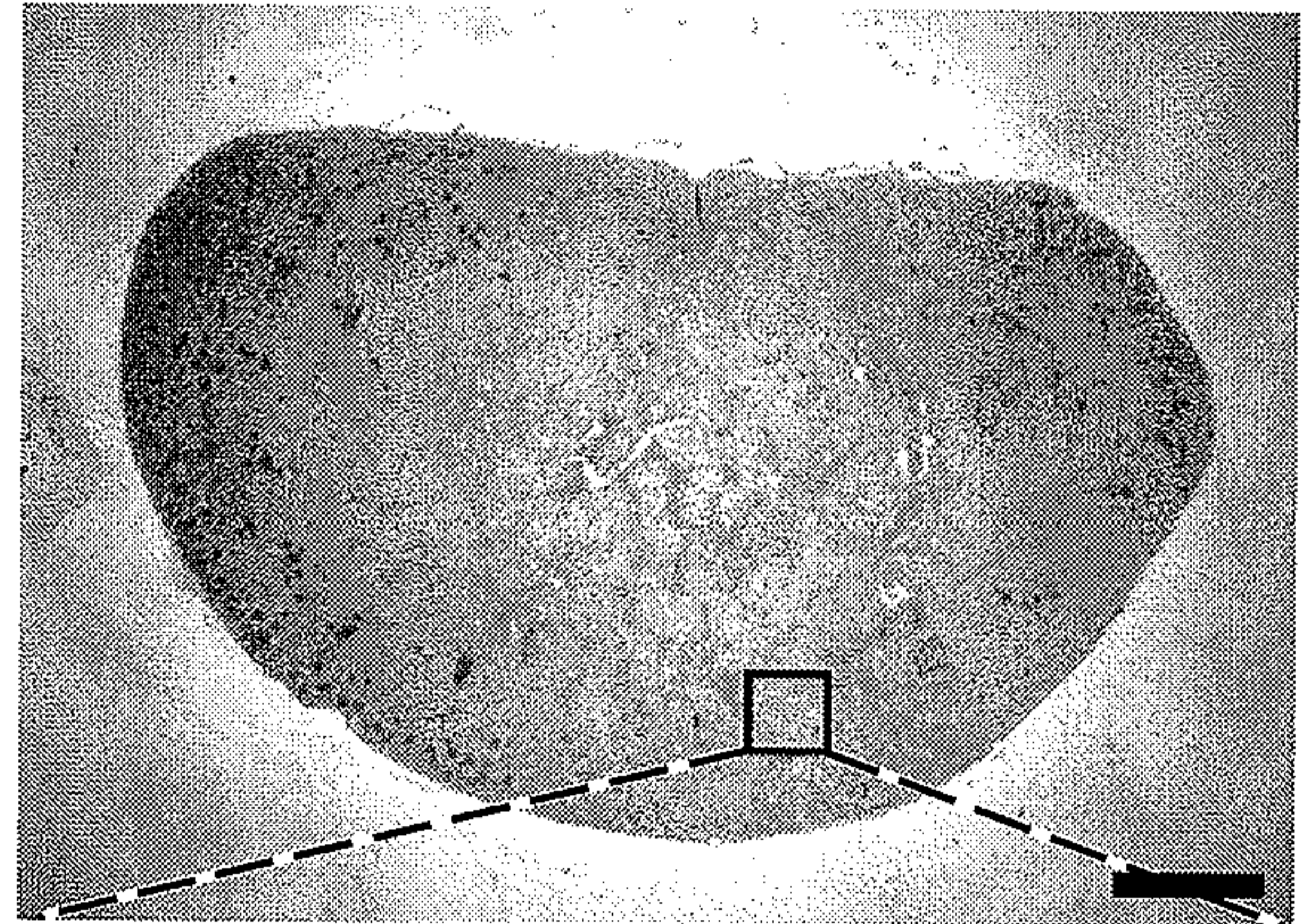


FIG.9C

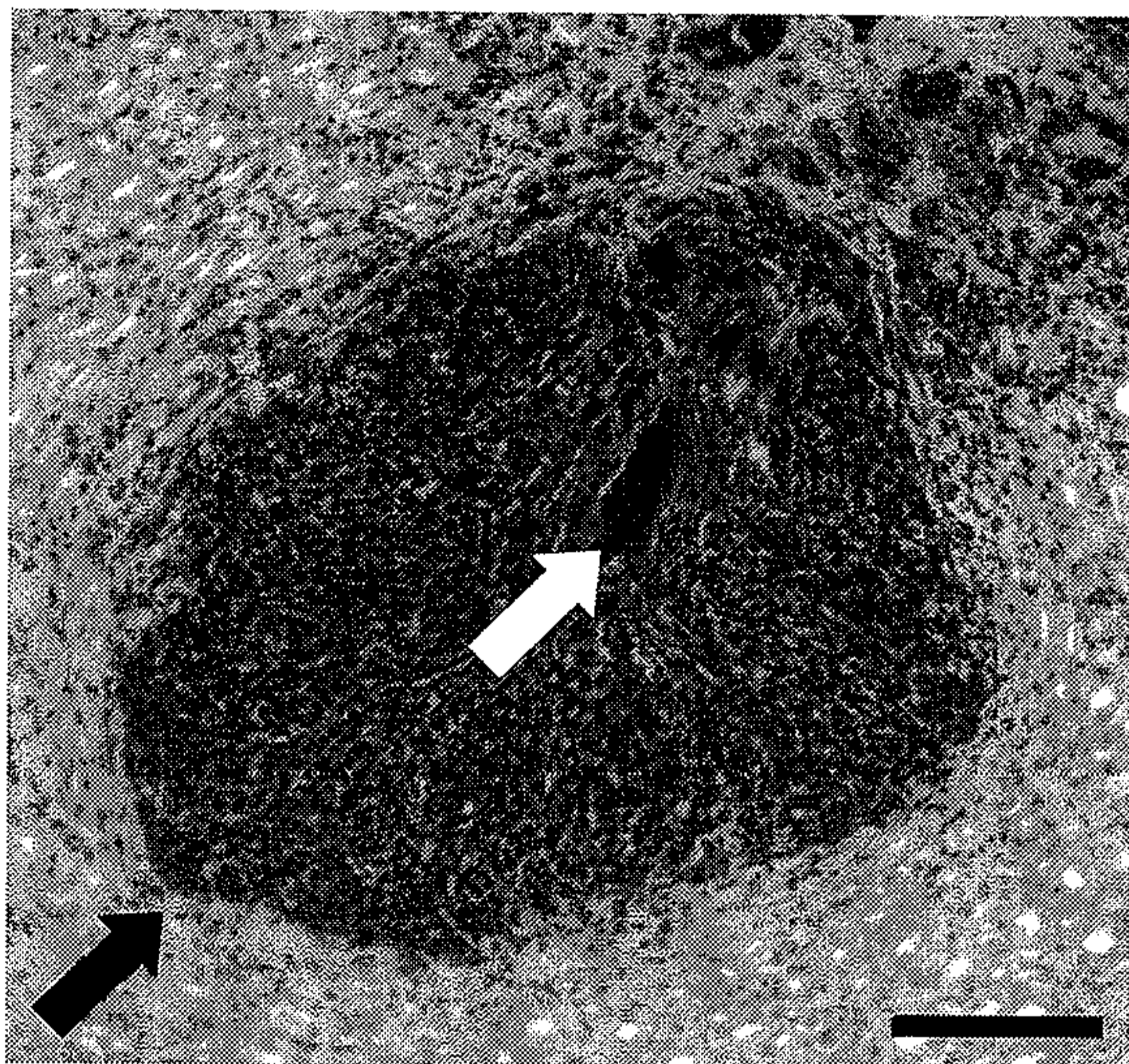


FIG.9B

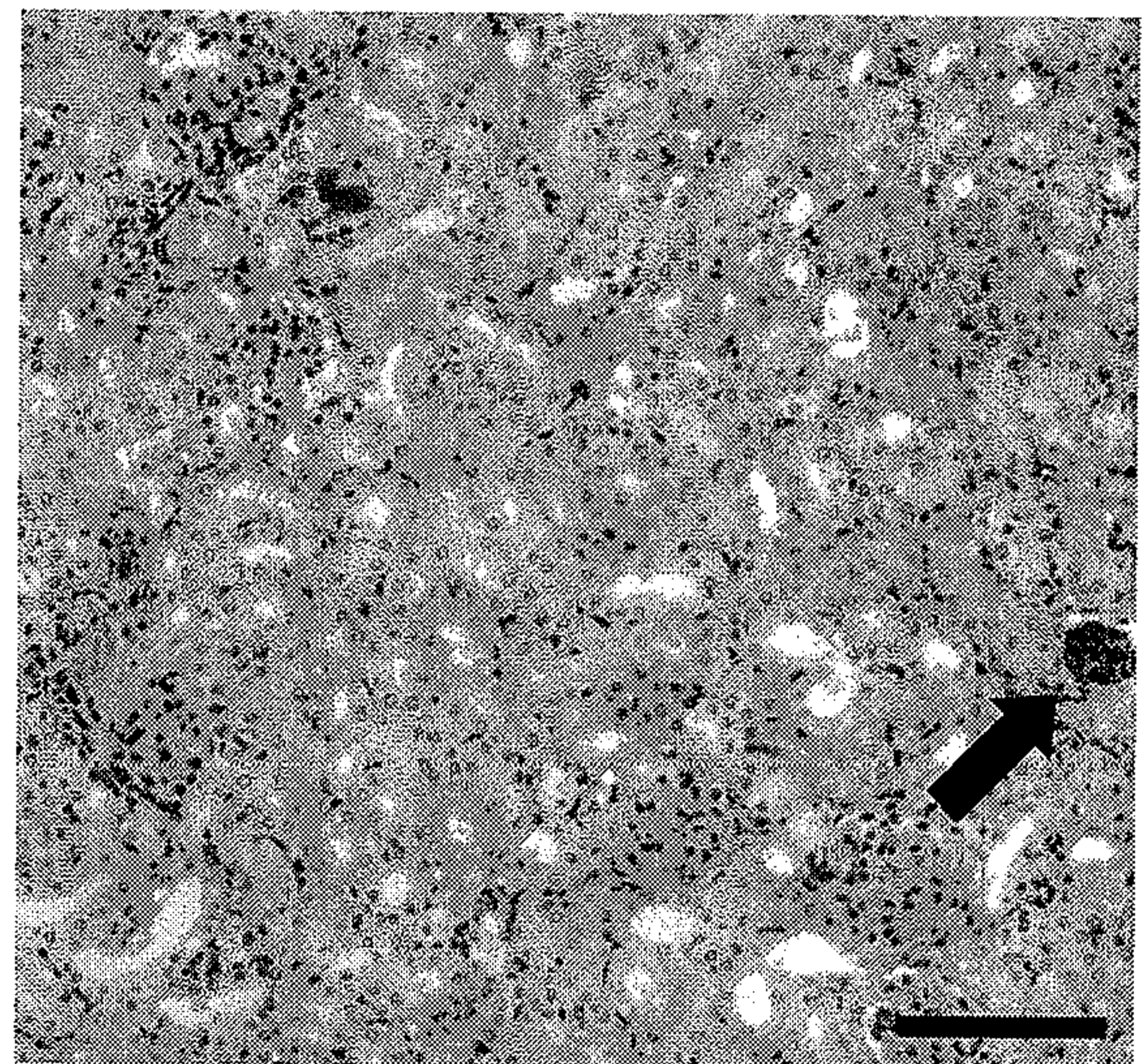


FIG.9D

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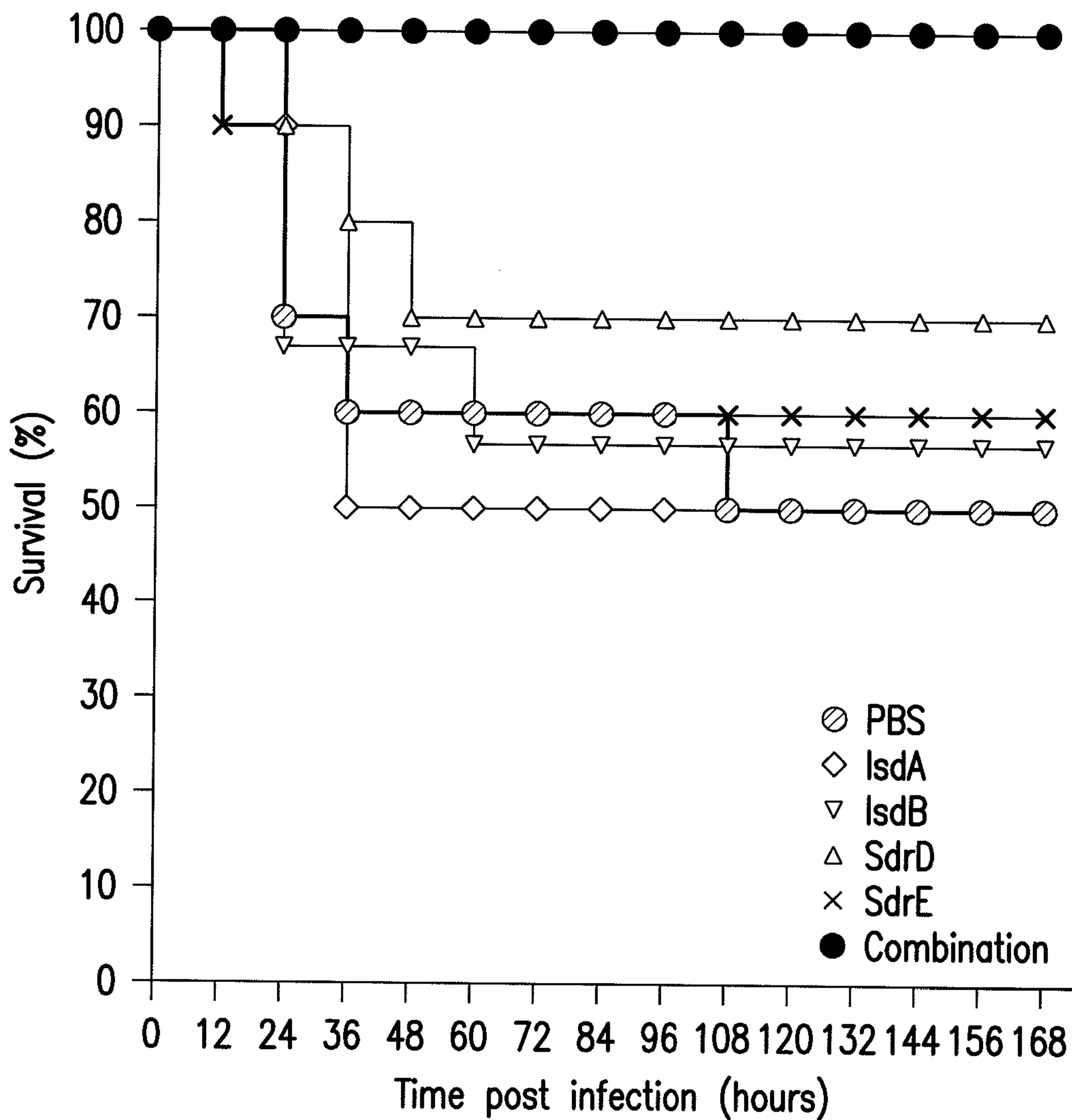


FIG.10

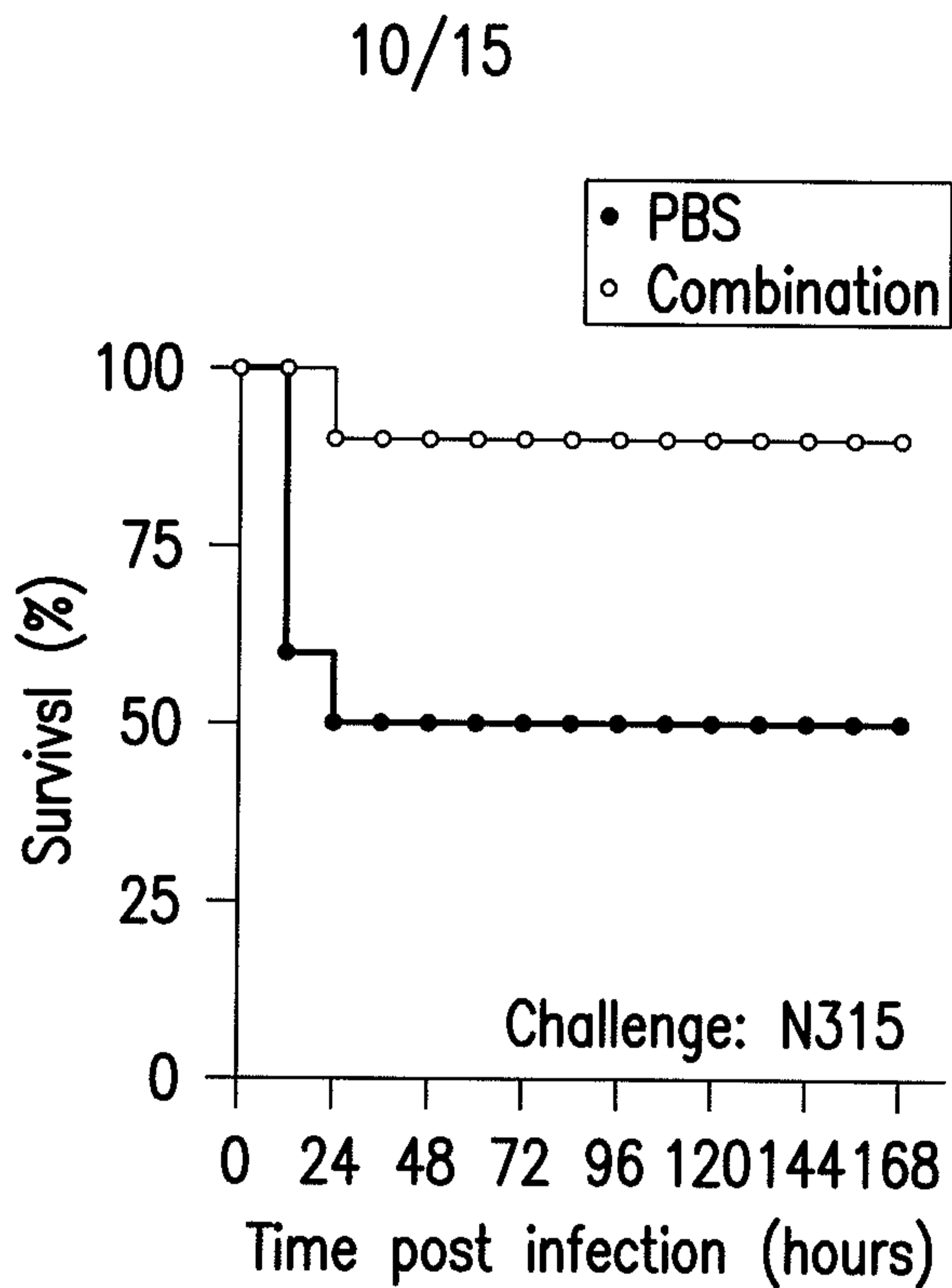


FIG. 11A

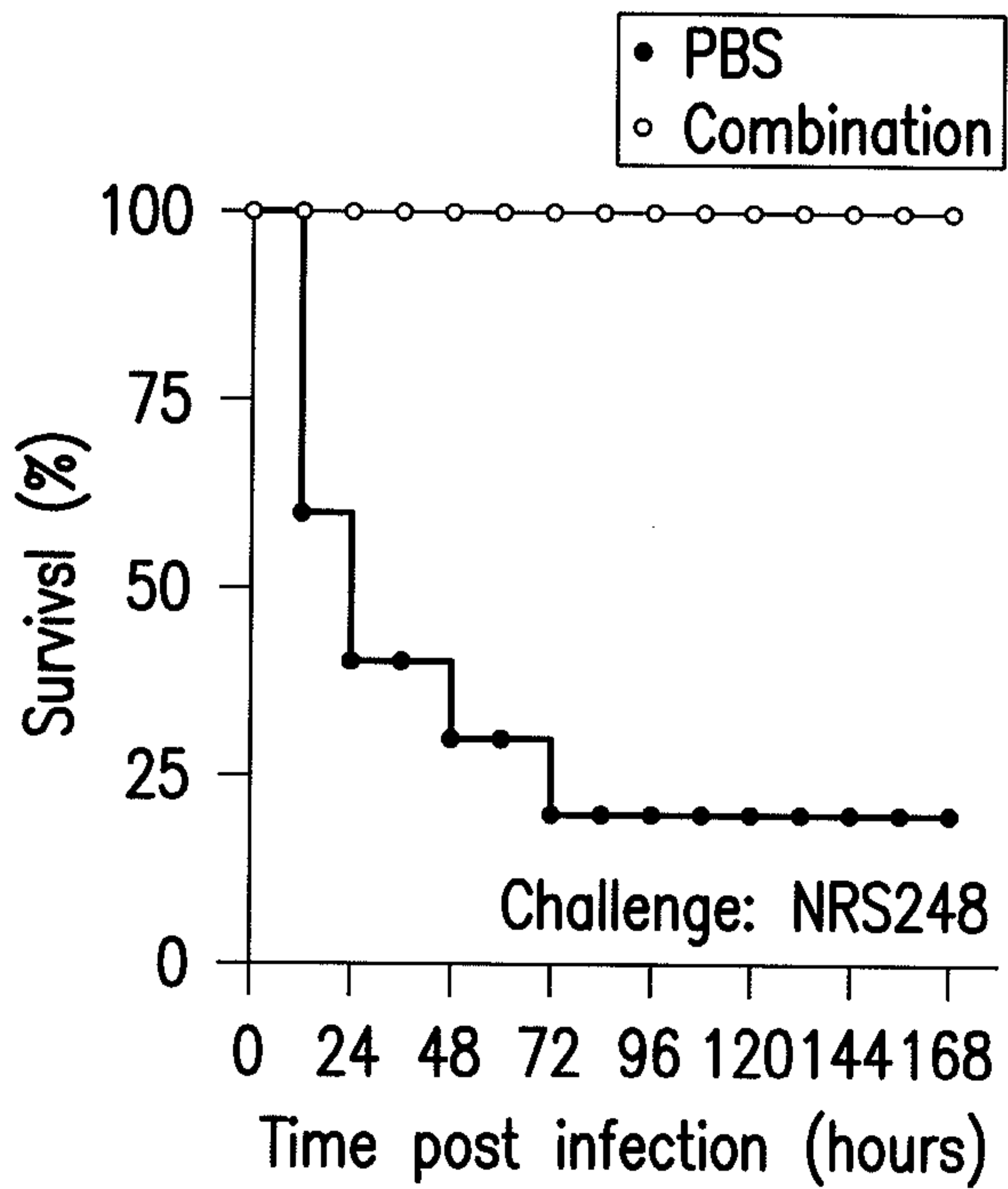


FIG. 11B

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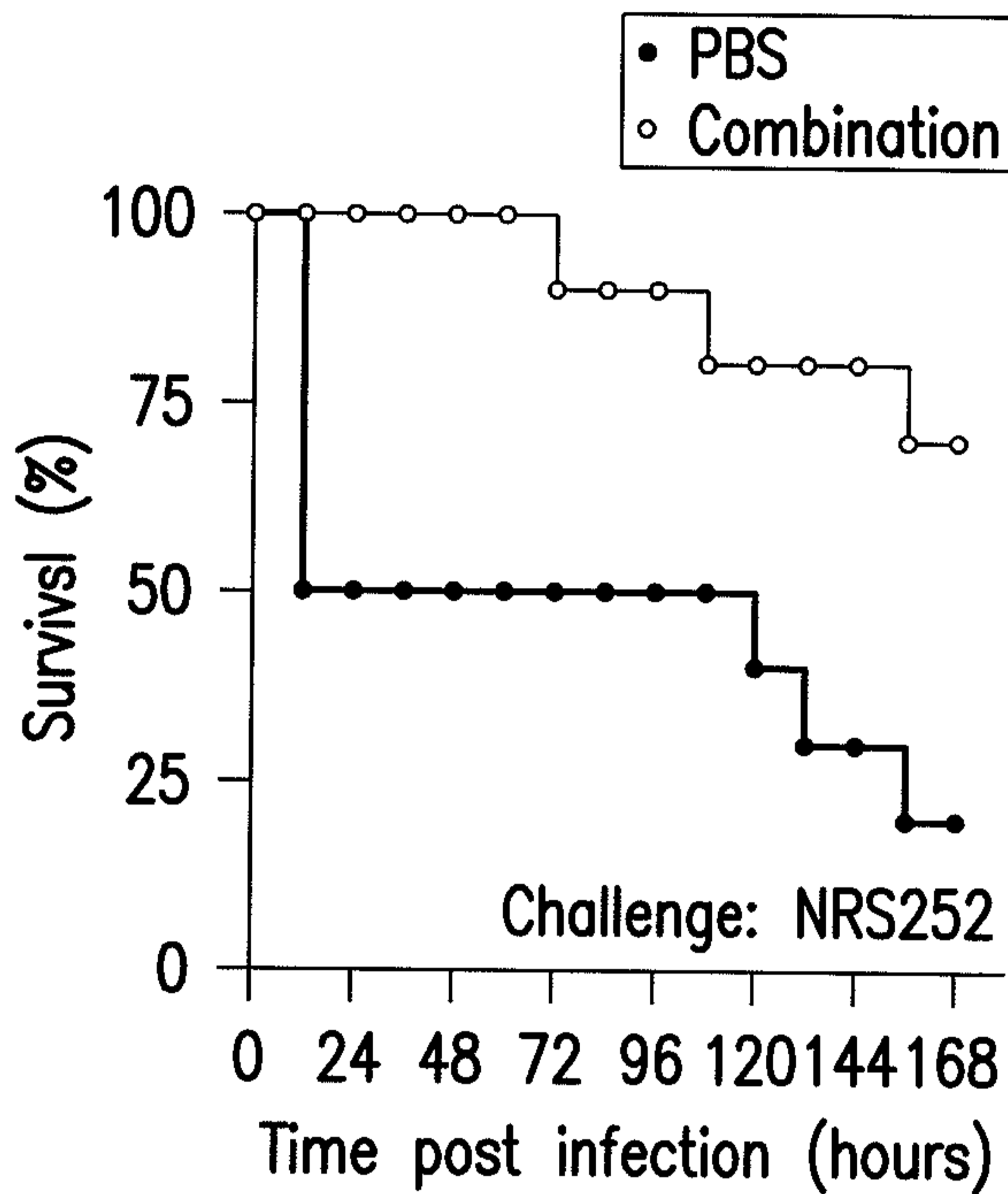


FIG. 11C

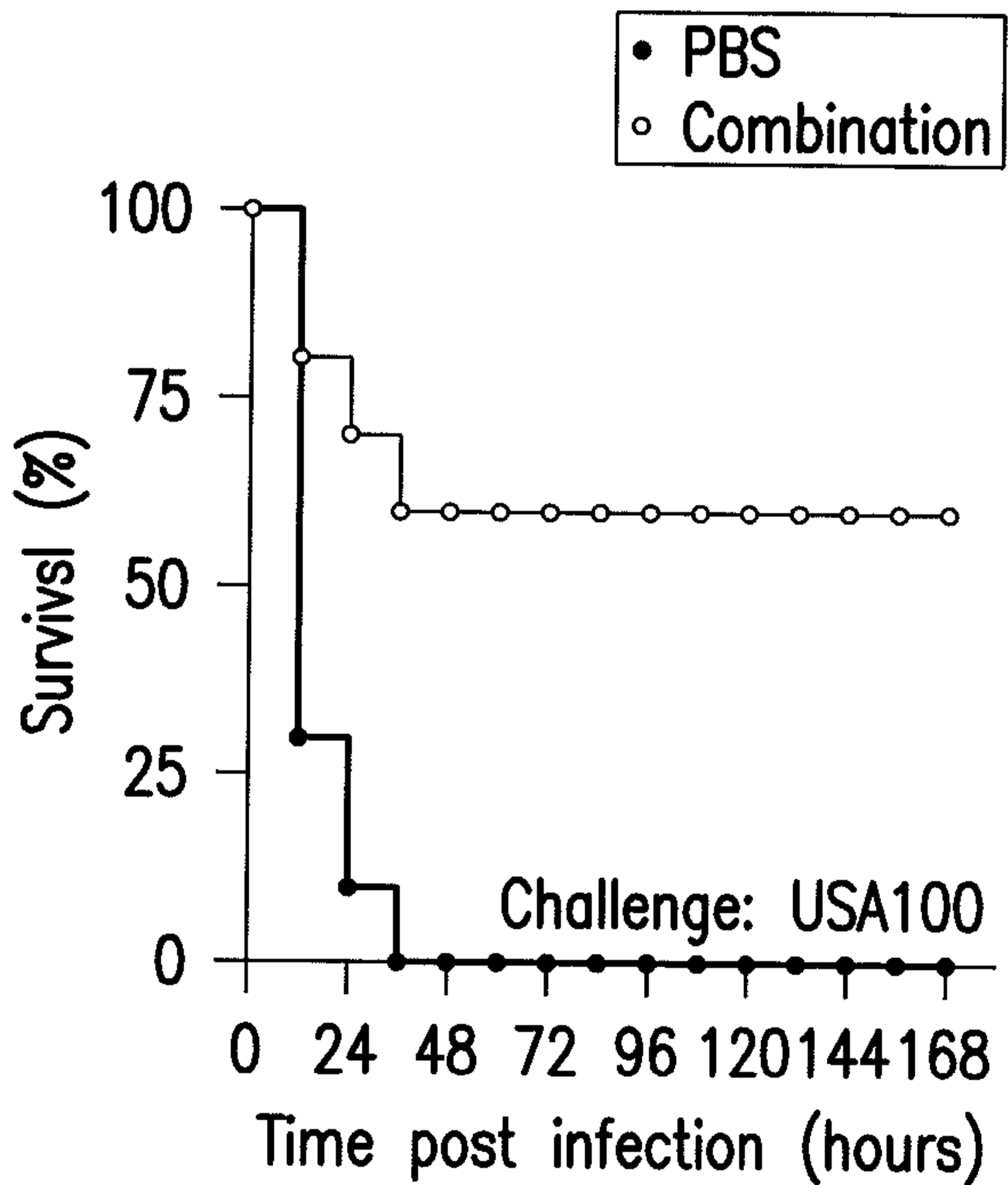


FIG. 11D

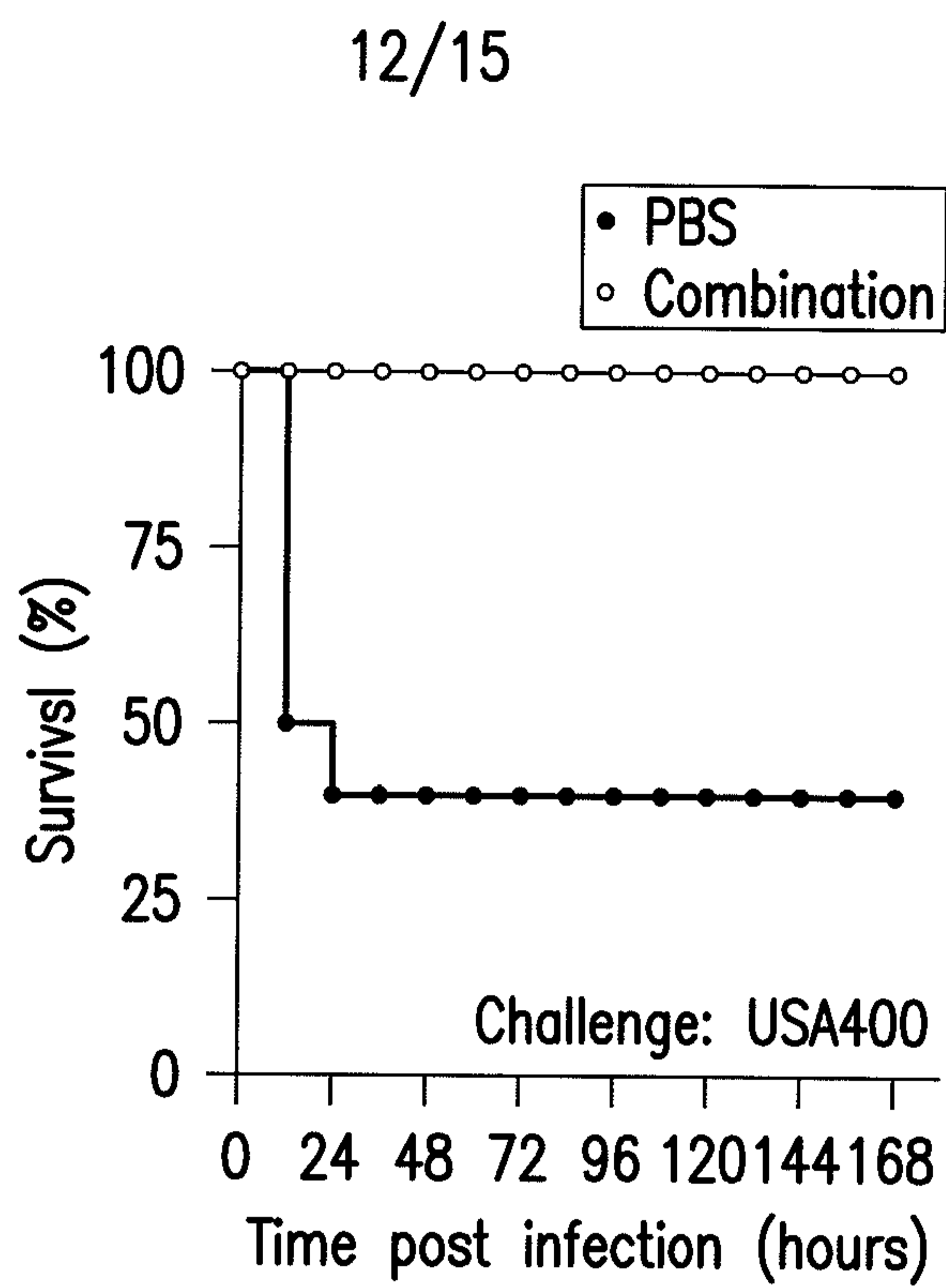


FIG. 11E



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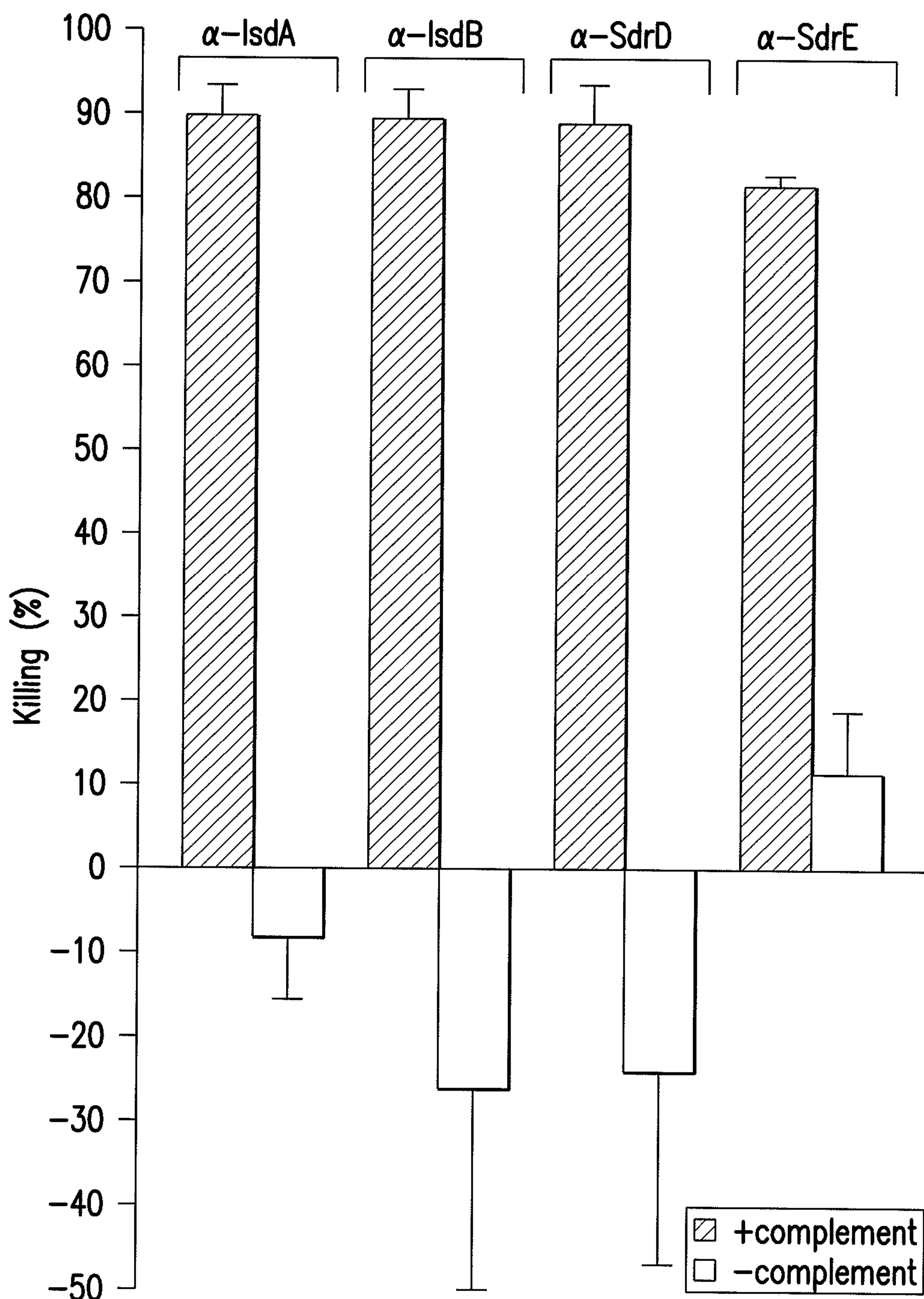


FIG. 12

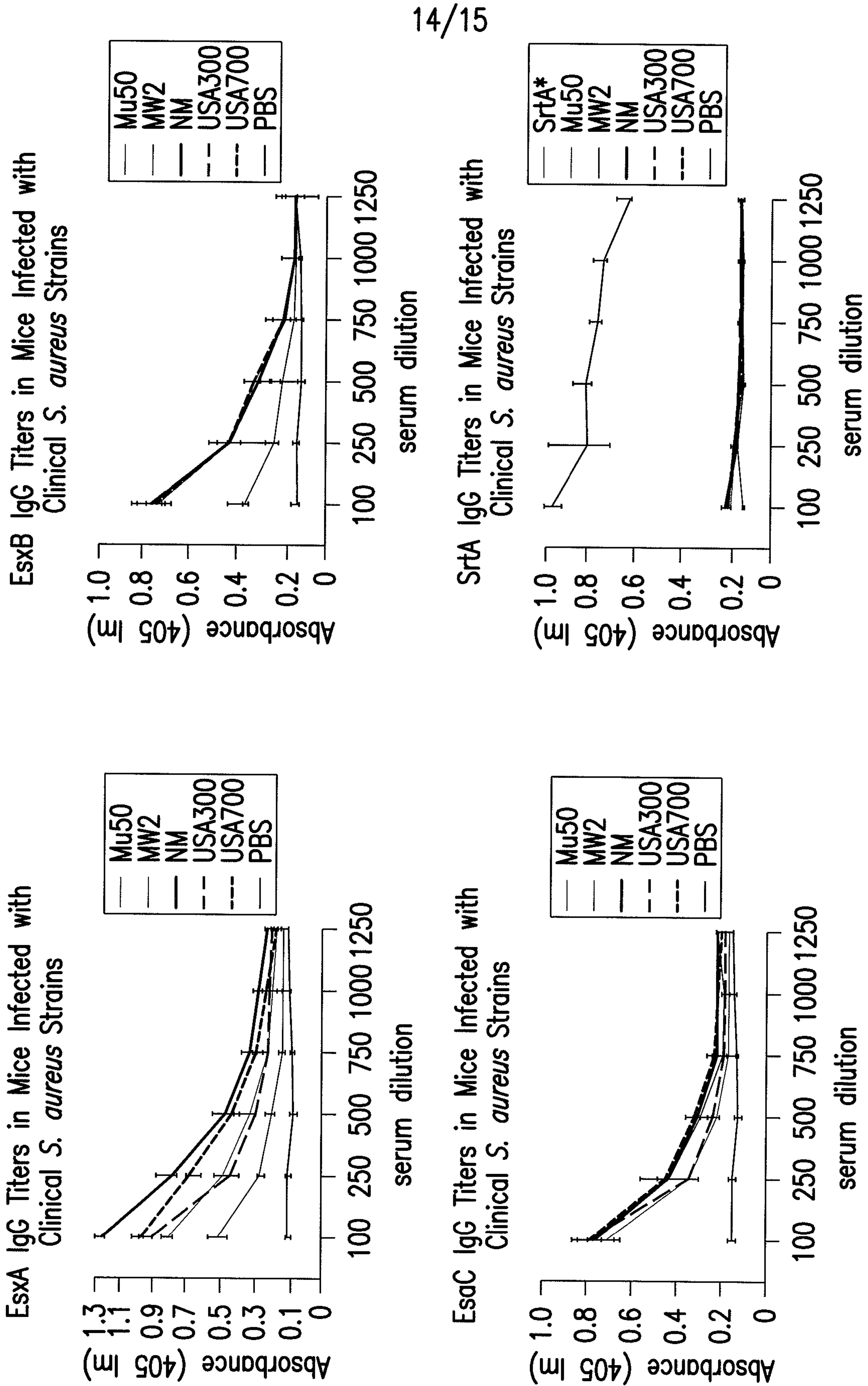


FIG. 13

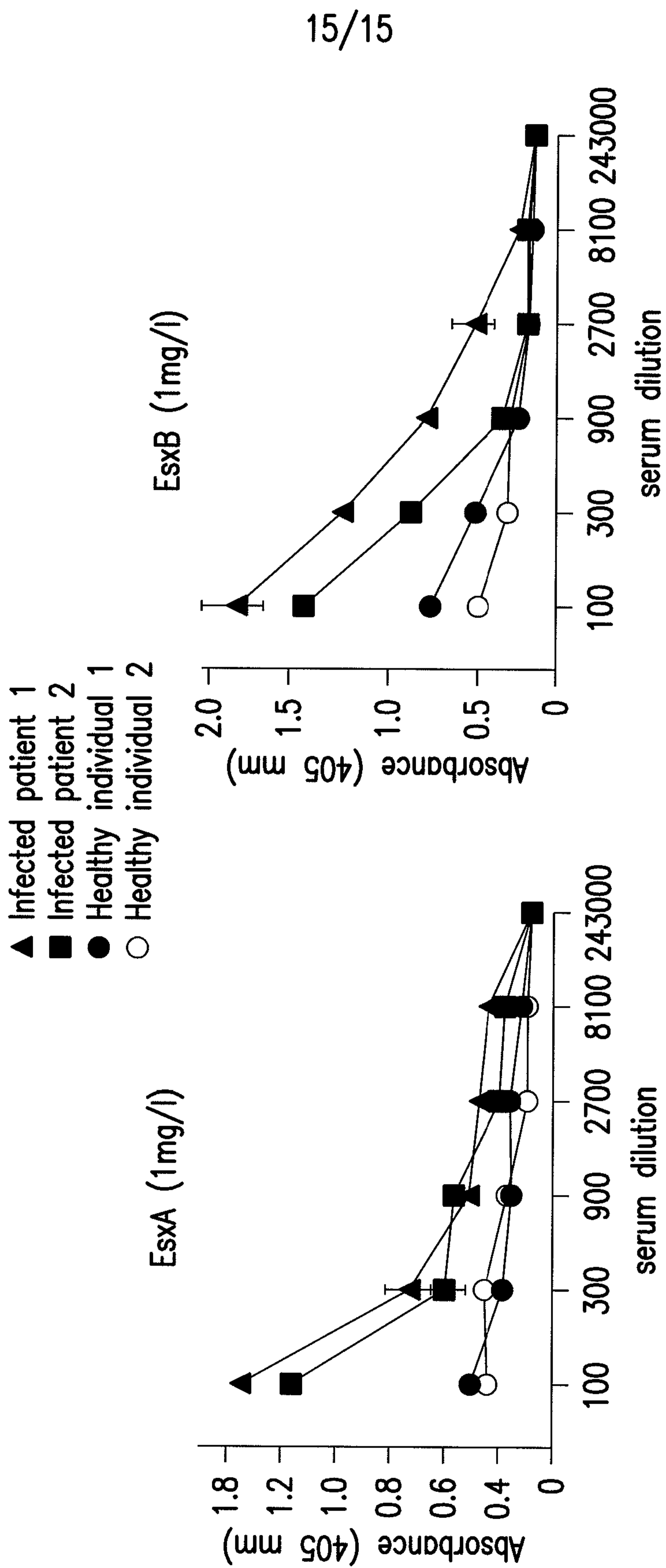


FIG.14