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(54) Titre : ANTI-INFECTIEUX A BASE DE PROTEINE ISD POUR UNE INFECTION A STAPHYLOCOCCUS AUREUS
(54) Title: STAPHYLOCOCCUS AUREUS ISD PROTEIN-BASED ANTI-INFECTIVES

(57) **Abrégé/Abstract:**

Iron-regulated surface determinant proteins IsdA, IsdB and IsdC from Staphylococcus aureus, as well as antibodies, antisense nucleic acids and siRNA specific to IsdA, IsdB or IsdC are used as vaccines and in method for treating or preventing a disease or condition associated with S. aureus infections Screening assays for identifying agents that inhibit or interfere with the expression level and/or function of IsdA, IsdB or IsdC are also described.



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(54) Title: STAPHYLOCOCCUS AUREUS ISD PROTEIN-BASED ANTI-INFECTIVES

(57) Abstract: Iron-regulated surface determinant proteins IsdA, IsdB and IsdC from Staphylococcus aureus, as well as antibodies, antisense nucleic acids and siRNA specific to IsdA, IsdB or IsdC are used as vaccines and in method for treating or preventing a disease or condition associated with S. aureus infections Screening assays for identifying agents that inhibit or interfere with the expression level and/or function of IsdA, IsdB or IsdC are also described.

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Staphylococcus aureus Isd Protein-based Anti-Infectives

Cross-Reference to Related Applications

This application claims priority to U.S. Provisional Application No. 60/621,921,
5 which was filed on October 25, 2004, the contents of which are hereby incorporated by
reference in their entirety.

Background

Iron is an absolute requirement for the growth of most microorganisms, with the
possible exceptions of lactobacilli (Archibald (1983) FEMS Microbiol. Lett. 19:29-32) and
10 *Borrelia burgdorferi* (Posey and Gherardini (2000) Science 288:1651-1653). Despite being
the fourth most abundant element on the Earth's crust, iron is frequently a growth-limiting
nutrient. In aerobic environments and at physiological pH, iron is present in the ferric
(Fe³⁺) state and forms insoluble hydroxide and oxyhydroxide precipitates. Mammals
overcome iron restriction by possessing high-affinity iron-binding glycoproteins such as
15 transferrin and lactoferrin that serve to solubilize and deliver iron to host cells (Weinberg
(1999) Emerg. Infect. Dis. 5:346-352). This results in a further restriction of free
extracellular iron, and accordingly, the concentration of free iron in the human body is
estimated to be 10⁻¹⁸ M, a concentration that is several orders lower than that required to
support a productive bacterial infection (Braun *et al.*, (1998) Bacterial iron transport:
20 mechanisms, genetics, and regulation, p. 67-145. In A. Sigel and H. Sigel (ed.), Metal Ions
in Biological Systems, vol. 35. Iron transport and storage in microorganisms, plants, and
animals. Marcel Dekker, Inc., New York). Sequestration of iron is an important innate
defense against bacterial infection (Skaar and Schneewind (2004) Microbes and Infect.
6:390-397).

25 To overcome iron restriction, bacteria have evolved several different mechanisms to
acquire this essential nutrient. For example, members of the *Pasteurellaceae* may express
receptors for the recognition of iron-loaded forms of transferrin and lactoferrin (Gray-Owen
and Schryvers, (1996) Trends Microbiol. 4:185-91). One of the most common iron
acquisition mechanisms, though, is through the use of low molecular weight, high-affinity
30 iron chelators, termed siderophores, and cognate cell envelope receptors that serve to
actively internalize ferric-siderophore complexes. Many siderophores are able to
successfully compete with transferrin and lactoferrin for host iron. Indeed, the expression

of ferric-siderophore uptake systems are critical virulence factors in bacteria such as septicemic *E. coli* (Williams (1979) Infect. Immun. 26:925-932), *Vibrio anguillarum* (Crosa *et al.*, (1980) Infect. Immun. 27:897-902), *Erwinia chrysanthemi* (Enard *et al.*, (1988) J. Bacteriol. 170:2419-2426) and *Pseudomonas aeruginosa* (Meyer *et al.*, (1996) Infect. Immun. 64:518-523).

Staphylococcus aureus (*S. aureus*) possesses several different iron-regulated ABC transporters, including those encoded by the *sstABCD* (Morrissey *et al.*, (2000) Infect. Immun. 68:6281-6288), *sirABC* (Heinrichs *et al.*, (1999) J. Bacteriol. 181:1436-1443; Dale *et al.*, (2004) J. Bacteriol., In press), *fhuCBG* (Sebulsky *et al.*, (2000) J. Bacteriol. 182:4394-4400), and *sbn* (Dale *et al.*, (2004) Infect. Immun. 72:29-37) operons. While the transported substrates are unknown for the *sst* and *sir* systems, the *fhuCBG* genes, in concert with *fhuD1* and *fhuD2* (Sebulsky and Heinrichs (2001) J. Bacteriol. 183:4994-5000), are involved in the acquisition of iron(III)-hydroxamate complexes. Several members of the staphylococci, including numerous coagulase-negative staphylococci (CoNS) and strains of *S. aureus*, produce siderophores. Two of these siderophores, staphyloferrin A (Konetschny-Rapp *et al.*, (1990) Eur. J. Biochem. 191:65-74; Meiwes *et al.*, (1990) FEMS Microbiol. Lett. 67:201-206) and staphyloferrin B (Dreschel *et al.*, (1993) BioMetals. 6:185-192; Haag *et al.*, (1994) FEMS Microbiol. Lett. 115:125-130), are of the polycarboxylate class, while the third, aureochelin (Courcol *et al.*, (1997) Infect. Immun. 65:1944-1948), is chemically uncharacterized. Further, an additional siderophore, encoded by the *sbn* operon, appears to be specific for *S. aureus* and may be a key determinant in the virulence of *S. aureus* in comparison to other CoNS strains (Dale *et al.*, (2004) Infect. Immun. 72:29-37).

The most abundant source of iron in the human body, however, is sequestered in heme-containing proteins (hemoproteins). Heme is a cyclic molecule that contains a single iron atom bound to four-ring nitrogen atoms of porphyrins. Hemoproteins are responsible for numerous cellular functions, and hemoglobin and myoglobin are the most abundant heme-containing proteins in mammals. As iron is essential for the survival of bacterial pathogens, bacteria have acquired several mechanisms to scavenge iron from free heme and hemoproteins. *S. aureus*, in particular, can acquire iron from heme or hemoproteins through the Iron-regulated surface determinant (Isd) system. The Isd system comprises cell-surface proteins that can bind and transport heme across the bacterial cell wall as well as at least one cytoplasmic protein that can extract iron from heme (Mazmanian *et al.*,

(2003) Science 299:906-909; Clarke *et al.*, (2004) Mol. Microbiol. 51:1509-1519). Further, Isd proteins, specifically IsdA appears to bind a broad spectrum of extracellular matrix proteins, including but not limited to, fibrinogen and fibronectin (Clarke *et al.*, (2004) Mol. Microbiol. 51:1509-1519) as well as transferrin (Taylor and Heinrichs (2002) Mol. Microbiol. 43:1603-1614) and hemin (Mazmanian *et al.*, (2003) Science 299:906-909).

S. aureus is a prevalent human pathogen that causes a wide range of infections ranging from minor skin and wound infections to more serious sequelae such as endocarditis, osteomyelitis and septicemia (Archer (1998) Clin. Infect. Dis. 26:1179-1181). The ability of *S. aureus* to invade and colonize many tissues may be ascribed to its capacity to express several virulence factors such as fibronectin-, elastin- and collagen-binding proteins that aid in tissue adherence, and multiple exotoxins and proteases that result in tissue destruction and bacterial dissemination. The ability of this bacterium to acquire iron during *in vivo* growth is also likely important to its pathogenesis, and several research groups have characterized several different genes whose products are involved in the binding and/or transport of host iron compounds (Mazmanian *et al.*, (2003) Science 299:906-9; Modun *et al.*, (1998) Infect. Immun. 66:3591-3596; Taylor and Heinrichs (2002) Mol. Microbiol. 43:1603-1614).

Initially, penicillin could be used to treat even the worst *S. aureus* infections. However, the emergence of penicillin-resistant strains of *S. aureus* has reduced the effectiveness of penicillin in treating *S. aureus* infections and most strains of *S. aureus* encountered in hospital infections today do not respond to penicillin. Penicillin-resistant strains of *S. aureus* produce a beta-lactamase, which converts penicillin to penicillinoic acid, and thereby destroys antibiotic activity. Furthermore, the beta-lactamase encoding gene often is propagated episomally, typically on a plasmid, and often is only one of several genes on an episomal element that, together, confer multidrug resistance.

Methicillins, introduced in the 1960s, largely overcame the problem of penicillin resistance in *S. aureus*. These compounds conserve the portions of penicillin responsible for antibiotic activity and modify or alter other portions that make penicillin a good substrate for inactivating lactamases. However, methicillin resistance has emerged in *S. aureus*, along with resistance to many other antibiotics effective against this organism, including aminoglycosides, tetracycline, chloramphenicol, macrolides and lincosamides. In fact, methicillin-resistant strains of *S. aureus* generally are multiply drug resistant. Methicillin-resistant *S. aureus* (MRSA) has become one of the most important nosocomial

pathogens worldwide and poses serious infection control problems. Today, many strains are multiresistant against virtually all antibiotics with the exception of vancomycin-type glycopeptide antibiotics. Drug resistance of *S. aureus* infections poses significant treatment difficulties, which are likely to get much worse unless new therapeutic agents are developed. Thus, there is an urgent unmet medical need for new and effective therapeutic agents to treat *S. aureus* infections.

Summary of the Invention

The present invention is based, at least in part, on the identification and characterization of Isd (iron-regulated surface determinant) proteins, IsdA, IsdB, and IsdC, which are part of the Isd system involved in the internalization of iron from heme and hemoproteins in *S. aureus*. IsdA, IsdB, and IsdC are expressed on the cell surface of *S. aureus* and are important for iron-restricted growth and survival *in vivo*. As a result, IsdA, IsdB, and IsdC proteins are attractive vaccine targets whose inhibition may lead to compromised bacterial growth *in vivo*. Further, IsdA, IsdB, and IsdC proteins are attractive drug targets that can be used in screening assays to identify *S. aureus* specific antibiotics.

In one aspect, the invention features Isd protein-based vaccines. In an exemplary embodiment, an Isd based vaccine comprises an IsdA polypeptide and a pharmaceutically acceptable carrier. In one embodiment, the IsdA polypeptide comprises the full-length amino acid sequence of SEQ ID NO: 3. In another embodiment, the IsdA polypeptide is a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 3. In another embodiment, the Isd vaccine comprises an IsdB polypeptide and a pharmaceutically acceptable carrier. In certain embodiments, the IsdB polypeptide comprises the full-length amino acid sequence of SEQ ID NO: 6. In another embodiment, the IsdB polypeptide is a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 6. In another embodiment, the Isd vaccine comprises an IsdC polypeptide and a pharmaceutically acceptable carrier. In certain embodiments, the IsdC polypeptide comprises the full-length amino acid sequence of SEQ ID NO: 9. In another embodiment, the IsdC polypeptide is a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 9. The vaccine composition may be formulated into an injectable formulation and may further comprise an adjuvant.

In another aspect, the invention features novel antibiotics including antibodies, antisense nucleic acids, and siRNAs that inhibit iron uptake in *Staphylococcus aureus* (*S.*

aureus). The invention features antibodies against IsdA, IsdB and/or IsdC. In certain embodiments, antibodies against an IsdA polypeptide may be generated against the full-length recombinant amino acid sequence of SEQ ID NO: 3 or against a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 3. Antibodies against an IsdB polypeptide be generated against the full-length recombinant amino acid sequence of SEQ ID NO: 6 or against a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 6. Antibodies against an IsdC polypeptide be generated against the full-length recombinant amino acid sequence of SEQ ID NO: 9 or against a peptide of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids of SEQ ID NO: 9. Antibodies against an Isd polypeptide may be monoclonal or polyclonal. Antibodies against an Isd polypeptide may be formulated into an injectable formulation and may be administered as anti-bacterial treatments.

In a further aspect, the invention features screening assays for identifying agents that inhibit or otherwise interfere with the expression level and/or function of any of the Isd proteins. In an exemplary embodiment, the invention features screening assays for agents that inhibit the expression and/or function of IsdA. In one embodiment, the assay is a binding assay and an agent that binds to an *isd* gene product and thereby interferes with its biochemical function is a candidate *S. aureus* specific antibiotic. In another embodiment, the assay is an expression assay and an agent that reduces the expression level of an Isd polypeptide is a candidate *S. aureus* specific antibiotic.

In a further aspect, Isd proteins may be expressed on Gram-positive bacteria, including but not limited to, *S. aureus*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, and *Bacillus anthracis*. Thus, vaccines and inhibitors that target Isd proteins, as described herein, may be used to treat numerous virulent Gram-positive bacterial strains that cause disease in mammals.

Further features and advantages of the instant disclosed inventions will now be discussed in conjunction with the following Detailed Description and Claims.

Brief Description of the Drawings

Figure 1 shows (A) the nucleic acid sequence encoding *IsdA* (SEQ ID NO: 1), (B) the reverse complement of SEQ ID NO: 1 (SEQ ID NO: 2), and (C) the corresponding amino acid sequence for IsdA (SEQ ID NO: 3).

Figure 2 shows (A) the nucleic acid sequence encoding *IsdB* (SEQ ID NO: 4), (B) the reverse complement of SEQ ID NO: 4 (SEQ ID NO: 5), and (C) the corresponding amino acid sequence for *IsdB* (SEQ ID NO: 6).

Figure 3 shows (A) the nucleic acid sequence encoding *IsdC* (SEQ ID NO: 7), (B) the reverse complement of SEQ ID NO: 7 (SEQ ID NO: 8), and (C) the corresponding amino acid sequence for *IsdC* (SEQ ID NO: 9).

Figure 4 is an SDS-PAGE gel that shows whole cell lysates from *S. aureus* grown in iron-rich media and iron-depleted media.

Figure 5 is a graph showing *S. aureus* counts recovered from kidneys of mice 6 days following injection 10^7 bacteria into the tail vein.

Figure 6 is a table showing that wild-type *S. aureus* *Isd* proteins bound to heme can survive under conditions of increased hydrogen peroxide (H_2O_2) compared to *S. aureus* strains where *isdA*, *isdB*, *isdC* were knocked-out (✓, indicates greater than 90% survival of bacteria).

Figures 7A and 7B are SDS-PAGE gels showing proteins from wild type and *isdA* knockout *S. aureus* (A) stained with coomassie (for total protein) and (b) stained with TMBZ (tetramethylbenzidine).

Detailed Description of the Invention

1. General

As described herein, the internalization of iron through the uptake of heme is a virulence property that may be attenuated when *isd* genes, such as *isdA*, *isdB*, and *isdC* are knocked out. Further, as described herein, heme-bound *Isd* proteins may serve an additional role in promoting *S. aureus* survival in the host. Heme-bound *Isd* proteins appear to serve as an oxidative buffer that protects cells from the detrimental effects of free radicals. Therefore, mutants lacking expression of *Isd* proteins are more susceptible to challenges with hydrogen peroxide whereas wild type *S. aureus* can survive in higher concentrations of hydrogen peroxide.

The *Isd* proteins, as described herein, are essential for *S. aureus* infection *in vivo* and are highly expressed during *S. aureus* infection. As *S. aureus* enters a host, it encounters an environment that is iron-limited and *Isd* protein expression is subsequently

up regulated. In the iron-limited host, Isd expression likely remains up regulated as the *S. aureus* scavenge for iron. IsdA, in particular, as described herein is immunodominant, since a 1:4000 dilution of serum from convalescent patients (*i.e.*, patients suffering from *S. aureus* infections) reacted positively in Western immunoblots with 4 micrograms of purified IsdA protein. Thus, Isd proteins are attractive targets for vaccine development. Antigenic peptides of Isd proteins may be used as vaccine targets to generate an effective immune response against *S. aureus*. Further, inhibiting the function of Isd proteins using an Isd specific antibody, antisense RNA, siRNA or small molecule inhibitor may be an effective way of attenuating the virulence of *S. aureus*.

The Isd proteins described herein are expressed on *S. aureus*. In further embodiments, Isd proteins may be expressed on other Gram-positive bacteria. Non-limiting examples of Gram-positive pathogens expressing Isd proteins include *S. aureus*, *Corynebacterium diphtheriae*, *Listeria monocytogenes*, and *Bacillus anthracis*. Thus, vaccines and inhibitors that target Isd proteins, as described herein, may be used to treat other virulent Gram-positive bacterial strains that cause disease in mammals.

2. Definitions

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

As used herein, the term “adjuvant” refers to a substance that elicits an enhanced immune response when used in combination with a specific antigen.

The term “agent” is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule (such as a nucleic acid, an antibody, a protein or portion thereof, *e.g.*, a peptide), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Screening assays described herein below may identify agents. Such agents may be inhibitors or antagonists of Isd mediated iron uptake in *Staphylococcus aureus*. The activity of such agents may render it suitable as a “therapeutic agent” which is a

biologically, physiologically, or pharmacologically active substance (or substances) that acts locally or systemically in a subject.

The terms “antagonist” or “inhibitor” refer to an agent that down regulates (*e.g.*, suppresses or inhibits) at least one bioactivity of a protein. An antagonist may be a compound which inhibits or decreases the interaction between a protein and another molecule, *e.g.*, a target peptide or enzyme substrate. An antagonist may also be a compound that down regulates expression of a gene or which reduces the amount of expressed protein present.

As used herein the term “antibody” refers to an immunoglobulin and any antigen-binding portion of an immunoglobulin (*e.g.*, IgG, IgD, IgA, IgM and IgE) *i.e.*, a polypeptide that contains an antigen-binding site, which specifically binds (“immunoreacts with”) an antigen. Antibodies can comprise at least one heavy (H) chain and at least one light (L) chain interconnected by at least one disulfide bond. The term “V_H” refers to a heavy chain variable region of an antibody. The term “V_L” refers to a light chain variable region of an antibody. In exemplary embodiments, the term “antibody” specifically covers monoclonal and polyclonal antibodies. A “polyclonal antibody” refers to an antibody, which has been derived from the sera of animals immunized with an antigen or antigens. A “monoclonal antibody” refers to an antibody produced by a single clone of hybridoma cells. Techniques for generating monoclonal antibodies include, but are not limited to, the hybridoma technique (see Kohler & Milstein (1975) *Nature* 256:495-497); the trioma technique; the human B-cell hybridoma technique (see Kozbor, *et al.* (1983) *Immunol. Today* 4:72), the EBV hybridoma technique (see Cole, *et al.*, 1985 In: *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) and phage display.

Polyclonal or monoclonal antibodies can be further manipulated or modified to generate chimeric or humanized antibodies. “Chimeric antibodies” are encoded by immunoglobulin genes that have been genetically engineered so that the light and heavy chain genes are composed of immunoglobulin gene segments belonging to different species. For example, substantial portions of the variable (V) segments of the genes from a mouse monoclonal antibody, *e.g.*, obtained as described herein, may be joined to substantial portions of human constant (C) segments. Such a chimeric antibody is likely to be less antigenic to a human than a mouse monoclonal antibody.

As used herein, the term “humanized antibody” (HuAb) refers to a chimeric antibody with a framework region substantially identical (*i.e.*, at least 85%) to a human

framework, having CDRs from a non-human antibody, and in which any constant region has at least about 85-90%, and preferably about 95% polypeptide sequence identity to a human immunoglobulin constant region. See, for example, PCT Publication WO 90/07861 and European Patent No. 0451216. All parts of such a HuAb, except possibly the CDRs,
5 are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. The term "framework region" as used herein, refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (*i.e.*, other than the CDRs) among different immunoglobulins in a single species, as defined by Kabat, *et al.* (1987) *Sequences of Proteins of Immunologic Interest*, 4th Ed.,
10 US Dept. Health and Human Services. Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably from immortalized B cells. The variable regions or CDRs for producing humanized antibodies may be derived from monoclonal antibodies capable of binding to the antigen, and will be produced in any convenient mammalian source, including mice, rats,
15 rabbits, or other vertebrates.

The term "antibody" also encompasses antibody fragments. Examples of antibody fragments include Fab, Fab', Fab'-SH, F(ab')₂, and Fv fragments; diabodies and any antibody fragment that has a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues, including without limitation: single-chain Fv (scFv)
20 molecules, single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific or
25 multivalent structures formed from antibody fragments. In an antibody fragment comprising one or more heavy chains, the heavy chain(s) can contain any constant domain sequence (*e.g.*, CH1 in the IgG isotype) found in a non-Fc region of an intact antibody, and/or can contain any hinge region sequence found in an intact antibody, and/or can contain a leucine zipper sequence fused to or situated in the hinge region sequence or the
30 constant domain sequence of the heavy chain(s). Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *et al.*, (1992) *J. Immunol.*, 148: 1547-1553 and the GCN4 leucine zipper described in U.S. Patent No. 6,468,532. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody and are typically produced by proteolytic

cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments).

An antibody “specifically binds” to an antigen or an epitope of an antigen if the antibody binds preferably to the antigen over most other antigens. For example, the
5 antibody may have less than about 50%, 20%, 10%, 5%, 1% or 0.1% cross-reactivity toward one or more other epitopes.

The term “conservative substitutions” refers to changes between amino acids of broadly similar molecular properties. For example, interchanges within the aliphatic group alanine, valine, leucine and isoleucine can be considered as conservative. Sometimes
10 substitution of glycine for one of these can also be considered conservative. Other conservative interchanges include those within the aliphatic group aspartate and glutamate; within the amide group asparagine and glutamine; within the hydroxyl group serine and threonine; within the aromatic group phenylalanine, tyrosine and tryptophan; within the basic group lysine, arginine and histidine; and within the sulfur-containing group methionine and
15 cysteine. Sometimes substitution within the group methionine and leucine can also be considered conservative. Preferred conservative substitution groups are aspartate-glutamate; asparagine-glutamine; valine-leucine-isoleucine; alanine-valine; valine-leucine-isoleucine-methionine; phenylalanine-tyrosine; phenylalanine-tyrosine-tryptophan; lysine-arginine; and histidine-lysine-arginine.

20 An “effective amount” is an amount sufficient to produce a beneficial or desired clinical result upon treatment. An effective amount can be administered to a patient in one or more doses. In terms of treatment, an effective amount is an amount that is sufficient to decrease an infection in a patient. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These factors include
25 age, sex and weight of the patient, the condition being treated, the severity of the condition and the form and effective concentration of the agent administered.

The term “epitope” refers to that region of an antigen to which an antibody binds preferentially and specifically. A monoclonal antibody binds preferentially to a single specific epitope of a molecule that can be molecularly defined. An epitope of a particular
30 protein may be constituted by a limited number of amino acid residues, e.g. 5 – 15 residues that are either in a linear or non-linear organization on the protein.

“Equivalent” when used to describe nucleic acids or nucleotide sequences refers to nucleotide sequences encoding functionally equivalent polypeptides. Equivalent nucleotide

sequences will include sequences that differ by one or more nucleotide substitution, addition or deletion, such as an allelic variant; and will, therefore, include sequences that differ due to the degeneracy of the genetic code. For example, nucleic acid variants may include those produced by nucleotide substitutions, deletions, or additions. The
5 substitutions, deletions, or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

“Homology” or alternatively “identity” refers to sequence similarity between two
10 peptides or between two nucleic acid molecules. Homology may be determined by comparing a position in each sequence, which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the
15 sequences. The term “percent identical” refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity may be determined by comparing a position in each sequence, which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site is
20 occupied by the same or a similar amino acid residue (*e.g.*, similar in steric and/or electronic nature), then the molecules may be referred to as homologous (similar) at that position. Expression as a percentage of homology, similarity, or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used,
25 including FASTA, BLAST, or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and may be used with, *e.g.*, default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences may be
30 determined by the GCG program with a gap weight of 1, *e.g.*, each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences. Other techniques for alignment are described in *Methods in Enzymology*, vol. 266: Computer Methods for Macromolecular Sequence Analysis (1996), ed. Doolittle,

Academic Press, Inc., a division of Harcourt Brace & Co., San Diego, California, USA. Preferably, an alignment program that permits gaps in the sequence is utilized to align the sequences. The Smith-Waterman is one type of algorithm that permits gaps in sequence alignments. See *Meth. Mol. Biol.* 70: 173-187 (1997). Also, the GAP program using the
 5 Needleman and Wunsch alignment method may be utilized to align sequences. An alternative search strategy uses MPSRCH software, which runs on a MASPAC computer. MPSRCH uses a Smith-Waterman algorithm to score sequences on a massively parallel computer. This approach improves the ability to pick up distantly related matches, and is especially tolerant of small gaps and nucleotide sequence errors. Nucleic acid-encoded
 10 amino acid sequences may be used to search both protein and DNA databases. Databases with individual sequences are described in *Methods in Enzymology*, ed. Doolittle, supra. Databases include Genbank, EMBL, and DNA Database of Japan (DDBJ).

As used herein, the term "infection" refers to an invasion and the multiplication of microorganisms such as *S. aureus* in body tissues, which may be clinically unapparent or
 15 result in local cellular injury due to competitive metabolism, toxins, intracellular replication or antigen antibody response. The infection may remain localized, subclinical and temporary if the body's defensive mechanisms are effective. A local infection may persist and spread by extension to become an acute, subacute or chronic clinical infection or disease state. A local infection may also become systemic when the microorganisms gain
 20 access to the lymphatic or vascular system.

The terms "iron-regulated surface determinant system" or "Isd system" as used herein, refers to the *S. aureus* Isd locus, which comprises numerous genes encoded by five different transcriptional units that, together, encode a putative heme uptake system. The five transcriptional units are *isdA*, *isdB*, *isdCDEFsrtBisdG*, *isdH*, and *isdI*. Transcription of
 25 *isd* genes is regulated by environment iron on the control of the Fur promoter. Four of the proteins encoded by the Isd locus, IsdA, IsdB, IsdC, and IsdH, are covalently anchored to the cell wall by an amide linkage between the C-terminal end of the polypeptide chain and peptidoglycan. IsdA, IsdB, and IsdH are characterized as having a C-terminal sorting signal referred to as an LPXTG motif (*i.e.*, a motif recognized by sortase A). IsdC is
 30 characterized as having a C-terminal sorting signal referred to as an NPQTN motif (*i.e.*, a motif recognized by sortase B in *S. aureus*). IsdA, IsdB, and IsdH are anchored to the cell wall by a sortase A (*srtA*), a membrane anchored transpeptidase that cleaves cell surface proteins at the LPXTG motif and catalyzes the formation of the amide bond between the

polypeptide and peptidoglycan. IsdC is anchored to the cell wall by sortase B (srtB), a transpeptidase similar to sortase A. Other proteins encoded by the Isd locus, include IsdD, IsdE, and IsdF, which are putative membrane translocation factors, and IsdG and IsdI, which are cytoplasmic heme-iron binding proteins, that may be involved in extracting iron
5 from heme.

“IsdA polypeptide” as used herein refers to iron-regulated surface determinant A. The sequence of IsdA polypeptide is as set forth in SEQ ID NO: 3 and is encoded by SEQ ID NO: 1. The term also encompasses any fragments, variants, analogs, agonists, chemical derivatives, functional derivatives or functional fragments of an IsdA polypeptide. “IsdA
10 immunogens” are IsdA polypeptides, which are capable of eliciting an immune response in a subject.

“IsdB polypeptide” as used herein refers to iron-regulated surface determinant B. The sequence of IsdB polypeptide is as set forth in SEQ ID NO: 6 and is encoded by SEQ ID NO: 4. The term also encompasses any fragments, variants, analogs, agonists, chemical
15 derivatives, functional derivatives or functional fragments of an IsdB polypeptide. “IsdB immunogens” are IsdB polypeptides, which are capable of eliciting an immune response in a subject.

“IsdC polypeptide” as used herein refers to iron-regulated surface determinant C. The sequence of IsdC polypeptide is as set forth in SEQ ID NO: 9 and is encoded by SEQ
20 ID NO: 7. The term also encompasses any fragments, variants, analogs, agonists, chemical derivatives, functional derivatives or functional fragments of an IsdC polypeptide. “IsdC immunogens” are IsdC polypeptides, which are capable of eliciting an immune response in a subject.

“Label” and “detectable label” refer to a molecule capable of detection including,
25 but not limited to radioactive isotopes, fluorophores, chemiluminescent moieties, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, ligands (e.g., biotin or haptens) and the like. “Fluorophore” refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of appropriate labels include fluorescein, rhodamine, dansyl, umbelliferone, Texas red,
30 luminol, NADPH, alpha- or beta-galactosidase and horseradish peroxidase.

As used herein with respect to genes, the term “mutant” refers to a gene, which encodes a mutant protein. As used herein with respect to proteins, the term “mutant” means a protein, which does not perform its usual or normal physiological role. *S. aureus*

polypeptide mutants may be produced by amino acid substitutions, deletions or additions. The substitutions, deletions, or additions may involve one or more residues. Especially preferred among these are substitutions, additions and deletions, which alter the properties and activities of a *S. aureus* protein.

5 The terms “polynucleotide”, and “nucleic acid” are used interchangeably to refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA,
10 ribosomal RNA, ribozymes, cDNA, antisense nucleic acids, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the
15 polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. The term “recombinant” polynucleotide means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin, which either does not occur in nature or is linked to another polynucleotide in a non-natural arrangement. An
20 “oligonucleotide” refers to a single stranded polynucleotide having less than about 100 nucleotides, less than about, *e.g.*, 75, 50, 25, or 10 nucleotides.

 The terms “polypeptide”, “peptide” and “protein” (if single chain) are used interchangeably herein to refer to polymers of amino acids. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino
25 acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term “amino acid” refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and
30 peptidomimetics.

 The term “small molecule” refers to a compound, which has a molecular weight of less than about 5 kD, less than about 2.5 kD, less than about 1.5 kD, or less than about 0.9 kD. Small molecules may be, for example, nucleic acids, peptides, polypeptides, peptide

nucleic acids, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention. The term "small organic molecule" refers to a small molecule that is often identified as being an organic or medicinal compound, and does not include molecules that are exclusively nucleic acids, peptides or polypeptides.

The term "specifically hybridizes" refers to detectable and specific nucleic acid binding. Polynucleotides, oligonucleotides and nucleic acids of the invention selectively hybridize to nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. Stringent conditions may be used to achieve selective hybridization conditions as known in the art and discussed herein. Generally, the nucleic acid sequence homology between the polynucleotides, oligonucleotides, and nucleic acids of the invention and a nucleic acid sequence of interest will be at least 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99%, or more. In certain instances, hybridization and washing conditions are performed under stringent conditions according to conventional hybridization procedures and as described further herein.

The terms "stringent conditions" or "stringent hybridization conditions" refer to conditions, which promote specific hybridization between two complementary polynucleotide strands so as to form a duplex. Stringent conditions may be selected to be about 5°C lower than the thermal melting point (T_m) for a given polynucleotide duplex at a defined ionic strength and pH. The length of the complementary polynucleotide strands and their GC content will determine the T_m of the duplex, and thus the hybridization conditions necessary for obtaining a desired specificity of hybridization. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a polynucleotide sequence hybridizes to a perfectly matched complementary strand. In certain cases it may be desirable to increase the stringency of the hybridization conditions to be about equal to the T_m for a particular duplex.

A variety of techniques for estimating the T_m are available. Typically, G-C base pairs in a duplex are estimated to contribute about 3°C to the T_m , while A-T base pairs are estimated to contribute about 2°C, up to a theoretical maximum of about 80-100°C. However, more sophisticated models of T_m are available in which G-C stacking interactions, solvent effects, the desired assay temperature and the like are taken into

account. For example, probes can be designed to have a dissociation temperature (T_d) of approximately 60°C, using the formula: $T_d = (((((3 \times \#GC) + (2 \times \#AT)) \times 37) - 562) / \#bp) - 5$; where #GC, #AT, and #bp are the number of guanine-cytosine base pairs, the number of adenine-thymine base pairs, and the number of total base pairs, respectively, involved in the formation of the duplex.

Hybridization may be carried out in 5xSSC, 4xSSC, 3xSSC, 2xSSC, 1xSSC or 0.2xSSC for at least about 1 hour, 2 hours, 5 hours, 12 hours, or 24 hours. The temperature of the hybridization may be increased to adjust the stringency of the reaction, for example, from about 25°C (room temperature), to about 45°C, 50°C, 55°C, 60°C, or 65°C. The hybridization reaction may also include another agent affecting the stringency, for example, hybridization conducted in the presence of 50% formamide increases the stringency of hybridization at a defined temperature.

The hybridization reaction may be followed by a single wash step, or two or more wash steps, which may be at the same or a different salinity and temperature. For example, the temperature of the wash may be increased to adjust the stringency from about 25°C (room temperature), to about 45°C, 50°C, 55°C, 60°C, 65°C, or higher. The wash step may be conducted in the presence of a detergent, e.g., 0.1 or 0.2% SDS. For example, hybridization may be followed by two wash steps at 65°C each for about 20 minutes in 2xSSC, 0.1% SDS, and optionally two additional wash steps at 65°C each for about 20 minutes in 0.2xSSC, 0.1% SDS.

Exemplary stringent hybridization conditions include overnight hybridization at 65°C in a solution comprising, or consisting of, 50% formamide, 10xDenhardt (0.2% Ficoll, 0.2% Polyvinylpyrrolidone, 0.2% bovine serum albumin) and 200 µg/ml of denatured carrier DNA, e.g., sheared salmon sperm DNA, followed by two wash steps at 65°C each for about 20 minutes in 2xSSC, 0.1% SDS, and two wash steps at 65°C each for about 20 minutes in 0.2xSSC, 0.1% SDS.

Hybridization may consist of hybridizing two nucleic acids in solution, or a nucleic acid in solution to a nucleic acid attached to a solid support, e.g., a filter. When one nucleic acid is on a solid support, a prehybridization step may be conducted prior to hybridization. Prehybridization may be carried out for at least about 1 hour, 3 hours or 10 hours in the same solution and at the same temperature as the hybridization solution (without the complementary polynucleotide strand).

Appropriate stringency conditions are known to those skilled in the art or may be determined experimentally by the skilled artisan. See, for example, Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-12.3.6; Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y; S. Agrawal (ed.) Methods in Molecular Biology, volume 20; Tijssen (1993) Laboratory Techniques in biochemistry and molecular biology-hybridization with nucleic acid probes, e.g., part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York; and Tibanyenda, N. *et al.*, Eur. J. Biochem. 139:19 (1984) and Ebel, S. *et al.*, Biochem. 31:12083 (1992).

10 The term "substantially homologous" when used in connection with a nucleic acid or amino acid sequences, refers to sequences which are substantially identical to or similar in sequence with each other, giving rise to a homology of conformation and thus to retention, to a useful degree, of one or more biological (including immunological) activities. The term is not intended to imply a common evolution of the sequences.

15 A "subject" refers to a male or female mammal, including humans.

A "variant" of an Isd polypeptide refers to a molecule, which is substantially similar to IsdA, IsdB, or IsdC. Variant peptides may be covalently prepared by direct chemical synthesis of the variant peptide, using methods well known in the art. Variants of Isd polypeptides may further include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. These variants may be prepared by site-directed mutagenesis, (as exemplified by Adelman *et al.*, *DNA* 2: 183 (1983)) of the nucleotides in the DNA encoding the peptide molecule thereby producing DNA encoding the variant and thereafter expressing the DNA in recombinant cell culture. The variants typically exhibit the same qualitative biological activity as wild type Isd polypeptides. It is known in the art that one may also synthesize all possible single amino acid substitutions of a known polypeptide (Geysen *et al.*, *Proc. Nat. Acad. Sci. (USA)* 18:3998-4002 (1984)). While the effects of different substitutions are not always additive, it is reasonable to expect that two favorable or neutral single substitutions at different residue positions in an Isd polypeptide can safely be combined without losing any Isd protein activity. Methods for the preparation of degenerate polypeptides are as described in Rutter, U.S. Pat. No. 5,010,175; Haughter *et al.*, *Proc. Nat. Acad. Sci. (USA)* 82:5131-5135 (1985); Geysen *et al.*, *Proc. Nat. Acad. Sci.*

(USA) 18:3998-4002 (1984); WO86/06487; and WO86/00991. In devising a substitution strategy, a person of ordinary skill would determine which residues to vary and which amino acids or classes of amino acids are suitable replacements. One may also take into account studies of sequence variations in families or naturally occurring homologous proteins. Certain amino acid substitutions are more often tolerated than others, and these are often correlated with similarities in size, charge, etc., between the original amino acid and its replacement. Insertions or deletions of amino acids may also be made, as described above. The substitutions are preferably conservative, see, e.g., Schulz et al., *Principle of Protein Structure* (Springer-Verlag, New York (1978)); and Creighton, *Proteins: Structure and Molecular Properties* (W. H. Freeman & Co., San Francisco (1983)); both of which are hereby incorporated by reference in their entireties.

A "chemical derivative" of an Isd polypeptide can contain additional chemical moieties not normally part of the IsdA, IsdB, or IsdC amino acid sequences. Such chemical modifications may be introduced into an Isd polypeptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Amino terminal residues can be reacted with succinic or other carboxylic acid anhydrides. Other suitable reagents for derivatizing alpha-amino-containing residues include amido-esters such as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalase reacted with glyoxylate. Specific modifications of tyrosyl residues per se have been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Carboxyl side groups such as aspartyl or glutamyl can be selectively modified by reaction with carbodiimides (R'N--C--N--R') such as 1-cyclohexy-3-[2-morpholinyl-(4-ethyl)] carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

A "vector" is a self-replicating nucleic acid molecule that transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that function primarily for insertion of a nucleic acid molecule into a cell, replication of vectors that function primarily for the replication of nucleic acid, and expression vectors that

function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the above functions. As used herein, "expression vectors" are defined as polynucleotides which, when introduced into an appropriate host cell, can be transcribed and translated into a polypeptide(s). An "expression system" usually connotes a
5 suitable host cell comprised of an expression vector that can function to yield a desired expression product.

3. *Isd* Genes

Three genes of the *Isd* locus, *isdA*, *isdB*, and *isdC*, encode cell surface proteins,
10 which are covalently anchored to the *S. aureus* cell wall. Figures 1-3 provide the nucleic acid sequences of *isdA* (SEQ ID NO: 1), *isdB* (SEQ ID NO: 4), and *isdC* (SEQ ID NO: 7).

Nucleic acids of the present invention may also comprise, consist of or consist essentially of any of the *isd* nucleotide sequences described herein. Yet other nucleic acids comprise, consist of or consist essentially of a nucleotide sequence that has at least about
15 70%, 80%, 90%, 95%, 98% or 99% identity or homology with an *isd* gene. Substantially homologous sequences may be identified using stringent hybridization conditions.

Isolated nucleic acids which differ from the nucleic acids of the invention due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the
20 same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the polypeptides of the invention will exist. One skilled in the art will appreciate that these variations in one or more nucleotides (from less than 1% up to
25 about 3 or 5% or possibly more of the nucleotides) of the nucleic acids encoding a particular protein of the invention may exist among a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

Nucleic acids encoding proteins which have amino acid sequences evolutionarily
30 related to a polypeptide disclosed herein are provided, wherein "evolutionarily related to", refers to proteins having different amino acid sequences which have arisen naturally (e.g., by allelic variance or by differential splicing), as well as mutational variants of the proteins of the invention which are derived, for example, by combinatorial mutagenesis.

Fragments of the polynucleotides of the invention encoding a biologically active portion of the subject polypeptides are also provided. As used herein, a fragment of a nucleic acid encoding an active portion of a polypeptide disclosed herein refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length amino acid sequence of a polypeptide of the invention, and which encodes a given polypeptide that retains at least a portion of a biological activity of the full-length Isd protein as defined herein, or alternatively, which is functional as a modulator of the biological activity of the full-length protein. For example, such fragments include a polypeptide containing a domain of the full-length protein from which the polypeptide is derived that mediates the interaction of the protein with another molecule (e.g., polypeptide, DNA, RNA, etc.).

Nucleic acids provided herein may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of such recombinant polypeptides.

A nucleic acid encoding an Isd polypeptide provided herein may be obtained from mRNA or genomic DNA from any organism in accordance with protocols described herein, as well as those generally known to those skilled in the art. A cDNA encoding a polypeptide of the invention, for example, may be obtained by isolating total mRNA from an organism, for example, a bacteria, virus, mammal, etc. Double stranded cDNAs may then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. A gene encoding a polypeptide of the invention may also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. In one aspect, methods for amplification of a nucleic acid of the invention, or a fragment thereof may comprise: (a) providing a pair of single stranded oligonucleotides, each of which is at least eight nucleotides in length, complementary to sequences of a nucleic acid of the invention, and wherein the sequences to which the oligonucleotides are complementary are at least ten nucleotides apart; and (b) contacting the oligonucleotides with a sample comprising a nucleic acid comprising the nucleic acid of the invention under conditions which permit amplification of the region located between the pair of oligonucleotides, thereby amplifying the nucleic acid.

Isd proteins may be expressed from recombinant vectors, host cells containing the recombinant vectors and methods of producing the encoded *S. aureus* polypeptides.

Appropriate vectors may be introduced into host cells using well-known techniques such as infection, transduction, transfection, transvection, electroporation and transformation. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation
5 generally will occur only in complementing host cells.

The vector may contain a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

10 Preferred vectors comprise cis-acting control regions to the polynucleotide of interest. Appropriate trans-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain embodiments, the vectors provide for specific expression, which may be inducible and/or cell type-specific. Particularly preferred among such vectors are those
15 inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova
20 viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late
25 promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs may further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the mature transcripts expressed by the constructs may preferably include a translation-initiating site at the beginning and a termination codon
30 (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for

eukaryotic cell culture and tetracycline, kanamycin, or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE9, pQE10 available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A available from Stratagene; pET series of vectors available from Novagen; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Among known bacterial promoters suitable for use in the present invention include the *E. coli* lacI and lacZ promoters, the T3, T5 and T7 promoters, the gpt promoter, the lambda PR and PL promoters, the trp promoter and the xyl/tet chimeric promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals (for example, Davis, *et al.*, *Basic Methods in Molecular Biology* (1986)).

Transcription of DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 nucleotides that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at nucleotides 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated polypeptide into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide, for example, the amino acid sequence KDEL. The signals may be endogenous to the polypeptide or they
5 may be heterologous signals.

Coding sequences for a polypeptide of interest may be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. The present invention contemplates an isolated nucleic acid comprising a nucleic acid of the invention and at least one heterologous sequence encoding a heterologous peptide linked in frame to
10 the nucleotide sequence of the nucleic acid of the invention so as to encode a fusion protein comprising the heterologous polypeptide. The heterologous polypeptide may be fused to (a) the C-terminus of the polypeptide encoded by the nucleic acid of the invention, (b) the N-terminus of the polypeptide, or (c) the C-terminus and the N-terminus of the polypeptide. In certain instances, the heterologous sequence encodes a polypeptide permitting the
15 detection, isolation, solubilization and/or stabilization of the polypeptide to which it is fused. In still other embodiments, the heterologous sequence encodes a polypeptide selected from the group consisting of a poly-His tag, myc, HA, GST, protein A, protein G, calmodulin-binding peptide, thioredoxin, maltose-binding protein, poly arginine, poly-His-Asp, FLAG, a portion of an immunoglobulin protein, and a transcytosis peptide.

20 Fusion expression systems can be useful when it is desirable to produce an immunogenic fragment of a polypeptide of the invention. For example, the VP6 capsid protein of rotavirus may be used as an immunologic carrier protein for portions of polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of a polypeptide of the invention to which
25 antibodies are to be raised may be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of the protein as part of the virion. The Hepatitis B surface antigen may also be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of a polypeptide of the
30 invention and the poliovirus capsid protein may be created to enhance immunogenicity (see, for example, EP Publication NO: 0259149; and Evans *et al.*, (1989) *Nature* 339:385; Huang *et al.*, (1988) *J. Virol.* 62:3855; and Schlienger *et al.*, (1992) *J. Virol.* 66:2).

Fusion proteins may facilitate the expression and/or purification of proteins. For example, a polypeptide of the invention may be generated as a glutathione-S-transferase (GST) fusion protein. Such GST fusion proteins may be used to simplify purification of a polypeptide of the invention, such as through the use of glutathione-derivatized matrices
5 (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, may allow purification of the expressed fusion protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader
10 sequence may then be subsequently removed by treatment with enterokinase to provide the purified protein (e.g., see Hochuli *et al.*, (1987) *J. Chromatography* 411: 177; and Janknecht *et al.*, *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in
15 accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene may be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR
20 amplification of gene fragments may be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which may subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons: 1992).

In other embodiments, nucleic acids of the invention may be immobilized onto a
25 solid surface, including, plates, microtiter plates, slides, beads, particles, spheres, films, strands, precipitates, gels, sheets, tubing, containers, capillaries, pads, slices, etc. The nucleic acids of the invention may be immobilized onto a chip as part of an array. The array may comprise one or more polynucleotides of the invention as described herein. In one embodiment, the chip comprises one or more polynucleotides of the invention as part
30 of an array of polynucleotide sequences.

Another aspect relates to the use of nucleic acids of the invention in "antisense therapy". As used herein, antisense therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize or otherwise bind

under cellular conditions with the cellular mRNA and/or genomic DNA encoding one of the polypeptides of the invention so as to inhibit expression of that polypeptide, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, antisense therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent transport agent, hybridization-triggered cleavage agent, etc. An antisense molecule can be a "peptide nucleic acid" (PNA). PNA refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least about 5 nucleotides in length linked to a peptide backbone of amino acid residues ending in lysine. The terminal lysine confers solubility to the composition." PNAs preferentially bind complementary single stranded DNA or RNA and stop transcript elongation, and may be pegylated to extend their lifespan in the cell.

An antisense construct of the present invention may be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the mRNA which encodes a polypeptide of the invention. Alternatively, the antisense construct may be an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences encoding a polypeptide of the invention. Such oligonucleotide probes may be modified oligonucleotides which are resistant to endogenous nucleases, e.g., exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by van der Krol *et al.*, (1988) *Biotechniques* 6:958-976; and Stein *et al.*, (1988) *Cancer Res* 48:2659-2668.

In a further aspect, double stranded small interfering RNAs (siRNAs), and methods for administering the same are provided. siRNAs decrease or block gene expression. While not wishing to be bound by theory, it is generally thought that siRNAs inhibit gene expression by mediating sequence specific mRNA degradation. RNA interference (RNAi)

is the process of sequence-specific, post-transcriptional gene silencing, particularly in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (Elbashir *et al.* Nature 2001; 411(6836): 494-8). Accordingly, it is understood that siRNAs and long dsRNAs having substantial sequence identity to all or a portion of a polynucleotide of the present invention may be used to inhibit the expression of a nucleic acid of the invention.

Alternatively, siRNAs that decrease or block the expression the *Isd* polypeptides described herein may be determined by testing a plurality of siRNA constructs against the target gene. Such siRNAs against a target gene may be chemically synthesized. The nucleotide sequences of the individual RNA strands are selected such that the strand has a region of complementarity to the target gene to be inhibited (i.e., the complementary RNA strand comprises a nucleotide sequence that is complementary to a region of an mRNA transcript that is formed during expression of the target gene, or its processing products, or a region of a (+) strand virus). The step of synthesizing the RNA strand may involve solid-phase synthesis, wherein individual nucleotides are joined end to end through the formation of internucleotide 3'-5' phosphodiester bonds in consecutive synthesis cycles.

Provided herein are siRNA molecules comprising a nucleotide sequence consisting essentially of a sequence of an *isd* nucleic acid as described herein. An siRNA molecule may comprise two strands, each strand comprising a nucleotide sequence that is at least essentially complementary to each other, one of which corresponds essentially to a sequence of a target gene. The sequence that corresponds essentially to a sequence of a target gene is referred to as the "sense target sequence" and the sequence that is essentially complementary thereto is referred to as the "antisense target sequence" of the siRNA. The sense and antisense target sequences may be from about 15 to about 30 consecutive nucleotides long; from about 19 to about 25 consecutive nucleotides; from about 19 to 23 consecutive nucleotides or about 19, 20, 21, 22 or 23 nucleotides long. The length of the sense and antisense sequences is determined so that an siRNA having sense and antisense target sequences of that length is capable of inhibiting expression of a target gene, preferably without significantly inducing a host interferon response.

SiRNA target sequences may be predicted using any of the algorithms provided on the world wide web at the mmcmanus with the extension web.mit.edu/mmcmanus/www/home1.2files/siRNAs.

The sense target sequence may be essentially or substantially identical to the coding or a non-coding portion, or combination thereof, of a target nucleic acid. For example, the sense target sequence may be essentially complementary to the 5' or 3' untranslated region, promoter, intron or exon of a target nucleic acid or complement thereof. It can also be
5 essentially complementary to a region encompassing the border between two such gene regions.

The nucleotide base composition of the sense target sequence can be about 50% adenines (As) and thymidines (Ts) and 50% cytidines (Cs) and guanosines (Gs). Alternatively, the base composition can be at least 50% Cs/Gs, e.g., about 60%, 70% or
10 80% of Cs/Gs. Accordingly, the choice of sense target sequence may be based on nucleotide base composition. Regarding the accessibility of target nucleic acids by siRNAs, such can be determined, e.g., as described in Lee *et al.* (2002) *Nature Biotech.* 19:500. This approach involves the use of oligonucleotides that are complementary to the target nucleic acids as probes to determine substrate accessibility, e.g., in cell extracts.
15 After forming a duplex with the oligonucleotide probe, the substrate becomes susceptible to RNase H. Therefore, the degree of RNase H sensitivity to a given probe as determined, e.g., by PCR, reflects the accessibility of the chosen site, and may be of predictive value for how well a corresponding siRNA would perform in inhibiting transcription from this target gene. One may also use algorithms identifying primers for polymerase chain reaction
20 (PCR) assays or for identifying antisense oligonucleotides for identifying first target sequences.

The sense and antisense target sequences are preferably sufficiently complementary, such that an siRNA comprising both sequences is able to inhibit expression of the target gene, i.e., to mediate RNA interference. For example, the sequences may be sufficiently
25 complementary to permit hybridization under the desired conditions, e.g., in a cell. Accordingly, the sense and antisense target sequences may be at least about 95%, 97%, 98%, 99% or 100% identical and may, e.g., differ in at most 5, 4, 3, 2, 1 or 0 nucleotides.

Sense and antisense target sequences are also preferably sequences that are not likely to significantly interact with sequences other than the target nucleic acid or
30 complement thereof. This can be confirmed by, e.g., comparing the chosen sequence to the other sequences in the genome of the target cell. Sequence comparisons can be performed according to methods known in the art, e.g., using the BLAST algorithm, further described herein. Of course, small scale experiments can also be performed to confirm that a

particular first target sequence is capable of specifically inhibiting expression of a target nucleic acid and essentially not that of other genes.

siRNAs may also comprise sequences in addition to the sense and antisense sequences. For example, an siRNA may be an RNA duplex consisting of two strands of RNA, in which at least one strand has a 3' overhang. The other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, an siRNA comprises sense and antisense sequences, each of which are on one RNA strand, consisting of about 19-25 nucleotides which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly may also enhance the nuclease resistance of the overhang at least in tissue culture medium. RNA strands of siRNAs may have a 5' phosphate and a 3' hydroxyl group.

In one embodiment, an siRNA molecule comprises two strands of RNA forming a duplex. In another embodiment, an siRNA molecule consists of one RNA strand forming a hairpin loop, wherein the sense and antisense target sequences hybridize and the sequence between the two target sequences is a spacer sequence that essentially forms the loop of the hairpin structure. The spacer sequence may be any combination of nucleotides and any length provided that two complementary oligonucleotides linked by a spacer having this sequence can form a hairpin structure, wherein at least part of the spacer forms the loop at the closed end of the hairpin. For example, the spacer sequence can be from about 3 to about 30 nucleotides; from about 3 to about 20 nucleotides; from about 5 to about 15 nucleotides; from about 5 to about 10 nucleotides; or from about 3 to about 9 nucleotides. The sequence can be any sequence, provided that it does not interfere with the formation of a hairpin structure. In particular, the spacer sequence is preferably not a sequence having any significant homology to the first or the second target sequence, since this might interfere with the formation of a hairpin structure. The spacer sequence is also preferably

not similar to other sequences, e.g., genomic sequences of the cell into which the nucleic acid will be introduced, since this may result in undesirable effects in the cell.

A person of skill in the art will understand that when referring to a nucleic acid, e.g., an RNA, the RNA may comprise or consist of naturally occurring nucleotides or of nucleotide derivatives that provide, e.g., more stability to the nucleic acid. Any derivative is permitted provided that the nucleic acid is capable of functioning in the desired fashion. For example, an siRNA may comprise nucleotide derivatives provided that the siRNA is still capable of inhibiting expression of the target gene.

For example, siRNAs may include one or more modified base and/or a backbone modified for stability or for other reasons. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulphur heteroatom. Moreover, siRNA comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, can be used in the invention. It will be appreciated that a great variety of modifications have been made to RNA that serve many useful purposes known to those of skill in the art. The term siRNA as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of siRNA, provided that it is derived from an endogenous template.

There is no limitation on the manner in which an siRNA may be synthesised. Thus, it may be synthesized *in vitro* or *in vivo*, using manual and/or automated procedures. *In vitro* synthesis may be chemical or enzymatic, for example using cloned RNA polymerase (e.g., T3, T7, SP6) for transcription of a DNA (or cDNA) template, or a mixture of both. SiRNAs may also be prepared by synthesizing each of the two strands, e.g., chemically, and hybridizing the two strands to form a duplex. *In vivo*, the siRNA may be synthesized using recombinant techniques well known in the art (see e.g., Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition (1989); DNA Cloning, Volumes I and II (D. N Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed, 1984); Nucleic Acid Hybridisation (B. D. Hames & S. J. Higgins eds. 1984); Transcription and Translation (B. D. Hames & S. J. Higgins eds. 1984); Animal Cell Culture (R. I. Freshney ed. 1986); Immobilised Cells and Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide to Molecular Cloning (1984); the series, Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J. H. Miller and M. P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), Immunochemical

Methods in Cell and Molecular Biology (Academic Press, London), Scopes, (1987), Protein Purification: Principles and Practice, Second Edition (Springer-Verlag, N.Y.), and Handbook of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell eds 1986). For example, bacterial cells can be transformed with an expression vector which
5 comprises the DNA template from which the siRNA is to be derived.

If synthesized outside the cell, the siRNA may be purified prior to introduction into the cell. Purification may be by extraction with a solvent (such as phenol/chloroform) or resin, precipitation (for example in ethanol), electrophoresis, chromatography, or a combination thereof. However, purification may result in loss of siRNA and may therefore
10 be minimal or not carried out at all. The siRNA may be dried for storage or dissolved in an aqueous solution, which may contain buffers or salts to promote annealing, and/or stabilization of the RNA strands.

The double-stranded structure may be formed by a single self-complementary RNA strand or two separate complementary RNA strands.

15 It is known that mammalian cells can respond to extracellular siRNA and therefore may have a transport mechanism for dsRNA (Asher *et al.* (1969) *Nature* 223 715-717). Thus, siRNA may be administered extracellularly into a cavity, interstitial space, into the circulation of a mammal, or introduced orally. Methods for oral introduction include direct mixing of the RNA with food of the mammal, as well as engineered approaches in which a
20 species that is used as food is engineered to express the RNA, then fed to the mammal to be affected. For example, food bacteria, such as *Lactococcus lactis*, may be transformed to produce the dsRNA (see WO93/17117, WO97/14806). Vascular or extravascular circulation, the blood or lymph systems and the cerebrospinal fluid are sites where the RNA may be injected.

25 RNA may be introduced into the cell intracellularly. Physical methods of introducing nucleic acids may also be used in this respect. siRNA may be administered using the microinjection techniques described in Zernicka-Goetz *et al.* (1997) *Development* 124, 1133-1137 and Wianny *et al.* (1998) *Chromosoma* 107, 430-439.

Other physical methods of introducing nucleic acids intracellularly include
30 bombardment by particles covered by the siRNA, for example gene gun technology in which the siRNA is immobilized on gold particles and fired directly at the site of wounding. Thus, the invention provides the use of an siRNA in a gene gun for inhibiting the expression of a target gene. Further, there is provided a composition suitable for gene gun

therapy comprising an siRNA and gold particles. An alternative physical method includes electroporation of cell membranes in the presence of the siRNA. This method permits RNAi on a large scale. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. siRNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or otherwise increase inhibition of the target gene.

Any known gene therapy technique can be used to administer the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of siRNA encoded by the expression construct. Thus, siRNA can also be produced inside a cell. Vectors, e.g., expression vectors that comprise a nucleic acid encoding one or the two strands of an siRNA molecule may be used for that purpose. The nucleic acid may further comprise an antisense sequence that is essentially complementary to the sense target sequence. The nucleic acid may further comprise a spacer sequence between the sense and the antisense target sequence. The nucleic acid may further comprise a promoter for directing expression of the sense and antisense sequences in a cell, e.g., an RNA Polymerase II or III promoter and a transcriptional termination signal. The sequences may be operably linked.

In one embodiment a nucleic acid comprises an RNA coding region (e.g., sense or antisense target sequence) operably linked to an RNA polymerase III promoter. The RNA coding region can be immediately followed by a pol III terminator sequence, which directs termination of RNA synthesis by pol III. The pol III terminator sequences generally have 4 or more consecutive thymidine ("T") residues. In a preferred embodiment, a cluster of 5 consecutive T residues is used as the terminator by which pol III transcription is stopped at the second or third T of the DNA template, and thus only 2 to 3 uridine ("U") residues are added to the 3' end of the coding sequence. A variety of pol III promoters can be used with the invention, including for example, the promoter fragments derived from H1 RNA genes or U6 snRNA genes of human or mouse origin or from any other species. In addition, pol III promoters can be modified/engineered to incorporate other desirable properties such as the ability to be induced by small chemical molecules, either ubiquitously or in a tissue-specific manner. For example, in one embodiment the promoter may be activated by tetracycline. In another embodiment the promoter may be activated by IPTG (lacI system).

siRNAs can be produced in cells by transforming cells with two nucleic acids, e.g., vectors, each nucleic acid comprising an expressing cassette, each expression cassette comprising a promoter, an RNA coding sequence (one being a sense target sequence and the other being an antisense target sequence) and a termination signal. Alternatively, a single nucleic acid may comprise these two expression cassettes. In yet another embodiment, a nucleic acid encodes a single stranded RNA comprising a sense target sequence linked to a spacer linked to an antisense target sequence. The nucleic acids may be present in a vector, such as an expression vector, e.g., a eukaryotic expression vector that allows expression of the sense and antisense target sequences in cells into which it is introduced.

Vectors for producing siRNAs are described, e.g., in Paul *et al.* (2002) *Nature Biotechnology* 29:505; Xia *et al.* (2002) *Nature Biotechnology* 20:1006; Zeng *et al.* (2002) *Mol. Cell* 9:1327; Thijn *et al.* (2002) *Science* 296:550; BMC Biotechnol. 2002 Aug 28;2(1):15; Lee *et al.* (2002) *Nature Biotechnology* 19: 500; McManus *et al.* (2002) *RNA* 8:842; Miyagishi *et al.* (2002) *Nature Biotechnology* 19:497; Sui *et al.* (2002) *PNAS* 99:5515; Yu *et al.* (2002) *PNAS* 99:6047; Shi *et al.* (2003) *Trends Genet.* 19(1):9; Gaudilliere *et al.* (2002) *J. Biol. Chem.* 277(48):46442; US2002/0182223; US 2003/0027783; WO 01/36646 and WO 03/006477. Vectors are also available commercially. For example, the pSilencer is available from Gene Therapy Systems, Inc. and pSUPER RNAi system is available from Oligoengine.

Also provided herein are compositions comprising one or more siRNA or nucleic acid encoding an RNA coding region of an siRNA. Compositions may be pharmaceutical compositions and comprise a pharmaceutically acceptable carrier. Compositions may also be provided in a device for administering the composition in a cell or in a subject. For example a composition may be present in a syringe or on a stent. A composition may also comprise agents facilitating the entry of the siRNA or nucleic acid into a cell.

In general, the oligonucleotides may be synthesized using protocols known in the art, for example, as described in Caruthers *et al.*, *Methods in Enzymology* (1992) 211:3-19; Thompson *et al.*, International PCT Publication No. WO 99/54459; Wincott *et al.*, *Nucl. Acids Res.* (1995) 23:2677-2684; Wincott *et al.*, *Methods Mol. Bio.*, (1997) 74:59; Brennan *et al.*, *Biotechnol. Bioeng.* (1998) 61:33-45; and Brennan, U.S. Pat. No. 6,001,311; each of which is hereby incorporated by reference in its entirety herein. In general, the synthesis of oligonucleotides involves conventional nucleic acid protecting and coupling groups, such as

dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a Expedite 8909 RNA synthesizer sold by Applied Biosystems, Inc. (Weiterstadt, Germany), using ribonucleoside phosphoramidites sold by ChemGenes Corporation (Ashland Technology Center, 200 Homer Avenue,
5 Ashland, MA 01721, USA). Alternatively, syntheses can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif., USA), or by methods such as those described in Usman *et al.*, *J. Am. Chem. Soc.* (1987) 109:7845; Scaringe *et al.*, *Nucl. Acids Res.* (1990) 18:5433; Wincott *et al.*, *Nucl. Acids Res.* (1990) 23:2677-2684; and Wincott *et al.*, *Methods Mol. Bio.* (1997) 74:59, each of which is hereby
10 incorporated by reference in its entirety.

The nucleic acid molecules of the present invention may be synthesized separately and dsRNAs may be formed post-synthetically, for example, by ligation (Moore *et al.*, *Science* (1992) 256:9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, *Nucl. Acids Res.* (1991) 19:4247; Bellon *et al.*, *Nucleosides &*
15 *Nucleotides* (1997) 16:951; and Bellon *et al.*, *Bioconjugate Chem.* (1997) 8:204; or by hybridization following synthesis and/or deprotection. The nucleic acid molecules can be purified by gel electrophoresis using conventional methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al.*, *supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

20 In another embodiment, the level of a particular mRNA or polypeptide in a cell is reduced by introduction of a ribozyme into the cell or nucleic acid encoding such. Ribozyme molecules designed to catalytically cleave mRNA transcripts can also be introduced into, or expressed, in cells to inhibit target gene expression (*see, e.g.*, Sarver *et al.*, 1990, *Science* 247:1222-1225 and U.S. Patent No. 5,093,246). One commonly used
25 ribozyme motif is the hammerhead, for which the substrate sequence requirements are minimal. Design of the hammerhead ribozyme is disclosed in Usman *et al.*, *Current Opin. Struct. Biol.* (1996) 6:527-533. Usman also discusses the therapeutic uses of ribozymes. Ribozymes can also be prepared and used as described in Long *et al.*, *FASEB J.* (1993) 7:25; Symons, *Ann. Rev. Biochem.* (1992) 61:641; Perrotta *et al.*, *Biochem.* (1992) 31:16-
30 17; Ojwang *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1992) 89:10802-10806; and U.S. Patent No. 5,254,678. Ribozyme cleavage of HIV-I RNA is described in U.S. Patent No. 5,144,019; methods of cleaving RNA using ribozymes is described in U.S. Patent No. 5,116,742; and methods for increasing the specificity of ribozymes are described in U.S.

Patent No. 5,225,337 and Koizumi *et al.*, *Nucleic Acid Res.* (1989) 17:7059-7071. Preparation and use of ribozyme fragments in a hammerhead structure are also described by Koizumi *et al.*, *Nucleic Acids Res.* (1989) 17:7059-7071. Preparation and use of ribozyme fragments in a hairpin structure are described by Chowrira and Burke, *Nucleic Acids Res.* (1992) 20:2835. Ribozymes can also be made by rolling transcription as described in Daubendiek and Kool, *Nat. Biotechnol.* (1997) 15(3):273-277.

Gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (i.e., the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells in the body. (See generally, Helene (1991) *Anticancer Drug Des.*, 6(6):569-84; Helene *et al.* (1992) *Ann. N.Y. Acad. Sci.*, 660:27-36; and Maher (1992) *Bioassays* 14(12):807-15).

In a further embodiment, RNA aptamers can be introduced into or expressed in a cell. RNA aptamers are specific RNA ligands for proteins, such as for Tat and Rev RNA (Good *et al.* (1997) *Gene Therapy* 4: 45-54) that can specifically inhibit their translation.

4. *Isd Polypeptides*

The *S. aureus* polypeptides, including IsdA (SEQ ID NO: 3), IsdB (SEQ ID NO: 6), and IsdC (SEQ ID NO: 9) (Figures 1-3), described herein, include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host cell, including for example, bacterial, yeast, higher plant, insect, and mammalian cells. In certain, embodiments, the polypeptides disclosed herein inhibit the function of Isd polypeptides.

Polypeptides may also comprise, consist of or consist essentially of any of the amino acid sequences described herein. Yet other polypeptides comprise, consist of or consist essentially of an amino acid sequence that has at least about 70%, 80%, 90%, 95%, 98% or 99% identity or homology with an Isd polypeptide. For example, polypeptides that differ from a sequence in a naturally occurring Isd protein in about 1, 2, 3, 4, 5 or more amino acids are also contemplated. The differences may be substitutions, *e.g.*, conservative substitutions, deletions or additions. The differences are preferably in regions that are not significantly conserved among different species. Such regions may be identified by aligning the amino acid sequences of Isd proteins from various species. These amino acids can be substituted, *e.g.*, with those found in another species. Other amino acids that may be substituted, inserted or

deleted at these or other locations can be identified by mutagenesis studies coupled with biological assays.

Proteins may also comprise one or more non-naturally occurring amino acids. For example, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into proteins. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, alpha-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, gamma-Abu, epsilon-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine, fluoro-amino acids, designer amino acids such as beta-methyl amino acids, Calpha-methyl amino acids, Nalpha-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Yet other proteins that are encompassed herein are those that comprise modified amino acids. Exemplary proteins are derivative proteins that may be one modified by glycosylation, pegylation, phosphorylation or any similar process that retains at least one biological function of the protein from which it was derived.

Proteins may be used as a substantially pure preparation, *e.g.*, wherein at least about 90% of the protein in the preparation are the desired protein. Compositions comprising at least about 50%, 60%, 70%, or 80% of the desired protein may also be used.

The *S. aureus* polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography and high performance liquid chromatography ("HPLC") is employed for purification. Proteins may be used as a substantially pure preparation, *e.g.*, wherein at least about 90% of the protein in the preparation are the desired protein. Compositions comprising at least about 50%, 60%, 70%, or 80% of the desired protein may also be used.

Proteins may be denatured or non-denatured and may be aggregated or non-aggregated as a result thereof. Proteins can be denatured according to methods known in the art.

In certain embodiments, an Isd polypeptide described herein may be a fusion protein containing a domain which increases its solubility and/or facilitates its purification, identification, detection, and/or structural characterization. Exemplary domains, include, for example, glutathione S-transferase (GST), protein A, protein G, calmodulin-binding peptide, thioredoxin, maltose binding protein, HA, myc, poly arginine, poly His, poly His-Asp or FLAG fusion proteins and tags. Additional exemplary domains include domains that alter protein localization *in vivo*, such as signal peptides, type III secretion system-targeting peptides, transcytosis domains, nuclear localization signals, etc. In various embodiments, a polypeptide of the invention may comprise one or more heterologous fusions. Polypeptides may contain multiple copies of the same fusion domain or may contain fusions to two or more different domains. The fusions may occur at the N-terminus of the polypeptide, at the C-terminus of the polypeptide, or at both the N- and C-terminus of the polypeptide. It is also within the scope of the invention to include linker sequences between a polypeptide of the invention and the fusion domain in order to facilitate construction of the fusion protein or to optimize protein expression or structural constraints of the fusion protein. In another embodiment, the polypeptide may be constructed so as to contain protease cleavage sites between the fusion polypeptide and polypeptide of the invention in order to remove the tag after protein expression or thereafter. Examples of suitable endoproteases, include, for example, Factor Xa and TEV proteases. A protein may also be fused to a signal sequence. For example, when prepared recombinantly, a nucleic acid encoding the peptide may be linked at its 5' end to a signal sequence, such that the protein is secreted from the cell.

In certain embodiments, polypeptides of the invention may be synthesized chemically, ribosomally in a cell free system, or ribosomally within a cell. Chemical synthesis of polypeptides of the invention may be carried out using a variety of art recognized methods, including stepwise solid phase synthesis, semi-synthesis through the conformationally-assisted re-ligation of peptide fragments, enzymatic ligation of cloned or synthetic peptide segments, and chemical ligation. Native chemical ligation employs a chemoselective reaction of two unprotected peptide segments to produce a transient thioester-linked intermediate. The transient thioester-linked intermediate then spontaneously undergoes a rearrangement to provide the full length ligation product having a native peptide bond at the ligation site. Full length ligation products are chemically identical to proteins produced by cell free synthesis. Full length ligation products may be refolded and/or oxidized, as allowed, to form native disulfide-containing protein molecules.

(see e.g., U.S. Patent Nos. 6,184,344 and 6,174,530; and Muir *et al.*, *Curr. Opin. Biotech.* (1993): vol. 4, p 420; Miller *et al.*, *Science* (1989): vol. 246, p 1149; Wlodawer *et al.*, *Science* (1989): vol. 245, p 616; Huang *et al.*, *Biochemistry* (1991): vol. 30, p 7402; Schnolzer, *et al.*, *Int. J. Pept. Prot. Res.* (1992): vol. 40, p 180-193; Rajarathnam *et al.*,
 5 *Science* (1994): vol. 264, p 90; R. E. Offord, "Chemical Approaches to Protein Engineering", in *Protein Design and the Development of New therapeutics and Vaccines*, J. B. Hook, G. Poste, Eds., (Plenum Press, New York, 1990) pp. 253-282; Wallace *et al.*, *J. Biol. Chem.* (1992): vol. 267, p 3852; Abrahmsen *et al.*, *Biochemistry* (1991): vol. 30, p 4151; Chang, *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91: 12544-12548; Schnolzer *et al.*,
 10 *Science* (1992): vol., 3256, p 221; and Akaji *et al.*, *Chem. Pharm. Bull.* (Tokyo) (1985) 33: 184).

In certain embodiments, it may be advantageous to provide naturally-occurring or experimentally-derived homologs of a polypeptide of the invention. Such homologs may function in a limited capacity as a modulator to promote or inhibit a subset of the biological
 15 activities of the naturally-occurring form of the polypeptide. Thus, specific biological effects may be elicited by treatment with a homolog of limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of the biological activities of a polypeptide of the invention. For instance, antagonistic homologs may be generated which interfere with the ability of the wild-type polypeptide of the
 20 invention to associate with certain proteins, but which do not substantially interfere with the formation of complexes between the native polypeptide and other cellular proteins.

Polypeptides may be derived from the full-length polypeptides of the invention. Isolated peptidyl portions of those polypeptides may be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such
 25 polypeptides. In addition, fragments may be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, proteins may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or may be divided into overlapping fragments of a desired length. The fragments may be produced (recombinantly or by chemical synthesis) and tested to
 30 identify those peptidyl fragments having a desired property, for example, the capability of functioning as a modulator of the polypeptides of the invention. In an illustrative embodiment, peptidyl portions of a protein of the invention may be tested for binding activity, as well as inhibitory ability, by expression as, for example, thioredoxin fusion

proteins, each of which contains a discrete fragment of a protein of the invention (see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/02502).

In another embodiment, truncated polypeptides may be prepared. Truncated polypeptides have from 1 to 20 or more amino acid residues removed from either or both the N- and C-termini. Such truncated polypeptides may prove more amenable to expression, purification or characterization than the full-length polypeptide. For example, truncated polypeptides may prove more amenable than the full-length polypeptide to crystallization, to yielding high quality diffracting crystals or to yielding an HSQC spectrum with high intensity peaks and minimally overlapping peaks. In addition, the use of truncated polypeptides may also identify stable and active domains of the full-length polypeptide that may be more amenable to characterization.

It is also possible to modify the structure of the polypeptides of the invention for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life, resistance to proteolytic degradation *in vivo*, etc.). Such modified polypeptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered "functional equivalents" of the polypeptides described in more detail herein. Such modified polypeptides may be produced, for instance, by amino acid substitution, deletion, or addition, which substitutions may consist in whole or part by conservative amino acid substitutions.

For instance, it is reasonable to expect that an isolated conservative amino acid substitution, such as replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, will not have a major affect on the biological activity of the resulting molecule. Whether a change in the amino acid sequence of a polypeptide results in a functional homolog may be readily determined by assessing the ability of the variant polypeptide to produce a response similar to that of the wild-type protein. Polypeptides in which more than one replacement has taken place may readily be tested in the same manner.

Methods of generating sets of combinatorial mutants of polypeptides of the invention are provided, as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g., homologs). The purpose of screening such combinatorial libraries is to generate, for example, homologs which may modulate the activity of a polypeptide of the invention, or alternatively, which possess novel activities altogether. Combinatorially-derived homologs may be generated which have a selective

potency relative to a naturally-occurring protein. Such homologs may be used in the development of therapeutics.

Likewise, mutagenesis may give rise to homologs which have intracellular half-lives dramatically different than the corresponding wild-type protein. For example, the altered protein may be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the protein. Such homologs, and the genes which encode them, may be utilized to alter protein expression by modulating the half-life of the protein. As above, such proteins may be used for the development of therapeutics or treatment.

In similar fashion, protein homologs may be generated by the present combinatorial approach to act as antagonists, in that they are able to interfere with the activity of the corresponding wild-type protein.

In a representative embodiment of this method, the amino acid sequences for a population of protein homologs are aligned, preferably to promote the highest homology possible. Such a population of variants may include, for example, homologs from one or more species, or homologs from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In certain embodiments, the combinatorial library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential protein sequences. For instance, a mixture of synthetic oligonucleotides may be enzymatically ligated into gene sequences such that the degenerate set of potential nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display).

There are many ways by which the library of potential homologs may be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence may be carried out in an automatic DNA synthesizer, and the synthetic genes may then be ligated into an appropriate vector for expression. One purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential protein sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang (1983) *Tetrahedron* 39:3; Itakura *et al.* (1981) *Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323;

Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.*, (1983) *Nucleic Acid Res.* 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott *et al.* (1990) *Science* 249:386-390; Roberts *et al.* (1992) *PNAS USA* 89:2429-2433; Devlin *et al.* (1990) *Science* 249: 404-406; Cwirla *et al.* (1990) *PNAS USA* 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis may be utilized to generate a combinatorial library. For example, protein homologs (both agonist and antagonist forms) may be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf *et al.* (1994) *Biochemistry* 33:1565-1572; Wang *et al.* (1994) *J. Biol. Chem.* 269:3095-3099; Balint *et al.* (1993) *Gene* 137:109-118; Grodberg *et al.* (1993) *Eur. J. Biochem.* 218:597-601; Nagashima *et al.* (1993) *J. Biol. Chem.* 268:2888-2892; Lowman *et al.* (1991) *Biochemistry* 30:10832-10838; and Cunningham *et al.*, (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin *et al.* (1993) *Virology* 193:653-660; Brown *et al.* (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight *et al.* (1982) *Science* 232:316); by saturation mutagenesis (Meyers *et al.* (1986) *Science* 232:613); by PCR mutagenesis (Leung *et al.* (1989) *Method Cell Mol Biol* 1:11-19); or by random mutagenesis (Miller *et al.* (1992) *A Short Course in Bacterial Genetics*, CSHL Press, Cold Spring Harbor, NY; and Greener *et al.* (1994) *Strategies in Mol Biol* 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated forms of proteins that are bioactive.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of protein homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected.

In an illustrative embodiment of a screening assay, candidate combinatorial gene products are displayed on the surface of a cell and the ability of particular cells or viral particles to bind to the combinatorial gene product is detected in a "panning assay". For instance, the gene library may be cloned into the gene for a surface membrane protein of a

bacterial cell (Ladner *et al.*, WO 88/06630; Fuchs *et al.*, (1991) *Bio/Technology* 9:1370-1371; and Goward *et al.*, (1992) *TIBS* 18:136-140), and the resulting fusion protein detected by panning, e.g. using a fluorescently labeled molecule which binds the cell surface protein, e.g., FITC-substrate, to score for potentially functional homologs. Cells may be visually
5 inspected and separated under a fluorescence microscope, or, when the morphology of the cell permits, separated by a fluorescence-activated cell sorter. This method may be used to identify substrates or other polypeptides that can interact with a polypeptide of the invention.

In similar fashion, the gene library may be expressed as a fusion protein on the
10 surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences may be expressed on the surface of infectious phage, thereby conferring two benefits. First, because these phage may be applied to affinity matrices at very high concentrations, a large number of phage may be screened at one time. Second, because each infectious phage displays the combinatorial gene product on its surface, if a particular
15 phage is recovered from an affinity matrix in low yield, the phage may be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins may be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner *et al.*, PCT publication WO 90/02909; Garrard *et al.*,
20 PCT publication WO 92/09690; Marks *et al.*, (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths *et al.*, (1993) *EMBO J.* 12:725-734; Clackson *et al.*, (1991) *Nature* 352:624-628; and Barbas *et al.*, (1992) *PNAS USA* 89:4457-4461). Other phage coat proteins may be used as appropriate.

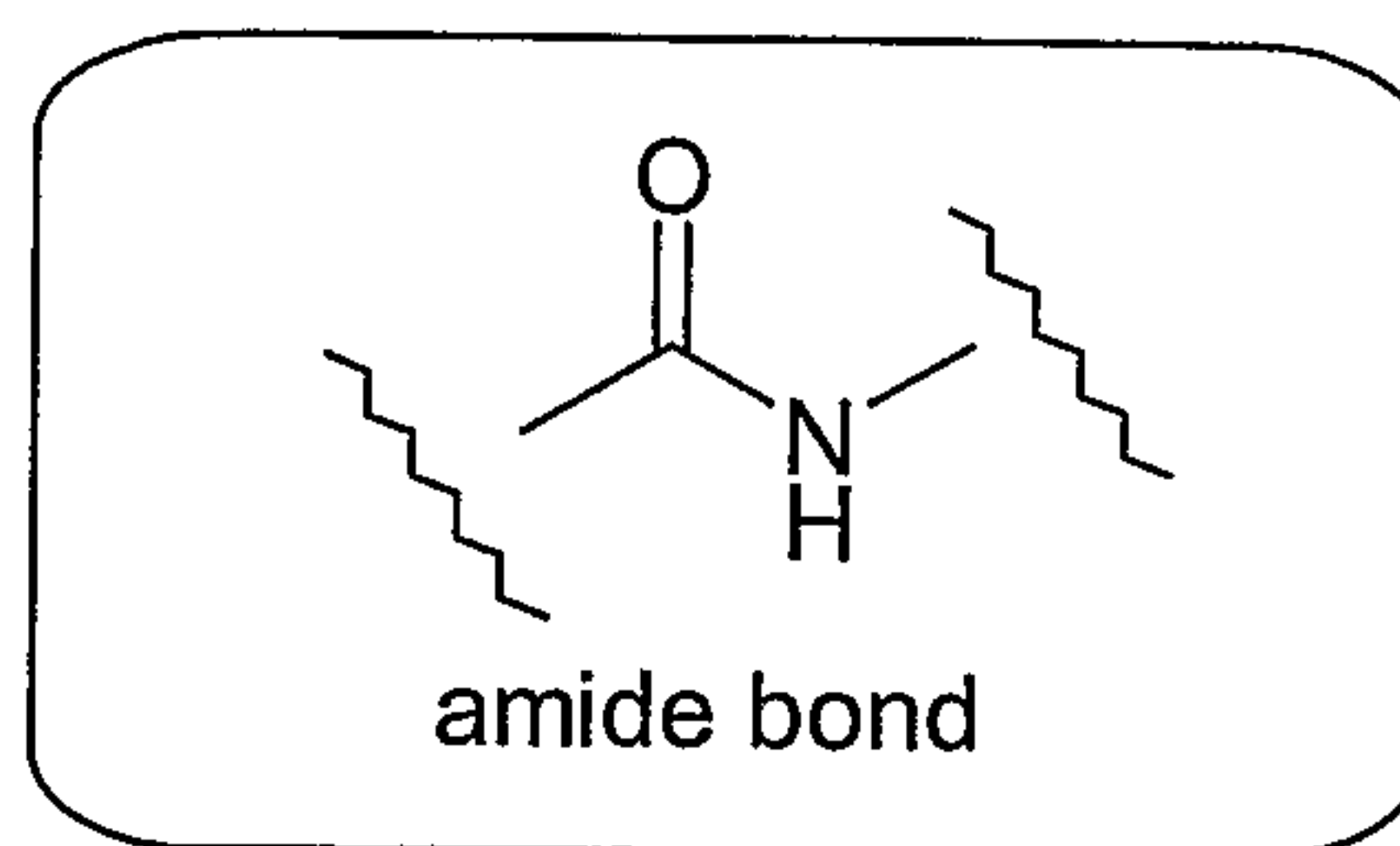
The polypeptides disclosed herein may be reduced to generate mimetics, e.g.
25 peptide or non-peptide agents, which are able to mimic binding of the authentic protein to another cellular partner. Such mutagenic techniques as described above, as well as the thioredoxin system, are also particularly useful for mapping the determinants of a protein which participates in a protein-protein interaction with another protein. To illustrate, the critical residues of a protein which are involved in molecular recognition of a substrate
30 protein may be determined and used to generate peptidomimetics that may bind to the substrate protein. The peptidomimetic may then be used as an inhibitor of the wild-type protein by binding to the substrate and covering up the critical residues needed for interaction with the wild-type protein, thereby preventing interaction of the protein and the

substrate. By employing, for example, scanning mutagenesis to map the amino acid residues of a protein which are involved in binding a substrate polypeptide, peptidomimetic compounds may be generated which mimic those residues in binding to the substrate.

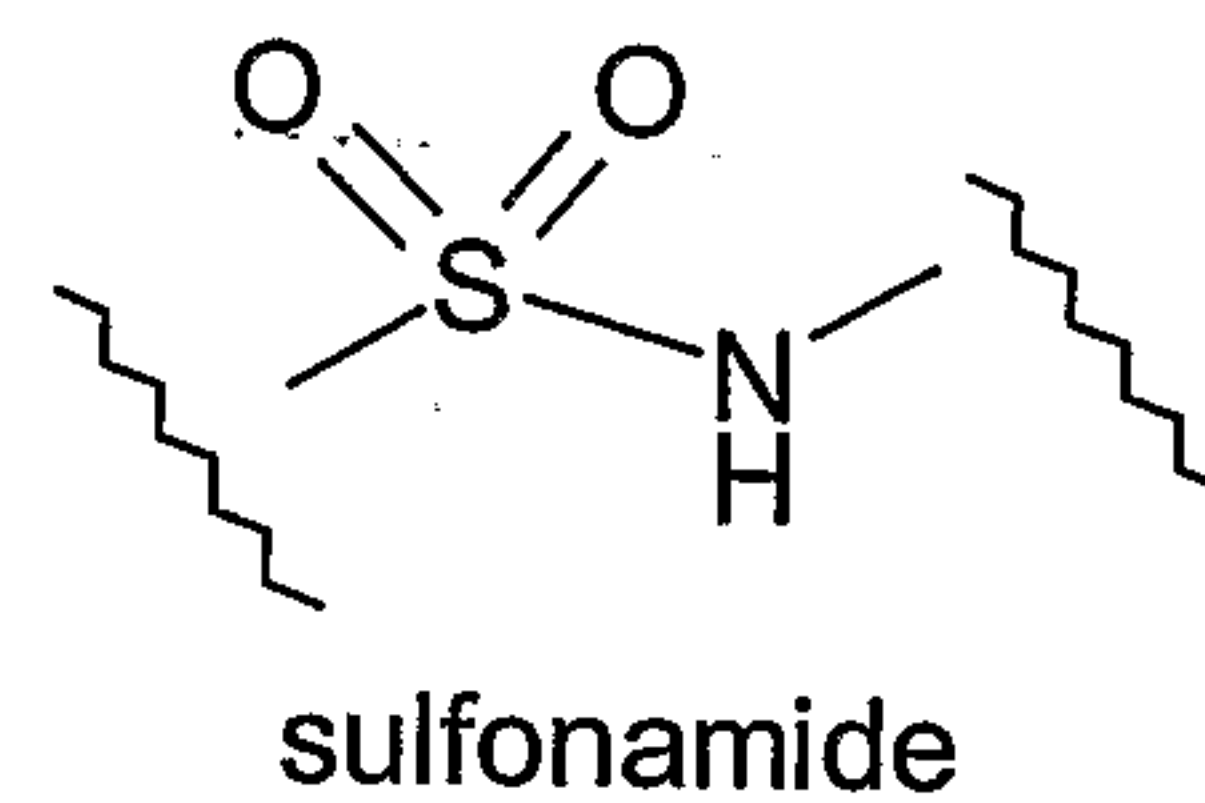
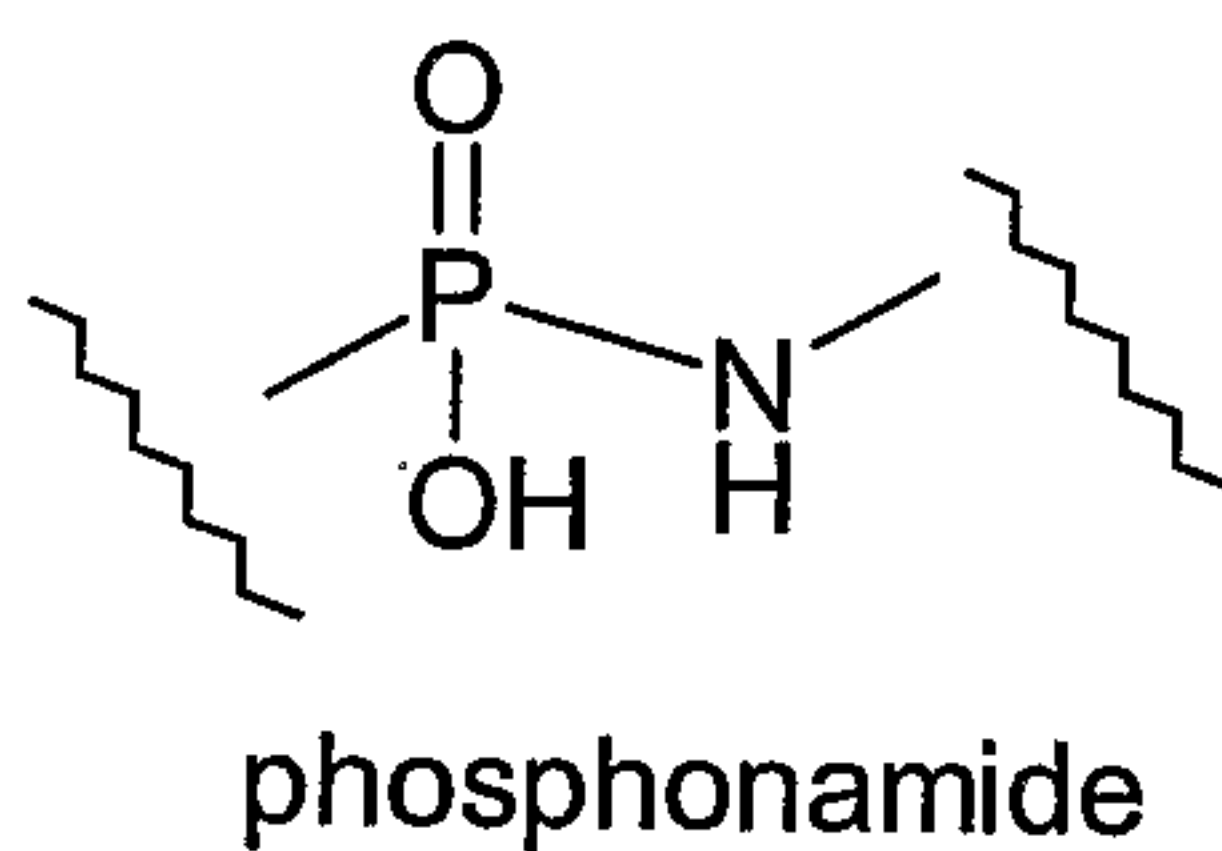
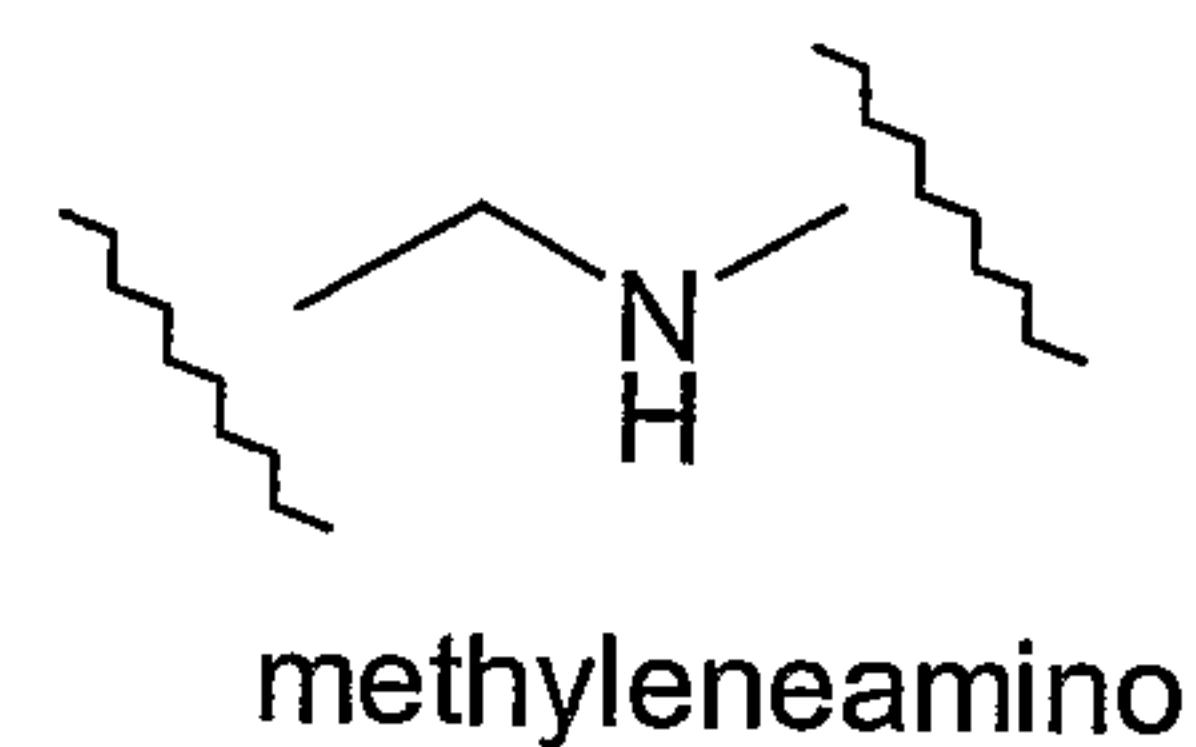
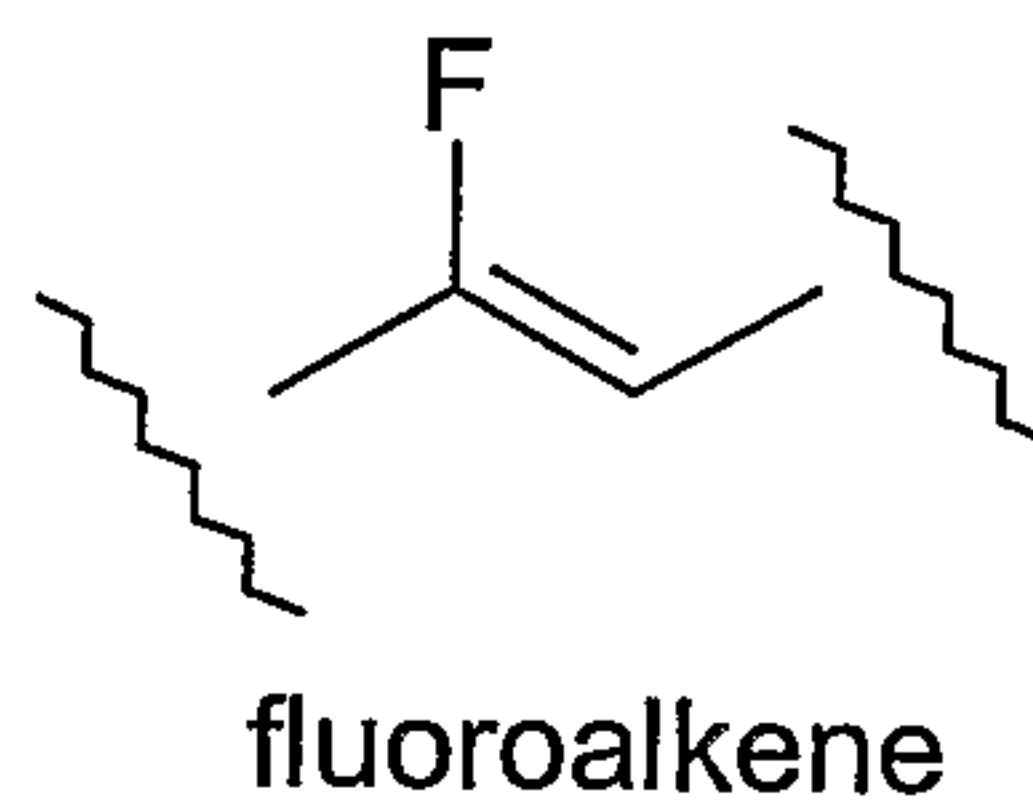
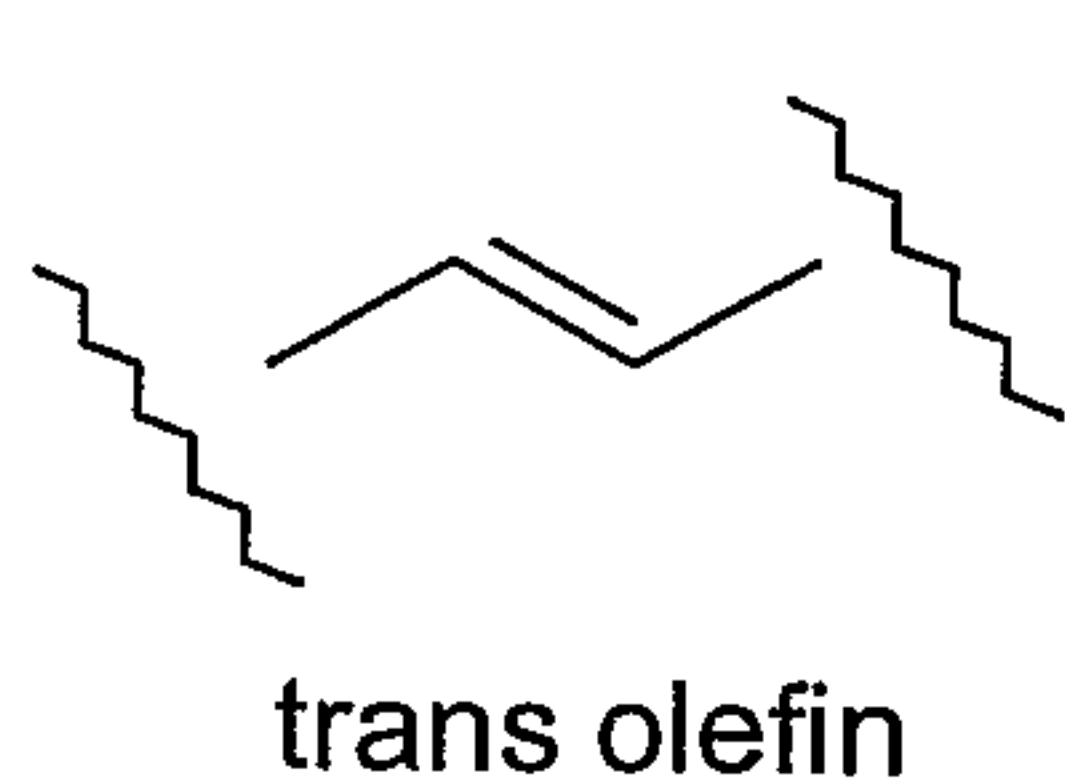
For instance, derivatives of the Isd proteins described herein may be chemically modified peptides and peptidomimetics. Peptidomimetics are compounds based on, or derived from, peptides and proteins. Peptidomimetics can be obtained by structural modification of known peptide sequences using unnatural amino acids, conformational restraints, isosteric replacement, and the like. The subject peptidomimetics constitute the continuum of structural space between peptides and non-peptide synthetic structures; peptidomimetics may be useful, therefore, in delineating pharmacophores and in helping to translate peptides into nonpeptide compounds with the activity of the parent peptides.

Moreover, mimetopes of the subject peptides can be provided. Such peptidomimetics can have such attributes as being non-hydrolyzable (e.g., increased stability against proteases or other physiological conditions which degrade the corresponding peptide), increased specificity and/or potency for stimulating cell differentiation. For illustrative purposes, non-hydrolyzable peptide analogs of such residues may be generated using benzodiazepine (e.g., see Freidinger *et al.*, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman *et al.*, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey *et al.*, in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson *et al.*, (1986) *J. Med. Chem.* 29:295; and Ewenson *et al.*, in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai *et al.*, (1985) *Tetrahedron Lett* 26:647; and Sato *et al.* (1986) *J Chem Soc Perkin Trans 1*:1231), and β -aminoalcohols (Gordon *et al.* (1985) *Biochem Biophys Res Commun* 126:419; and Dann *et al.* (1986) *Biochem Biophys Res Commun* 134:71).

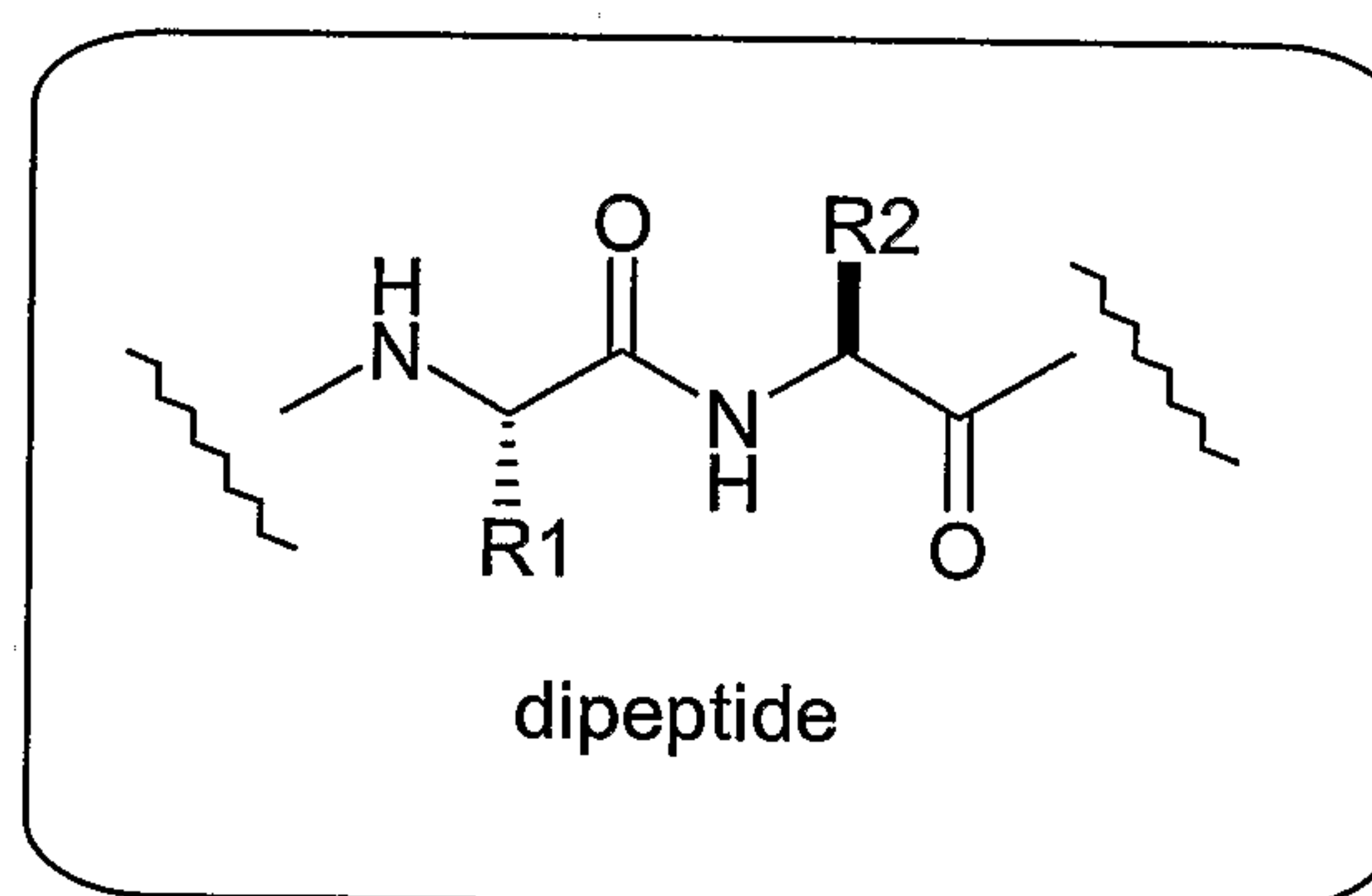
In addition to a variety of sidechain replacements which can be carried out to generate peptidomimetics, the description specifically contemplates the use of conformationally restrained mimics of peptide secondary structure. Numerous surrogates have been developed for the amide bond of peptides. Frequently exploited surrogates for the amide bond include the following groups (i) trans-olefins, (ii) fluoroalkene, (iii) methyleneamino, (iv) phosphonamides, and (v) sulfonamides.



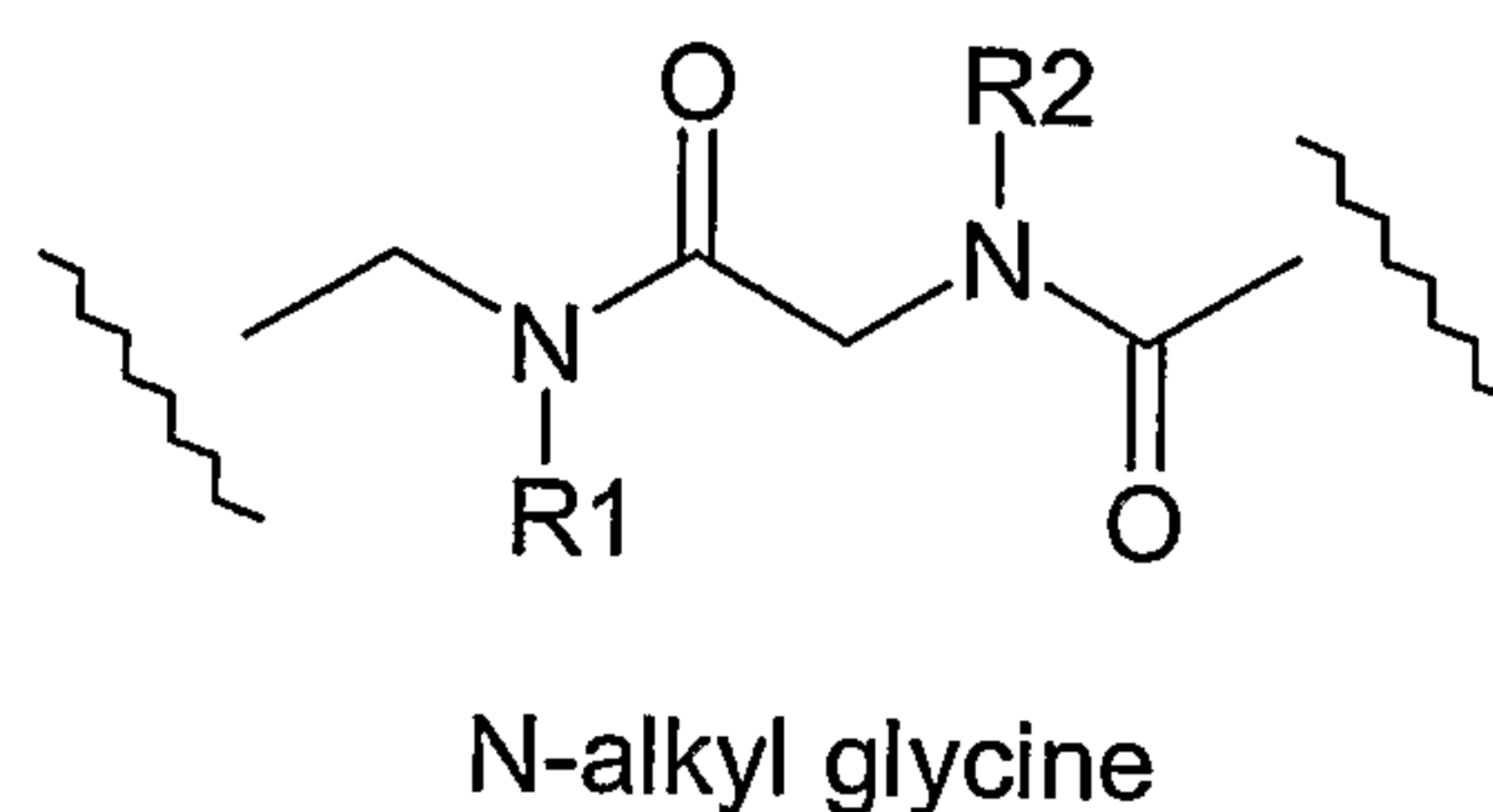
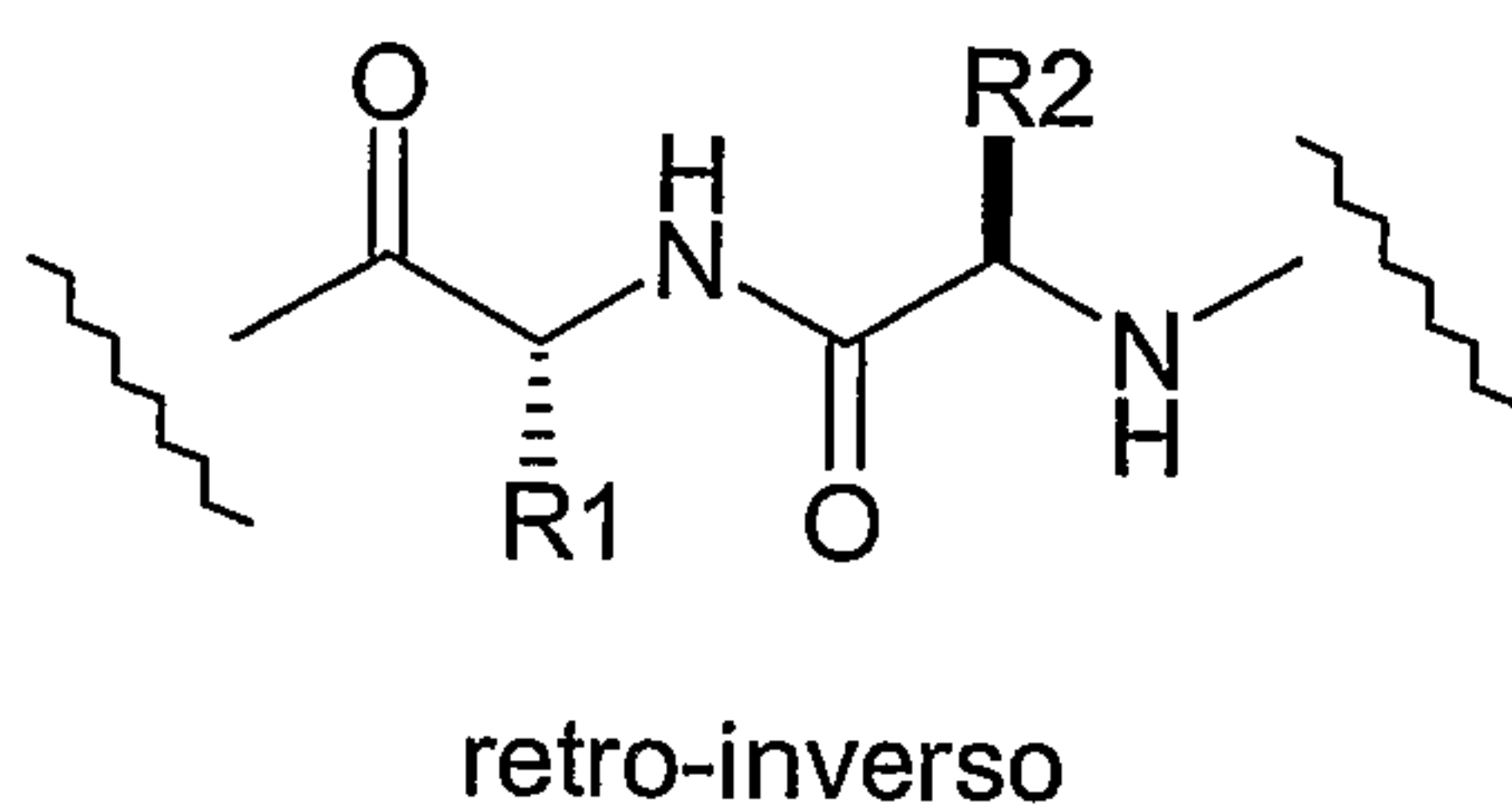
Examples of Surrogates:



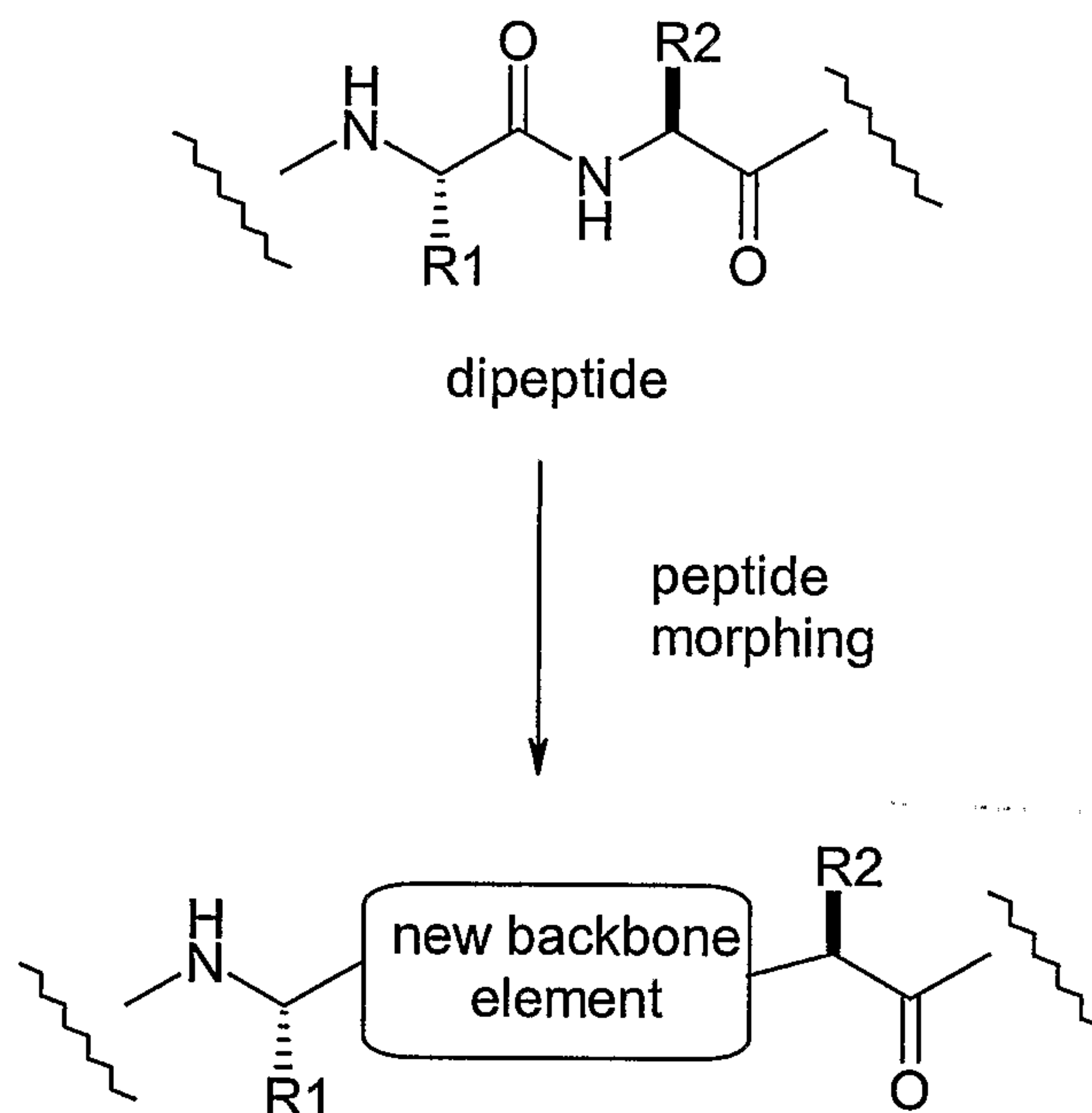
Additionally, peptidomimetics based on more substantial modifications of the backbone of a peptide can be used. Peptidomimetics which fall in this category include (i) retro-inverso analogs, and (ii) N-alkyl glycine analogs (so-called peptoids).



Examples of analogs:



Furthermore, the methods of combinatorial chemistry are being brought to bear, on the development of new peptidomimetics. For example, one embodiment of a so-called “peptide morphing” strategy focuses on the random generation of a library of peptide analogs that comprise a wide range of peptide bond substitutes.



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In an exemplary embodiment, the peptidomimetic can be derived as a retro-inverso analog of the peptide. Such retro-inverso analogs can be made according to the methods known in the art, such as that described by the Sisto *et al.* U.S. Patent 4,522,752. A retro-inverso analog can be generated as described, e.g., in WO 00/01720. It will be understood that a mixed peptide, e.g., including some normal peptide linkages, may be generated. As a general guide, sites which are most susceptible to proteolysis are typically altered, with less susceptible amide linkages being optional for mimetic switching. The final product, or intermediates thereof, can be purified by HPLC.

Peptides may comprise at least one amino acid or every amino acid that is a D stereoisomer. Other peptides may comprise at least one amino acid that is reversed. The amino acid that is reversed may be a D stereoisomer. Every amino acid of a peptide may be reversed and/or every amino acid may be a D stereoisomer.

In another illustrative embodiment, a peptidomimetic can be derived as a retro-enantio analog of a peptide. Retro-enantio analogs such as this can be synthesized with commercially available D-amino acids (or analogs thereof) and standard solid- or solution-phase peptide-synthesis techniques, as described, e.g., in WO 00/01720. The final product may be purified by HPLC to yield the pure retro-enantio analog.

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In still another illustrative embodiment, trans-olefin derivatives can be made for the subject peptide. Trans-olefin analogs can be synthesized according to the method of Y.K. Shue *et al.* (1987) *Tetrahedron Letters* 28:3225 and as described in WO 00/01720. It is further possible to couple pseudodipeptides synthesized by the above method to other pseudodipeptides, to make peptide analogs with several olefinic functionalities in place of amide functionalities.

Still another class of peptidomimetic derivatives include the phosphonate derivatives. The synthesis of such phosphonate derivatives can be adapted from known synthesis schemes. See, for example, Loots *et al.* in *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118); Petrillo *et al.* in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium, Pierce Chemical Co. Rockland, IL, 1985).

Many other peptidomimetic structures are known in the art and can be readily adapted for use in the subject peptidomimetics. To illustrate, a peptidomimetic may incorporate the 1-azabicyclo[4.3.0]nonane surrogate (see Kim *et al.* (1997) *J. Org. Chem.* 62:2847), or an *N*-acyl piperazic acid (see Xi *et al.* (1998) *J. Am. Chem. Soc.* 120:80), or a 2-substituted piperazine moiety as a constrained amino acid analogue (see Williams *et al.* (1996) *J. Med. Chem.* 39:1345-1348). In still other embodiments, certain amino acid residues can be replaced with aryl and bi-aryl moieties, e.g., monocyclic or bicyclic aromatic or heteroaromatic nucleus, or a biaromatic, aromatic-heteroaromatic, or biheteroaromatic nucleus.

The subject peptidomimetics can be optimized by, e.g., combinatorial synthesis techniques combined with high throughput screening.

Moreover, other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of inhibiting cell survival and/or tumor growth. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional

structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. The predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

“Peptides, variants and derivatives thereof” or “peptides and analogs thereof” are included in “peptide therapeutics” and is intended to include any of the peptides or modified forms thereof, e.g., peptidomimetics, described herein. Preferred peptide therapeutics decrease cell survival or increase apoptosis. For example, they may decrease cell survival or increase apoptosis by a factor of at least about 2 fold, 5 fold, 10 fold, 30 fold or 100 fold, as determined, e.g., in an assay described herein.

The activity of an Isd protein, fragment, or variant thereof may be assayed using an appropriate substrate or binding partner or other reagent suitable to test for the suspected activity as described below.

In another embodiment, the activity of a polypeptide may be determined by assaying for the level of expression of RNA and/or protein molecules. Transcription levels may be determined, for example, using Northern blots, hybridization to an oligonucleotide array or by assaying for the level of a resulting protein product. Translation levels may be determined, for example, using Western blotting or by identifying a detectable signal produced by a protein product (e.g., fluorescence, luminescence, enzymatic activity, etc.). Depending on the particular situation, it may be desirable to detect the level of transcription and/or translation of a single gene or of multiple genes.

Alternatively, it may be desirable to measure the overall rate of DNA replication, transcription and/or translation in a cell. In general this may be accomplished by growing the cell in the presence of a detectable metabolite which is incorporated into the resultant DNA, RNA, or protein product. For example, the rate of DNA synthesis may be determined by growing cells in the presence of BrdU which is incorporated into the newly synthesized DNA. The amount of BrdU may then be determined histochemically using an anti-BrdU antibody.

In other embodiments, polypeptides of the invention may be immobilized onto a solid surface, including, microtiter plates, slides, beads, films, etc. The polypeptides of the invention may be immobilized onto a “chip” as part of an array. An array, having a

plurality of addresses, may comprise one or more polypeptides of the invention in one or more of those addresses. In one embodiment, the chip comprises one or more polypeptides of the invention as part of an array of polypeptide sequences.

In other embodiments, polypeptides of the invention may be immobilized onto a solid surface, including, plates, microtiter plates, slides, beads, particles, spheres, films, strands, precipitates, gels, sheets, tubing, containers, capillaries, pads, slices, etc. The polypeptides of the invention may be immobilized onto a "chip" as part of an array. An array, having a plurality of addresses, may comprise one or more polypeptides of the invention in one or more of those addresses. In one embodiment, the chip comprises one or more polypeptides of the invention as part of an array.

5. *Isd Vaccines*

IsdA, IsdB, and IsdC polypeptides are cell surface proteins expressed by *S. aureus* and are essential for full virulence *in vivo* (shown using a mouse model of kidney infection). Further, IsdA is immunodominant as anti-IsdA antibodies are detected in convalescent human sera. Thus, the IsdA, IsdB, and/or IsdC polypeptides may be used as a vaccine therapy to treat *S. aureus* infections.

IsdA, IsdB, and/or IsdC polypeptides or polynucleotides may be formulated into a vaccine and administered to a subject to induce an immune response (e.g. cellular or humoral) against IsdA, IsdB, and/or IsdC in that subject.

An exemplary IsdA protein for inclusion in a vaccine is the full length IsdA polypeptide or an IsdA peptide. In certain embodiments, recombinant IsdA protein will be used in a vaccine. In alternate embodiments, IsdB or IsdC protein used as a vaccine may be full-length IsdB or IsdC, a peptide fragment of IsdB or IsdC, or recombinant IsdB or IsdC protein.

Isd peptides that are antigenic and used as a vaccine may be identified using a variety of methods. In one approach, peptides containing antigenic sequences may be selected on the basis of generally accepted criteria of potential antigenicity and/or exposure. Such criteria include the hydrophilicity and relative antigenic index, as determined by surface exposure analysis of proteins. The determination of appropriate criteria is well-known to one of skill in the art, and has been described, for example, by Hopp *et al.*, *Proc Natl Acad Sci USA* 1981; 78: 3824-8; Kyte *et al.*, *J Mol Biol* 1982; 157: 105-32; Emini, *J Virol* 1985; 55: 836-9; Jameson *et al.*, *CA BIOS* 1988; 4: 181-6; and Karplus *et al.*,

Naturwissenschaften 1985; 72: 212-3. Amino acid domains predicted by these criteria to be surface exposed may be selected preferentially over domains predicted to be more hydrophobic.

Portions of IsdA, IsdB and/or IsdC determined to be antigenic may be chemically synthesized by methods known in the art from individual amino acids. Suitable methods for synthesizing protein fragments are described by Stuart and Young in "Solid Phase Peptide Synthesis," Second Edition, Pierce Chemical Company (1984).

Alternatively, antigenic linear epitope(s) of IsdA, IsdB or IsdC may be identified by minotope analysis with a corresponding Isd antibody. Briefly, for mimotope analysis a polypeptide will be subdivided into overlapping fragments. For example, overlapping 15 amino acid peptides will be synthesized to cover the entire length of the full-length polypeptide. Each 15 amino acid peptide may overlap by three amino acids. Alternatively, 15 amino acid peptide fragments may be designed in tandem order to cover the entire linear amino acid sequence. Each peptide may then be biotinylated and allowed to bind to streptavidin-coated wells in 96-well plates. The reactivity of various antisera may be detected by enzyme-linked immunosorbent assay (ELISA). After blocking non-specific binding, an anti-Isd antibody may be added to each well, followed by the sequential addition of peroxidase-conjugated secondary antibody, and peroxidase substrate. Anti-Isd antibodies may be affinity purified anti-full-length recombinant IsdA or affinity purified anti-IsdA peptide. Alternatively, anti-Isd antibodies may be against IsdB or IsdC. The optical density of each well may be read at 450 nm and duplicate or triplicate wells may be averaged. The average value obtained from a similar ELISA using control serum (i.e., preimmune serum) may be subtracted from the test immunoglobulin values and the resultant values may be plotted to determine which linear epitopes are recognized by the immunoglobulin(s).

Further, competitive binding assays using synthetic peptides representing linear epitopes may be used to determine antigenic fragments. In certain embodiments, antigenic fragments may inhibit uptake of labeled iron.

Also provided herein are DNA vaccines comprising nucleotide sequences, which encode IsdA, IsdB, and/or IsdC peptides. Exemplary DNA vaccines encode two or more IsdA peptides. Alternate DNA vaccines may encode two or more IsdB or IsdC peptides or any combination of two or more IsdA, IsdB, or IsdC peptides. The efficacy of candidate vaccines (peptide or DNA) may be tested in appropriate animal models such as rats, mice,

guinea pigs, monkeys and baboons. A protective or positive effect of the vaccine should be reflected by reduced fertility in the experimental animals.

Nucleic acids encoding IsdA, IsdB, or IsdC immunogens may be obtained by polymerase chain reaction (PCR), amplification of gene segments from genomic DNA, cDNA, RNA (e.g. by RT-PCR), or cloned sequences. PCR primers are chosen, based on the known sequences of the genes or cDNA, so that they result in the amplification of relatively unique fragments. Computer programs may be used in the design of primers with required specificity and optimal amplification purposes. See e.g., Oligo version 5.0 (National Biosciences). Factors which apply to the design and selection of primers for amplification are described for example, by Rylchik, W. (1993) "Selection of Primers for Polymerase Chain Reaction." In *Methods in Molecular Biology*, vol. 15, White B. ed., Humana Press, Totowa, N.J. Sequences may be obtained from GenBank or other public sources. Alternatively, the nucleic acids of this invention may also be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such synthesizers are commercially available from Biosearch, Applied Biosystems, etc). Suitable cloning vectors for expressing Isd polypeptides in a host or in a cell may be constructed according to standard techniques as described above.

Isd immunogens may alternatively be prepared from enzymatic cleavage of intact Isd polypeptides. Examples of proteolytic enzymes include, but are not limited to, trypsin, chymotrypsin, pepsin, papain, V8 protease, subtilisin, plasmin, and thrombin. Intact polypeptides can be incubated with one or more proteinases simultaneously or sequentially. Alternatively, or in addition, intact Isd polypeptides can be treated with disulfide reducing agents. Peptides may then be separated from each other by techniques known in the art, including but not limited to, gel filtration chromatography, gel electrophoresis, and reverse-phase HPLC.

6. Isd Antibodies and Uses thereof

To produce antibodies against IsdA, IsdB, and/or IsdC, host animals may be injected with full-length Isd polypeptides or with Isd peptides. Hosts may be injected with peptides of different lengths encompassing a desired target sequence. For example, peptide antigens that are at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145 or 150 amino acids may be used. Alternatively, if a portion of an Isd protein defines an epitope, but is too short to be

antigenic, it may be conjugated to a carrier molecule in order to produce antibodies. Some suitable carrier molecules include keyhole limpet hemocyanin, Ig sequences, TrpE, and human or bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragments with a cysteine
5 residue on the carrier molecule.

In addition, antibodies to three-dimensional epitopes, i.e., non-linear epitopes, may also be prepared, based on, e.g., crystallographic data of proteins. Antibodies obtained from that injection may be screened against the short antigens of IsdA, IsdB or IsdC. Antibodies prepared against an Isd peptide may be tested for activity against that peptide as well as the
10 full length Isd protein. Antibodies may have affinities of at least about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M or 10^{-12} M toward the Isd peptide and/or the full length Isd protein.

Suitable cells for the DNA sequences and host cells for antibody expression and secretion can be obtained from a number of sources, including the American Type Culture
15 Collection ("*Catalogue of Cell Lines and Hybridomas*" 5th edition (1985) Rockville, Md., U.S.A.).

Polyclonal and monoclonal antibodies may be produced by methods known in the art. Monoclonal antibodies may be produced by hybridomas prepared using known procedures including the immunological method described by Kohler and Milstein, Nature
20 1975; 256: 495-7; and Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon *et al.*, Eds. Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); as well as by the recombinant DNA method described by Huse *et al.*, Science (1989) 246: 1275-81.

25 Methods of antibody purification are well known in the art. See, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. Purification methods may include salt precipitation (for example, with ammonium sulfate), ion exchange chromatography (for example, on a cationic or anionic exchange column run at neutral pH and eluted with step gradients of increasing ionic
30 strength), gel filtration chromatography (including gel filtration HPLC), and chromatography on affinity resins such as protein A, protein G, hydroxyapatite, and anti-antibody. Antibodies may also be purified on affinity columns according to methods known in the art.

Other embodiments include functional equivalents of antibodies, and include, for example, chimerized, humanized, and single chain antibodies as well as fragments thereof. Methods of producing functional equivalents are disclosed in PCT Application WO 93/21319; European Patent Application No. 239,400; PCT Application WO 89/09622; 5 European Patent Application 388,745; and European Patent Application EP 332,424.

Functional equivalents include polypeptides with amino acid sequences substantially the same as the amino acid sequence of the variable or hypervariable regions of the antibodies of the invention. "Substantially the same" amino acid sequence is defined herein as a sequence with at least 70%, preferably at least about 80%, and more preferably 10 at least 90% homology to another amino acid sequence as determined by the FASTA search method in accordance with Pearson and Lipman, (1988) Proc Natl Acad Sci USA 85: 2444-8.

Chimerized antibodies may have constant regions derived substantially or exclusively from human antibody constant regions and variable regions derived 15 substantially or exclusively from the sequence of the variable region from a mammal other than a human. Humanized antibodies may have constant regions and variable regions other than the complement determining regions (CDRs) derived substantially or exclusively from the corresponding human antibody regions and CDRs derived substantially or exclusively from a mammal other than a human.

20 Suitable mammals other than a human may include any mammal from which monoclonal antibodies may be made. Suitable examples of mammals other than a human may include, for example, a rabbit, rat, mouse, horse, goat, or primate.

Antibodies to IsdA, IsdB or IsdC may be prepared as described above use as an anti-infective. In other embodiments, antibodies that recognize functional Isd fragments may 25 also be used in random peptide phage display technology (Eidne *et al.*, *Biol Reprod.* 63(5):1396-402 (2000)). Briefly, fifteen or twelve-mer random peptide phage display libraries can be used to determine peptides that might interact with functional Isd peptides by competitive displacement of Fab fragments of Isd antibodies. For this, fixed *S. aureus* cells are allowed to adhere to wells in multiwell plates, and immunostaining for IsdA, IsdB 30 or IsdC may then be evaluated in the absence and presence of unique and random peptides expressed by the phage library. Once the competitive peptides are identified by amino acid sequence analysis, increased amounts of peptide can be synthesized and used as alternative molecular antagonists to antibodies directed against functional fragments. Another

alternative is to screen small molecule libraries for their ability to competitively displace Fab fragments to functional IsdA, IsdB, or IsdC fragments. Molecular antagonists identified in this manner may be used to neutralize the effect of antibodies generated by an immune response to the Isd polypeptide or polynucleotide vaccine.

5 In a further embodiment, the antibodies to IsdA, IsdB, or IsdC (whole antibodies or antibody fragments) may be conjugated to a biocompatible material, such as polyethylene glycol molecules (PEG) according to methods well known to persons of skill in the art to increase the antibody's half-life. See for example, U.S. Patent No. 6,468,532. Functionalized PEG polymers are available, for example, from Nektar Therapeutics.

10 Commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl

15 carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the polypeptide, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives

20 include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc.

7. *Pharmaceutical Compositions*

25 Purified IsdA, IsdB, or IsdC polypeptides and nucleic acids may be formulated and introduced as a vaccine through oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, intravaginal, and via scarification (i.e., scratching through the top layers of skin, e.g., using a bifurcated needle) or any other standard route of immunization. Isd polypeptides may further be orally delivered as a vaccine by enteric-

30 coated capsules, which will dissolve in the gut and taken up by antigen presenting cells in Peyer's patches. Oral delivery of Isd polypeptides may supplement injections of Isd polypeptides.

Further, *S. aureus* anti-Isd antibodies, *isd* antisense nucleic acids and siRNAs, as described herein may be administered by various means, depending on their intended use, as is well known in the art. For example, if such *S. aureus* antagonist compositions are to be administered orally, they may be formulated as tablets, capsules, granules, powders or
5 syrups. Alternatively, formulations of the present invention may be administered parenterally as injections (intravenous, intramuscular or subcutaneous), drop infusion preparations or suppositories. For application by the ophthalmic mucous membrane route, compositions of the present invention may be formulated as eyedrops or eye ointments. These formulations may be prepared by conventional means, and, if desired, the
10 compositions may be mixed with any conventional additive, such as an excipient, a binder, a disintegrating agent, a lubricant, a corrigent, a solubilizing agent, a suspension aid, an emulsifying agent or a coating agent.

In formulations of the subject invention, wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release
15 agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants may be present in the formulated agents.

Subject compositions may be suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal, aerosol and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well
20 known in the art of pharmacy. The amount of composition that may be combined with a carrier material to produce a single dose vary depending upon the subject being treated, and the particular mode of administration.

Methods of preparing these formulations include the step of bringing into association compositions of the present invention with the carrier and, optionally, one or
25 more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association agents with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or
30 tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia), each containing a predetermined amount of a subject composition thereof as an active ingredient.

Compositions of the present invention may also be administered as a bolus, electuary, or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the subject composition is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, acetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the subject composition moistened with an inert liquid diluent. Tablets, and other solid dosage forms, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the subject composition, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol,

benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

5 Suspensions, in addition to the subject composition, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

10 Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing a subject composition with one or more suitable non-irritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the body cavity and release the active agent. Formulations, which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in
15 the art to be appropriate.

 Dosage forms for transdermal administration of a subject composition includes powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active component may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants, which may be
20 required.

 The ointments, pastes, creams and gels may contain, in addition to a subject composition, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

25 Powders and sprays may contain, in addition to a subject composition, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays may additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

30 Compositions of the present invention may alternatively be administered by aerosol. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing the compound. A non-aqueous (*e.g.*, fluorocarbon propellant) suspension could be used. Sonic nebulizers may be used because they minimize exposing

the agent to shear, which may result in degradation of the compounds contained in the subject compositions.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of a subject composition with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular subject composition, but typically include non-ionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

10 In addition, Isd based vaccines may be administered parenterally as injections (intravenous, intramuscular or subcutaneous). The vaccine compositions of the present invention may optionally contain one or more adjuvants. Any suitable adjuvant can be used, such as aluminum hydroxide, aluminum phosphate, plant and animal oils, and the like, with the amount of adjuvant depending on the nature of the particular adjuvant employed. In addition, the anti-infective vaccine compositions may also contain at least one stabilizer, such as carbohydrates such as sorbitol, mannitol, starch, sucrose, dextrin, and glucose, as well as proteins such as albumin or casein, and buffers such as alkali metal phosphates and the like. Preferred adjuvants include the SynerVax™ adjuvant.

Pharmaceutical compositions of this invention suitable for parenteral administration 20 comprise a subject composition in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or 25 suspending or thickening agents.

Examples of suitable aqueous and non-aqueous carriers, which may be employed in the pharmaceutical compositions of the invention, include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity may be maintained, for example, by the use of coating materials, such as lecithin, 30 by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

Further, Isd immunogens or Isd antibodies of the present invention may be encapsulated in liposomes and administered via injection. Commercially available liposome delivery systems are available from Novavax, Inc. of Rockville, Md., commercially available under the name Novasomes™. These liposomes are specifically
5 formulated for immunogen or antibody delivery. In an embodiment of the invention, Novasomes™ containing Isd peptides or antibody molecules bound to the surface of these non-phospholipid positively charged liposomes may be used.

The pharmaceutical compositions described herein may be used to prevent or treat conditions or diseases resulting from *S. aureus* infections including, but not limited to a
10 furuncle, chronic furunculosis, impetigo, acute osteomyelitis, pneumonia, endocarditis, scalded skin syndrome, toxic shock syndrome, and food poisoning.

8. Exemplary screening assays for inhibitors of Isd

In general, agents or compounds capable of reducing pathogenic virulence by
15 interfering with iron-regulated surface determinants (Isd) can be identified using the instant disclosed assays to screen large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries. Those skilled in the field of drug discovery and development will understand that the precise source of agents (*e.g.*, test extracts or compounds) is not critical to the screening procedures of the invention. Accordingly,
20 virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such agents, extracts, or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (*e.g.*, semi-synthesis or total
25 synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources,
30 including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, Fla.), and PharmnaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, for example, by standard extraction and fractionation methods. Furthermore, if

desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (*e.g.*, taxonomic dereplication, biological
5 dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-pathogenic activity should be employed whenever possible.

When a crude extract is found to have an anti-pathogenic or anti-virulence activity, or a binding activity, further fractionation of the positive lead extract is necessary to isolate
10 chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having anti-pathogenic activity. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If
15 desired, compounds shown to be useful agents for the treatment of pathogenicity are chemically modified according to methods known in the art.

Potential inhibitors or antagonists of *Isd* encoded polypeptides may include organic molecules, peptides, peptide mimetics, polypeptides, and antibodies that bind to a nucleic acid sequence or polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also include small molecules that bind to and occupy the binding site
20 of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Other potential antagonists include antisense molecules.

Further, *S. aureus* anti-*Isd* antagonists as identified by the screening assays described herein may be administered by various means, depending on their intended use,
25 as described above.

8.1 Interaction Assays

Purified and recombinant *IsdA*, *IsdB*, and *IsdC* polypeptides may be used to develop assays to screen for agents that bind to an *Isd* gene product, and disrupt a protein-protein
30 interaction. Potential inhibitors or antagonists of *IsdA*, *IsdB*, or *IsdC* may include small organic molecules, peptides, polypeptides, peptide mimetics, and antibodies that bind to

either IsdA, IsdB, or IsdC and thereby reduce or extinguish its activity.

In certain embodiments, an agent may be identified that binds to an Isd polypeptide and inhibits the uptake of iron comprising the steps of (i) contacting the Isd polypeptide with an appropriate interacting molecule in the presence of an agent under conditions
5 permitting the interaction between the Isd polypeptide and the interacting molecule in the absence of an agent, and (ii) determining the level of interaction between the Isd polypeptide and the interacting molecule, wherein a different level of interaction between the Isd polypeptide and the interacting molecule in the presence of the agent relative to the absence of the agent indicate that the agent inhibits the interaction between the Isd
10 polypeptide and the interacting molecule.

In another embodiment, an agent may be identified that disrupts the interaction between an Isd polypeptide and an interacting molecule. In an exemplary binding assay, a reaction mixture may be generated to include at least a biologically active portion of either IsdA, IsdB, or IsdC, an agent(s) of interest, and an appropriate interacting molecule. An
15 exemplary interacting molecule may be a hemoprotein, heme, transferrin, fibrinogen or fibronectin. In an exemplary embodiment, the agent of interest is an antibody against a particular Isd polypeptide. Binding of an antibody to an Isd polypeptide may inhibit the function of the Isd polypeptide in binding heme or a hemoprotein. Detection and quantification of an interaction of a particular Isd polypeptide with an appropriate
20 interacting molecule provides a means for determining an agent's efficacy at inhibiting the interaction. The efficacy of the agent can be assessed by generating dose response curves from data obtained using various concentrations of the test agent. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, the interaction of a particular Isd polypeptide with an appropriate interacting molecule may be
25 quantitated in the absence of the test agent.

Interaction between a particular Isd polypeptide and an appropriate interacting molecule may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example, detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled polypeptides, by
30 immunoassay, or by chromatographic detection.

The measurement of the interaction of a particular Isd protein with the appropriate interacting molecule may be observed directly using surface plasmon resonance technology in optical biosensor devices. This method is particularly useful for measuring interactions

with larger (>5 kDa) polypeptides and can be adapted to screen for inhibitors of the protein-protein interaction.

Alternatively, it will be desirable to immobilize a particular Isd polypeptide or the appropriate interacting molecule to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a particular Isd protein to the interacting molecule for example, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/IsdA (GST/IsdA) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with, for example, an ³⁵S-labeled interacting molecule, and the test agent, and the mixture incubated under conditions conducive to complex formation, for example, at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (*e.g.*, beads placed in scintillant), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of interacting molecule found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins and other molecules on matrices are also available for use in the subject assay. For instance, either a particular Isd protein or the appropriate interacting molecule can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated IsdA, IsdB, or IsdC can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with either IsdA, IsdB, or IsdC but which do not interfere with the interaction between the polypeptide and the interacting molecule, can be derivatized to the wells of the plate, and IsdA, IsdB, or IsdC may be trapped in the wells by antibody conjugation. As above, preparations of an interacting molecule and a test compound may be incubated in the polypeptide-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated in the

presence or absence of a test agent. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the interacting molecule or enzyme-linked assays, which rely on detecting an enzymatic activity associated with the
5 interacting molecule.

For example, an enzyme can be chemically conjugated or provided as a fusion protein with the interacting molecule. To illustrate, the interacting molecule can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the
10 enzyme, for example, 3,3'-diamino-benzadine tetrahydrochloride or 4-chloro-1-naphthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig *et al.* (1974) J. Biol. Chem. 249:7130).

15 8.2 Expression assays

In a further embodiment, antagonists of iron uptake may affect the expression of *isdA*, *isdB*, and *isdC* nucleic acid or protein. In this screen, *S. aureus* cells may be treated with a compound(s) of interest, and then assayed for the effect of the compound(s) on *isdA*, *isdB*, and *isdC* nucleic acid or protein expression.

20 In certain embodiments, an agent may be identified that inhibits the expression of an Isd polypeptide in *Staphylococcus aureus* comprising the step of (i) culturing a wild type *Staphylococcus aureus* strain in the presence or absence of said agent; and(ii) comparing the expression of Isd polypeptides wherein a greater reduction in the expression of Isd polypeptides in cells treated with said agent indicates that said agent inhibits the expression
25 of Isd polypeptides in *Staphylococcus aureus*.

In an alternate embodiment, an agent may be identified that inhibits the expression of an *isd* nucleic acid in *Staphylococcus aureus* comprising the step of (i) culturing a wild type *Staphylococcus aureus* strain in the presence or absence of said agent; and (ii) comparing the expression of *isd* nucleic acids wherein a greater reduction in the expression
30 of *isd* nucleic acids in cells treated with said agent indicates that said agent inhibits the expression of *isd* nucleic acids in *Staphylococcus aureus*.

For example, total RNA can be isolated from *S. aureus* cells cultured in the presence or absence of test agents, using any suitable technique such as the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski *et al.* (1987) *Anal. Biochem.* 162:156-159. The expression of *isdA*, *isdB*, or *isdC* may then be assayed by any appropriate method such as Northern blot analysis, the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Northern blot analysis can be performed as described in Harada *et al.* (1990) *Cell* 63:303-312. Briefly, total RNA is prepared from *S. aureus* cells cultured in the presence of a test agent. For the Northern blot, the RNA is denatured in an appropriate buffer (such as glyoxal/dimethyl sulfoxide/sodium phosphate buffer), subjected to agarose gel electrophoresis, and transferred onto a nitrocellulose filter. After the RNAs have been linked to the filter by a UV linker, the filter is prehybridized in a solution containing formamide, SSC, Denhardt's solution, denatured salmon sperm, SDS, and sodium phosphate buffer. A *S. aureus isdA*, *isdB*, or *isdC* DNA sequence may be labeled according to any appropriate method (such as the ³²P-multiprimered DNA labeling system (Amersham)) and used as probe. After hybridization overnight, the filter is washed and exposed to x-ray film. Moreover, a control can also be performed to provide a baseline for comparison. In the control, the expression of *isdA*, *isdB*, or *isdC* in *S. aureus* may be quantitated in the absence of the test agent.

Alternatively, the levels of mRNA encoding IsdA, IsdB, and IsdC polypeptides may also be assayed, for *e.g.*, using the RT-PCR method described in Makino *et al.* (1990) *Technique* 2:295-301. Briefly, this method involves adding total RNA isolated from *S. aureus* cells cultured in the presence of a test agent, in a reaction mixture containing a RT primer and appropriate buffer. After incubating for primer annealing, the mixture can be supplemented with a RT buffer, dNTPs, DTT, RNase inhibitor and reverse transcriptase. After incubation to achieve reverse transcription of the RNA, the RT products are then subject to PCR using labeled primers. Alternatively, rather than labeling the primers, a labeled dNTP can be included in the PCR reaction mixture. PCR amplification can be performed in a DNA thermal cycler according to conventional techniques. After a suitable number of rounds to achieve amplification, the PCR reaction mixture is electrophoresed on a polyacrylamide gel. After drying the gel, the radioactivity of the appropriate bands may be quantified using an imaging analyzer. RT and PCR reaction ingredients and conditions,

reagent and gel concentrations, and labeling methods are well known in the art. Variations on the RT-PCR method will be apparent to the skilled artisan. Other PCR methods that can detect the nucleic acid of the present invention can be found in PCR Primer: A Laboratory Manual (Dieffenbach *et al.* eds., Cold Spring Harbor Lab Press, 1995). A control can also
5 be performed to provide a baseline for comparison. In the control, the expression of *isdA*, *isdB*, or *isdC* in *S. aureus* may be quantitated in the absence of the test agent.

Alternatively, the expression of IsdA, IsdB, and IsdC polypeptides may be quantitated following the treatment of *S. aureus* cells with a test agent using antibody-based methods such as immunoassays. Any suitable immunoassay can be used, including,
10 without limitation, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A
15 immunoassays.

For example, IsdA, IsdB, or IsdC polypeptides can be detected in a sample obtained from *S. aureus* cells treated with a test agent, by means of a two-step sandwich assay. In the first step, a capture reagent (*e.g.*, either a IsdA, IsdB, or IsdC antibody) is used to capture the specific polypeptide. The capture reagent can optionally be immobilized on a
20 solid phase. In the second step, a directly or indirectly labeled detection reagent is used to detect the captured marker. In one embodiment, the detection reagent is an antibody. The amount of IsdA, IsdB, or IsdC, polypeptide present in *S. aureus* cells treated with a test agent can be calculated by reference to the amount present in untreated *S. aureus* cells.

Suitable enzyme labels include, for example, those from the oxidase group, which
25 catalyze the production of hydrogen peroxide by reacting with substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides enzymes, other suitable labels include radioisotopes, such as iodine (^{125}I , ^{121}I), carbon (^{14}C),
30 sulphur (^{35}S), tritium (^3H).

Examples of suitable fluorescent labels include a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, and a fluorescamine label.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase. Examples of chemiluminescent labels include a luminol label, an isoluminol label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, and an aequorin label.

10 **Exemplification**

The invention, having been generally described, may be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention in any way.

15

Example 1: Expression of IsdA, IsdB and IsdC proteins

IsdA, IsdB, and IsdC proteins are expressed under iron-limiting conditions as shown in Figure 4 (*S. aureus* – Fe). The SDS-PAGE gel shown in Figure 4 illustrates that the IsdA, IsdB, and IsdC proteins are three of the most predominant iron regulated proteins expressed by *S. aureus*. These proteins are not expressed when the *S. aureus* cells are cultured in iron-rich media (*S. aureus* + Fe) and are, therefore, by inference likely all highly expressed *in vivo*.

Overexpression of IsdA, IsdB, and IsdC, as well as IsdE, as fusions in *E. coli* results in highly colored lysates. Absorptions and magnetic circular dichroism spectroscopy was used to confirm that this coloration was due to the ability of the proteins to scavenge different forms of protoporphyrin and heme from within the *E. coli* cytoplasm, confirming their role in heme binding.

Example 2: Generation of *isd* gene knockout mutants

Further, the coding regions of *isdA*, *isdB* and *isdC* were interrupted individually to generate strains that contain a single mutation in each of the *isd* genes. The *isdA* coding

region was interrupted by inserting a cassette encoding resistance to tetracycline. The *isdB* coding region was interrupted by inserting a cassette encoding resistance to erythromycin. The *isdC* coding region was interrupted by inserting a cassette encoding resistance to kanamycin. Each mutation was then moved into the same genetic background using phage transduction procedures and selected for using the appropriate resistance as described in Sebulsky *et al.*, (2001) J. Bacteriol. 183:4994-5000. Further, strains containing mutations knocking out two or more of the *isd* genes (*e.g.*, a strain mutated in *isdA*, *isdB*, and *isdC* may also be generated.

10 Example 3: Survival studies in the mouse model of kidney infection

Female Swiss-Webster mice, weighing 25 g, were purchased from Charles River Laboratories Canada, Inc., and housed in microisolator cages. Bacteria were grown overnight in Tryptic Soy Broth (TSB), harvested and washed three times in sterile saline. Pilot experiments demonstrated that *S. aureus* Newman colonized mice better in this model than did RN6390, and that the optimal amount of *S. aureus* Newman to inject into the tail vein to obtain an acute, but non-lethal kidney infection was 1×10^7 CFU. Bacteria, suspended in sterile saline, were administered intravenously via the tail vein. The number of viable bacteria injected were confirmed by plating serial dilutions of the inoculum on TSB. On day six post-injection, mice were sacrificed and kidneys were aseptically removed. Using a PowerGen 700 Homogenizer, kidneys were homogenized for 45 seconds in sterile PBS containing 0.1% Triton X-100 and homogenate dilutions were plated on TSB-agar to enumerate viable bacteria. Data presented are the log CFU recovered per mouse.

Results indicate that mutations in either *IsdA* alone, or in a strain carrying mutations in all of *IsdA*, *IsdB*, and *IsdC* attenuate *S. aureus* virulence using a murine kidney abscess model of *S. aureus* infection. Interestingly, after 6 days post-infection, recovered mutant bacteria are 90% decreased from the numbers recovered from the wildtype, thus indicating that these proteins, when expressed on the bacterial cell surface, play an essential role in the fitness of the bacteria during infection. This also indicates then that inhibition of these proteins *in vivo* could either prevent infection by *Isd*-expressing bacteria (*i.e.*, in the case of an *Isd*-based vaccine) or could result in clearance of the *Isd*-expressing bacteria once infection was initiated.

Example 4: Survival of *S. aureus* under increasing hydrogen peroxide concentration

Isd proteins bound to heme appear to act as an oxidative buffer that protects *S. aureus* cells from the detrimental effects of free radicals. A direct comparison of Newman strains incubated in the presence of heme to Newman strains deleted for IsdA, IsdB, and IsdC incubated in the presence of heme shows that mutant cells were not able to survive increased concentrations of hydrogen peroxide (Figure 6). Thus, mutants lacking the expression of several Isd proteins are more susceptible to challenge with hydrogen peroxide.

Figure 7 shows the expression of IsdA plus and minus heme in both wild type *S. aureus* and *S. aureus isdA::km^c* run on an SDS-PAGE gel stained with (A) Coomassie and (B) TMBZ (tetramethylbenzidine). Catalase activity associated with the heme-bound form of IsdA cleaves the TMBZ compound to yield a colored reaction product. Thus, heme-bound IsdA has catalase activity that may help resist the oxidative killing by phagocytes.

Example 5: Isd Vaccines

A vaccine comprising recombinant IsdA polypeptide can establish protective immunity in mice against systemic and localized *S. aureus* infection. Recombinant IsdA protein may be prepared using standard techniques. Groups of 12-15 Swiss-Webster mice (25 g) can be used for all immunization experiments and injected intraperitoneally (IP). Mice can be boosted with subsequent injections at various different time points. Sera can be monitored over the course of the experiment for anti-IsdA antibody titres. On approximately day 30, mice can be challenged intravenously with 1×10^7 *S. aureus* and monitored for a further 7 days. We have previously shown that injection with this number of live organisms results in non-fatal kidney infections. Mice can be sacrificed at various time points post infection to monitor the number of organisms infecting the kidney tissue. Passive immunization experiments can also be performed using sera collected from previously immunized mice to examine their effectiveness at preventing infection in other groups of mice. Similar immunization experiments can be conducted with IsdB and IsdC polypeptides.

Example 6: IsdA, IsdB, and IsdC Antibodies**A. Preparation of Monoclonal antibodies against full-length Isd proteins**

BALB/c mice can be immunized initially via intraperitoneal injections with full-length recombinant IsdA, IsdB, or IsdC and later boosted similarly with native IsdA, IsdB, or IsdC approximately six weeks later. The mice can be immunized with an appropriate adjuvant. Mouse serum can be obtained approximately ten days after the second injection and then tested for anti-HRP activity via ELISA. The mice whose serum exhibits high levels of anti-HRP activity can be chosen for cell fusion. Spleens can be collected from these mice and cell suspensions prepared by perfusion with Dulbecco's Modified Eagle Medium (DMEM).

Spleen cell suspension containing B-lymphocytes and macrophages can be prepared by perfusion of the spleen. The cell suspension can be washed and collected by centrifugation; myeloma cells can also be washed in this manner. Live cells can be counted and the cells can be placed into a 37°C water bath. One mL of 50% polyethylene glycol (PEG) can be added to DMEM. The Balb/c spleen cells can be fused with SP 2/0-Ag 14 mouse myeloma cells by PEG and the resultant hybridomas can be grown in hypoxanthine (H), aminopterin (A) and thymidine (T) (HAT) selected tissue culture media plus 20% fetal calf serum. The surviving cells can be allowed to grow to confluence. The spent culture medium can be checked for antibody titer, specificity, and affinity. The cells can be incubated in the PEG for one to 1.5 minutes at 37°C, after which the PEG was diluted by the slow addition of DMEM media. The cells can be pelleted and 35 to 40 mL of DMEM containing 10% fetal bovine serum may be added. The cells can then be dispensed into tissue culture plates and incubated overnight in a 37°C, 5% CO₂, humidified incubator.

The next day, DMEM-FCS containing hypoxanthine (H), aminopterin (A) and thymidine (T) medium (HAT medium) can be added to each well. The concentration of HAT in the medium to be added can be twice the final concentration required, i.e., $H_{\text{final}} = 1 \text{ times } 10^{-4}\text{M}$; $A_{\text{final}} = 4 \text{ times } 10^{-7}\text{M}$; and $T_{\text{final}} = 1.6 \text{ times } 10^{-5}\text{M}$.

Subsequently, the plates can be incubated with HAT medium every three to four days for two weeks. Fused cells can be then cultured in DMEM-FCS containing HAT medium. As fused cells become 1/2 to 3/4 confluent on the bottom of the wells, supernatant tissue culture fluid can be taken and tested for IsdA, IsdB, or IsdC specific antibodies by ELISA. Positive wells can be cloned by limiting dilution over macrophage or thymocyte feeder plates, and cultured in DMEM-FCS. Cloned wells can be tested and

recloned three times before a statistically significant monoclonal antibody can be obtained. Spent culture media can be tested from the antibody-producing clones.

B. Preparation of Polyclonal antibodies against full-length Isd proteins

5 Unconjugated purified recombinant IsdA, IsdB and/of IsdC can be used as an antigen to immunize two rabbits. Briefly, 1 mg of recombinant IsdA, IsdB, or IsdC can be resuspended in 1 ml of phosphate buffered saline and emulsified with an equal volume of Complete Freund's Adjuvant and approximately 1 ml (half of the total volume) can be injected into each rabbit intraperitoneally. A second and third immunization can follow two
10 and three weeks later, using Incomplete Freund's Adjuvant. Sera may be tested using enzyme-linked immunosorbent assays (ELISA) to determine recombinant IsdA, IsdB or IsdC specific antibody titers. A nti-recombinant IsdA, IsdB, and/or IsdC c ontaining sera that exhibits high titer based on ELISA results can be purified by affinity chromatography on a Sepharose column conjugated with corresponding recombinant Isd polypeptide. Anti-
15 recombinant IsdA, IsdB, and IsdC immunoglobulin can be tested for the ability to attenuate the virulence of *S. aureus* infection.

Example 7: Expression Assays

Assays to screen for agents that disrupt the expression of IsdA in *S. aureus* can be
20 conducted as follows. Wild type *S. aureus* cells can be cultured overnight in tryptic soy broth (TSB) (Difco) in the presence or a bsence of a test agent. Following 24 hours of culture, the cells can be washed in 1X PBS (phosphate buffered saline) and then lysed at 37°C using 10 µg of lysostaphin in STE (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The cell lysates can then be transferred to anti-IsdA antibody precoated
25 plates and incubated for 45 to 60 minutes at room temperature. As a control, cell lysates from untreated *S. aureus* cells can be used. After three washes with water, a secondary antibody conjugated to either alkaline phosphatase (AP) or horseradish peroxidase (HRP) can be added and incubated for one hour. The plate can then be washed to separate the bound from the free antibody complex. A chemiluminescent substrate (alkaline
30 phosphatase or Super Signal luminol solution from Pierce for horseradish peroxidase) can be used to detect bound antibody. A microplate luminometer can be used to detect the chemiluminescent signal. The a bsence of the signal i n samples of cell lysates obtained

from cells treated with test agent may indicate that the test agent inhibits the expression of IsdA. Similar expression assays may also be conducted for IsdB and IsdC.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis *et al.* U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Antibodies: A Laboratory Manual*, and *Animal Cell Culture* (R. I. Freshney, ed. (1987)), *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Incorporation by Reference

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A vaccine comprising an IsdA (SEQ ID NO: 3) polypeptide and a pharmaceutically acceptable carrier.
2. The vaccine of claim 1, wherein the vaccine is within an injectable formulation.
- 5 3. The vaccine of claim 1, which further comprises an adjuvant.
4. A vaccine comprising an IsdB (SEQ ID NO: 6) polypeptide and a pharmaceutically acceptable carrier.
5. The vaccine of claim 4, wherein the vaccine is within an injectable formulation.
6. The vaccine of claim 4, which further comprises an adjuvant.
- 10 7. A vaccine comprising an IsdC (SEQ ID NO: 9) polypeptide and a pharmaceutically acceptable carrier.
8. The vaccine of claim 7, wherein the vaccine is within an injectable formulation.
9. The vaccine of claim 7, which further comprises an adjuvant.
10. A pharmaceutical composition comprising an effective anti-bacterial amount of an
15 antibody that binds to IsdA (SEQ ID NO: 3) and a pharmaceutically acceptable carrier.
11. A pharmaceutical composition comprising an effective anti-bacterial amount of an antibody that binds to IsdB (SEQ ID NO: 6) and a pharmaceutically acceptable carrier.
- 20 12. A pharmaceutical composition comprising an effective anti-bacterial amount of an antibody that binds to IsdC (SEQ ID NO: 9) and a pharmaceutically acceptable carrier.
13. A pharmaceutical composition comprising a nucleic acid that is antisense to SEQ ID NO: 1, SEQ ID NO: 4, or SEQ ID NO: 7 and a pharmaceutically acceptable carrier.
- 25 14. A pharmaceutical composition comprising an siRNA molecule that comprises a nucleic acid of ID NO: 1, SEQ ID NO: 4, or SEQ ID NO: 7 and a pharmaceutically acceptable carrier.
15. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising

administering to the subject an effective amount of a vaccine in any of claims 1, 4, or 7.

16. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising administering to the subject an effective amount of a pharmaceutical composition of claim 10.
17. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising administering to the subject an effective amount of a pharmaceutical composition of claim 11.
18. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising administering to the subject an effective amount of a pharmaceutical composition of claim 12.
19. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising administering to the subject an effective amount of a pharmaceutical composition of claim 13.
20. A method for treating or preventing a disease or condition that is caused or contributed to by infection of *Staphylococcus aureus* in a subject comprising administering to the subject an effective amount of a pharmaceutical composition of claim 14.
21. A method for identifying an agent that binds to a Isd polypeptide and inhibits the uptake of iron comprising,
 - (i) contacting the Isd polypeptide with an appropriate interacting molecule in the presence of an agent under conditions permitting the interaction between the Isd polypeptide and the interacting molecule in the absence of an agent; and
 - (ii) determining the level of interaction between the Isd polypeptide and the interacting molecule, wherein a different level of interaction between the Isd polypeptide and the interacting molecule in the presence of the agent relative to

the absence of the agent indicate that the agent inhibits the interaction between the Isd polypeptide and the interacting molecule.

22. The method of claim 21, wherein the Isd polypeptide is selected from the group consisting of *Staphylococcus aureus* IsdA, IsdB, and IsdC.

5 23. A method for identifying an agent that inhibits the expression of a polypeptide selected from the group consisting of IsdA, IsdB, and IsdC polypeptide in *Staphylococcus aureus* comprising:

(i) culturing a wild type *Staphylococcus aureus* strain in the presence or absence of said agent; and

10 (ii) comparing the expression of Isd polypeptides wherein a greater reduction in the expression of Isd polypeptides in cells treated with said agent indicates that said agent inhibits the expression of Isd polypeptides in *Staphylococcus aureus*.

24. A method for identifying an agent that inhibits the expression of a nucleic acid selected from the group consisting of an *isdA*, *isdB* and *isdC* nucleic acid in
15 *Staphylococcus aureus* comprising:

(i) culturing a wild type *Staphylococcus aureus* strain in the presence or absence of said agent; and

(ii) comparing the expression of *isd* nucleic acids wherein a greater reduction in the expression of *isd* nucleic acids in cells treated with said agent indicates that said
20 agent inhibits the expression of *isd* nucleic acids in *Staphylococcus aureus*.

Figure 1**(A)**

1 atgacaaaac attattttaa cagtaagtat caatcagaac aacgttcac agctatgaaa
 5 61 aagattacaa tgggtacagc atctatcatt ttaggttccc ttgtatacat aggcgagac
 121 agccaacaag tcaatgcggc aacagaagct acgaacgcaa ctaataatca aagcacacaa
 181 gtttctcaag caacatcaca accaattaat ttccaagtgc aaaaagatgg ctcttcagag
 241 aagtcacaca tggatgacta tatgcaacac cctggtaaag taattaaaca aaataataaa
 301 tattatttcc aaaccgtgtt aaacaatgca tcattctgga aagaatacaa attttacaat
 10 361 gcaaacaatc aagaattagc aacaactgtt gttaacgata ataaaaaagc ggatactaga
 421 acaatcaatg ttgcagttga acctggatat aagagcttaa ctactaaagt acatattgtc
 481 gtgccacaaa ttaattacaa tcatagatat actacgcatt tggaatttga aaaagcaatt
 541 cctacattag ctgacgcagc aaaaccaaac aatgttaaac cggttcaacc aaaaccagct
 601 caacctaaaa cacctactga gcaaactaaa ccagttcaac ctaaagttga aaaagttaa
 15 661 cctactgtaa ctacaacaag caaagttgaa gacaatcact ctactaaagt tgtaagtact
 721 gacacaacaa aagatcaaac taaaacacaa actgctcata cagttaaaac agcacaaact
 781 gctcaagaac aaaataaagt tcaaacacct gttaaagatg ttgcaacagc gaaatctgaa
 841 agcaacaatc aagctgtgaa tgataataaa tcacaacaaa ctaacaaagt taaaaacat
 901 aacgaaacgc ctaacaagc atctaaagct aaagaattac caaaaactgg ttttaacttca
 20 961 gttgataact ttattagcac agttgccttc gcaacacttg cccttttagg ttcattatct
 1021 ttattacttt tcaaaagaaa agaactctaa taa (SEQ ID NO: 1)

(B)

ttatttagattcttttcttttgaaaagtaataaagataatgaacctaaaagggaagtg
 25 tgcaagggaactgtgctaataaagttatcaactgaagttaaaccagtttttggttaattc
 tttagcttttagatgcttggttaggcgtttcggttatgttttgtaactttgtagtttg
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 aacaggtgtttgaactttattttgcttctgagcagtttggtgctgttttaactgtatgagc
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 30 gtcttcaactttgcttggttagttacagtaggtttaactttttcaacttttaggttgaa
 tgggttagtttgctcagtaggtgttttaggttgagctgggttttggttgaaaccggttaac
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 cttatatccaggttcaactgcaacattgattgttctagtatccgcttttttattatcggt
 35 aacaacagttgttgctaattcttgattgtttgcattgtaaaatttgattctttccagaa
 tgatgcattgtttaacacggtttggaaataatatttattttgtttaattactttacc
 aggggtgttgcatatagtcacatcatgtgtgacttctctgaagagccatctttttgcacttg
 gaaattaattgggttgatgttgcttgagaaacttggtgtgctttgattattagttgcgtt
 cgtagcttctgttgccgcattgacttggttggtgtgctgctgcgcctatgtatacaagggaacc
 40 taaaatgatagatgctgtacccattgtaattctttttcatagctgatgaacgttggttctga
 ttgatacttactgtttaataatgttttgat (SEQ ID NO: 2)

(C)

1 MTKHYLNSKY QSEQRSSAMK KITMGTSII LGSLVYIGAD SQQVNAATEA
 45 51 TNATNNQSTQ VSQATSQPIN FQVQKDGSS KSHMDDYMQH PGKVIKQNNK
 101 YYFQTVLNN SFWKEYKFYN ANNQELATTV VNDNKKADTR TINVAVEPGY
 151 KSLTTKVHIV VPQINYNHRY TTHLEFEKAI PTLADAAKPN NVKPVQPKPA
 201 QPKTPTEQTK PVQPKVEKVK PTVTTTSKVE DNHSTKVST DTTKDQTKTQ
 251 TAHTVKTAQT AQEQNKVQTP VKDVATAKSE SNNQAVSDNK SQQTNKVTKH
 50 301 NETPKQASKA KELPKTGLTS VDNFISTVAF ATLALLGSL LLLFKRKESK
 (SEQ ID NO: 3)

Figure 2**(A)**

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 5 61 gttgcatctg tagcgattag tacactttta ttattaatgt caaatggcga agcacaagca
 121 gcagctgaag aaacaggtgg tacaaataca gaagcacaac caaaaactga agcagttgca
 181 agtccaacaa caacatctga aaaagctcca gaaactaaac cagtagctaa tgctgtctca
 241 gtatctaata aagaagttga ggcccctact tctgaaacaa aagaagctaa agaagttaaa
 301 gaagttaaag cccctaagga aacaaaagca gttaaaccag cagcaaaagc cactaacaat
 10 361 acatataccta ttttgaatca ggaacttaga gaagcgatta aaaaccctgc aataaaagat
 421 aaagatcata ggcacacaaa ctctcgtcca attgattttg aaatgaaaaa agaaaatggg
 481 gagcaacaat tttatcatta tgccagctct gttaaaccctg ctagagttat tttcactgat
 541 tcaaaaaccag aaattgaatt aggattacaa tcaggtcaat tttggagaaa atttgaagtt
 601 tatgaaggtg acaaaaagtt gccaatataa ttagtatcat acgatactgt taaagattac
 15 661 gcttacattc gcttctctgt ttcaaatgga acaaaaagccg ttaaaattgt aagttcaact
 721 cacttcaata acaagaaga aaaatacgat tacacattaa tggaattcgc acaaccaatt
 781 tataacagtg cagataaatt caaaactgaa gaagattata aagctgaaaa attattagcg
 841 ccatataaaa aagcgaaaac actagaaaga caagtttatg aattaaataa aattcaagat
 901 aaacttcctg aaaaattaaa ggctgagtac aagaagaaat tagaggatac aaagaaagct
 20 961 ttagatgagc aagtgaatc agctattact gaattccaaa atgtacaacc aacaaatgaa
 1021 aaaatgactg atttacaaga taaaaatat gttgtttatg aaagtgttga gaataacgaa
 1081 tctatgatgg atacttttgt taaacaccct attaaaacag gtatgcttaa cggcaaaaaa
 1141 tatatgggtc tggaaactac taatgacgat tactggaaag atttcatggg tgaagggtcaa
 1201 cgtgttagaa ctataagcaa agatgctaaa aataatacta gaacaattat tttcccatat
 25 1261 gttgaaggta aaactctata tgatgctatc gttaaagttc acgtaaaaac gattgattat
 1321 gatggacaat accatgtcag aatcgttgat aaagaagcat ttacaaaagc caataccgat
 1381 aaatctaaca aaaaagaaca acaagataac tcagctaaga aggaagctac tccagctacg
 1441 cctagcaaac caacaccatc acctgttgaa aaagaatcac aaaaacaaga cagccaaaaa
 1501 gatgacaata aacaattacc aagtgttgaa aaagaaaatg acgcatctag tgagtcaggt
 30 1561 aaagacaaaa cgctgctac aaaaccaact aaagggtgaag tagaatcaag tagtacaact
 1621 ccaactaagg tagtatctac gactcaaaat gttgcaaaac caacaactgc ttcatacaaaa
 1681 acaacaaaag atgttggtca aacttcagca ggttctagcg aagcaaaaaga tagtgctcca
 1741 ttacaaaaag caaacattaa aaacacaaat gatggacaca ctcaaagcca aaacaataaa
 1801 aatacacaag aaaataaagc aaaatcatta ccacaaactg gtgaagaatc aaataaagat
 35 1861 atgacattac cattaatggc attactagct ttaagtagca tcgttgcatt cgtattacct
 1921 agaaaacgta aaaactaa (SEQ ID NO: 4)

(B)

40 ttagtttttacgtttttctaggtaatacgaatgcaacgatgctacttaaagctagtaatgc
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 aaaagtatccatcatagattcggtattctcaacactttcataaacaacataattttgtatc
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5 ttctttgttattgaagtgagttgaacttacaattttaacggcttttgttccatttgaaac
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10 tgggtgcgctatgatctttatcttttattgcagggtttttaatcgcttctcctaagttcctg
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15 acctgttcttcagctgctgcttgcttcgccatttgacattaataaaaagtgtact
aatcgctacagatgcaacgcctagtgatgactttctaattgaataaaatgatttaaatc
tttttgctggtttgttcat (SEQ ID NO: 5)

(C)

20	1	MNKQQKEFKS	FYSIRKSSLG	VASVAISTLL	LLMSNGEAQA	AAEETGGTNT
	51	EAQPKTEAVA	SPTTTSEKAP	ETKPVANAVS	VSNKEVEAPT	SETKEAKEVK
	101	EVKAPKETKA	VKPAAKATNN	TYPILNQELR	EAIKNPAIKD	KDHSAPNSRP
	151	IDFEMKKENG	EQQFYHYASS	VKPARVIFTD	SKPEIELGLQ	SGQFWRKFEV
	201	YEGDKKLPIK	LVSYDTVVDY	AYIRFSVSNG	TKAVKIVSST	HFNNKEEKYD
25	251	YTLMEFAQPI	YNSADKFKTE	EDYKAEKLLA	PYKKAKTLER	QVYELNKIQD
	301	KLPEKLKAEY	KKKLEDTKKA	LDEQVKSALT	EFQNVQPTNE	KMTDLQDTKY
	351	VVYESVENNE	SMMDTFVKHP	IKTGMLNGKK	YVMETTNDD	YWKDFMVEGQ
	401	RVRTISKDAK	NNRTIIFPY	VEGKTLYDAI	VKVHVKTIDY	DGQYHVRIVD
	451	KEAFTKANTD	KSNKKEQQDN	SAKKEATPAT	PSKPTPSPVE	KESQKQDSQK
30	501	DDNKQLPSVE	KENDASSESG	KDKTPATKPT	KGEVESSSTT	PTKVVSTTQN
	551	VAKPTTASSK	TTKDVVQTS	GSSEAKDSAP	LQKANIKNTN	DGHTQSQNNK
	601	NTQENKAKSL	PQTGEESNKD	MTLPLMALLA	LSSIVAFVLP	RKRKN

(SEQ ID NO: 6)

Figure 3**(A)**

5 atgaaaaatatt ttaaaagttt ttaatacaac gatttttagcg ttaattatca tcatcgcgac
attcagtaat tctgcaaagt cgcagatag cggtagcttg aattatgagg ttacaaata
caataccaat gacacgtcaa ttgctaata ctattttaat aaaccggcaa agtacattaa
gaaaaatggg aaattgtatg ttcaaataac tgtcaaccac agtcattgga ttactggaat
gagtatcgaa ggacataaag aaaatattat tagtaaaaac actgccaaag atgaacgcac
10 ttctgaattt gaagtaagta agttgaacgg taaaatagat ggaaaaattg acgtttatat
cgatgaaaaa gtaaatggaa agccattcaa atatgaccat cattacaaca ttacatataa
atttaattgga ccaactgatg tagcagggtgc taatgcacca ggtaaagatg ataaaaattc
tgcttcagggt agtgacaaag gatctgatgg aacgactact ggtcaaagtg aatctaatag
ttcgaataaa gacaaagtag aaaatccaca aacaaatgct ggtacacctg catatatata
tgcaatacca gttgcatcct tagcattatt aatcgcaatc acattgtttg ttagaaaaaa
15 atctaaaggc aatgtggaat aa (SEQ ID NO: 7)

(B)

ttattccacattgccttttagatttttttctaacaacaatgtgattgcgattaataatgc
taaggatgcaactggattgcatatatatgcagggtgtaccagcatttgtttgtggatt
20 ttctactttgtctttattcgaactattagattcactttgaccagtagtcgttccatcaga
tcctttgtcactacctgaagcagaatttttatcatctttacctgggtgcatttagcacctgc
tacatcagttgggtccattaaatttatatgtaatggtgtaatgatgggtcatatttgatgg
ctttccatttactttttcatcgatataaacgtcaatttttccatctattttaccgttcaa
cttacttacttcaaattcagaagtgcgttcatctttggcagtggttttactaataatatt
25 ttctttatgtccttcgatactcattccagtaatccaatgactgtggttgacagttatttg
aacatacaatttaccatttttcttaatgtactttgccgggtttattaaaatagtcattagc
aattgacgtgtcattgggtattgtatttgtaaacctcataattcaaagtaccgctatctgc
ggcatttgcagaattactgaatgtcgcgatgatgataattaacgctaaaatcgttgtatt
aaaaactttttaaataatttttcat (SEQ ID NO: 8)
30

(C)

1 MKNILKVFNT TILALIIIIA TFSNSANAAD SGTLYNEYVK YNTNDTSIAN
51 DYFNKPAKYI KKNGLKLYVQI TVNHSHWITG MSIEGHKENI ISKNTAKDER
101 TSEFEVSKLN GKIDGKIDVY IDEKVNGKPF KYDHHYNITY KFNGPTDVAG
35 151 ANAPGKDDKN SASGSDKGS DTTTGQSESN SSNKDKVENP QTNAGTPAYI
201 YAIPVASLAL LIAITLFVRK KSKGNVE (SEQ ID NO: 9)

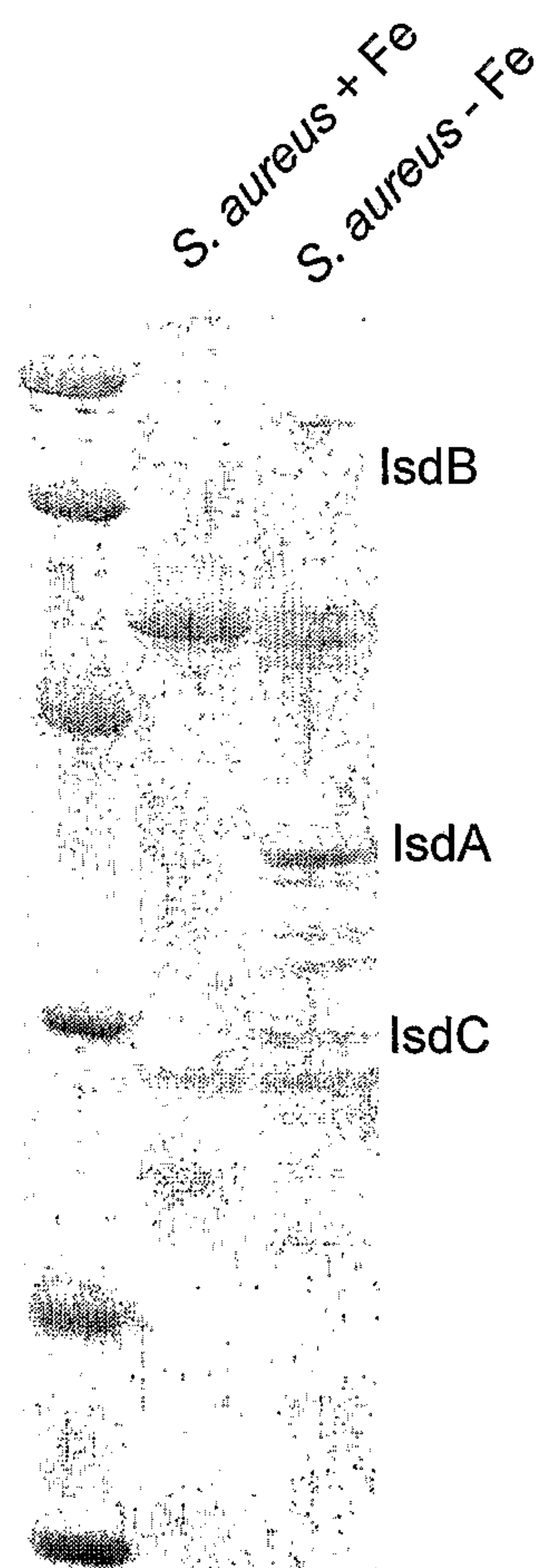
Figure 4

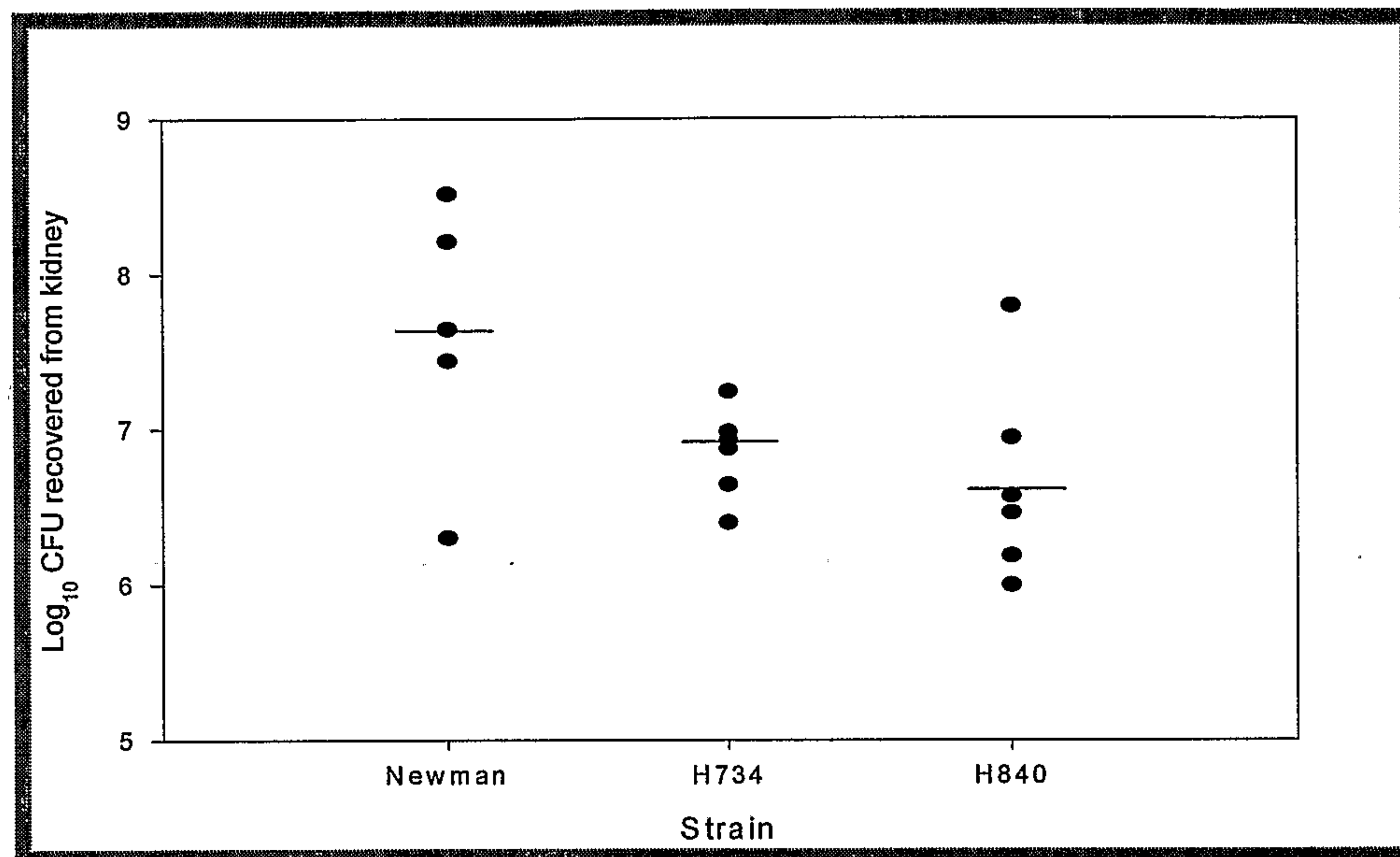
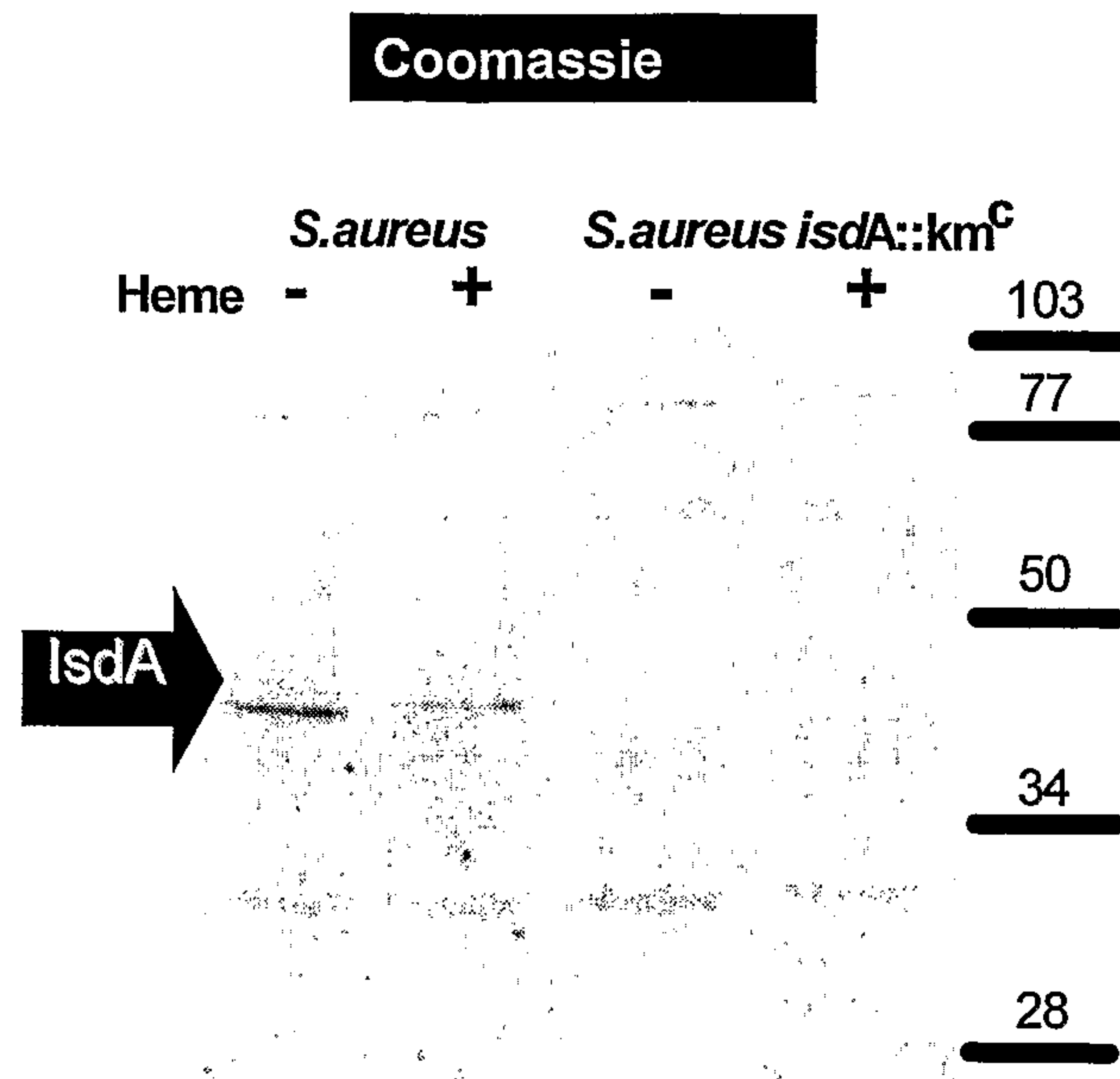
Figure 5

Figure 6

H₂O₂ (μM)	Newman + heme	Newman <i>ΔisdA ΔisdB</i> <i>ΔisdC</i> + heme
1000		
500	✓	
250	✓	
125	✓	
62.5	✓	
31.2	✓	✓
15.6	✓	✓
7.6	✓	✓
3.8	✓	✓
0	✓	✓

Figure 7

(A)



(B)

