CONJUGATION OF *STAPHYLOCOCCUS AUREUS* TYPE 5 AND TYPE 8 CAPSULAR POLYSACCHARIDES

This application claims the benefit of U.S. Provisional Application Serial No. 61/247,518, filed on 30th September 2009, which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

This invention is in the field of conjugating bacterial capsular saccharides, particularly *Staphylococcus aureus* type 5 or type 8 capsular polysaccharides, to carriers in order to form glycoconjugates. The glycoconjugates are useful for immunisation.

BACKGROUND ART

The capsular saccharides of bacteria have been used for many years in vaccines against capsulated bacteria. As saccharides are T-independent antigens, however, they are poorly immunogenic. Conjugation to a carrier can convert T-independent antigens into T-dependent antigens, thereby enhancing memory responses and allowing protective immunity to develop. The most effective saccharide vaccines are therefore based on glycoconjugates, and the prototype conjugate vaccine was against *Haemophilus influenzae* type b ('Hib') [e.g. see chapter 14 of ref. 97].

Another bacterium for which conjugate vaccines have been described is *Staphylococcus aureus* (*S.aureus*). Various polysaccharides have been isolated from *S.aureus* for use in glycoconjugates. Two polysaccharides of particular interest are the type 5 and type 8 capsular polysaccharides. Approximately 60% of human *S.aureus* strains are type 8 and approximately 30% are type 5. Much of the work on type 5 and type 8 conjugates has been performed by Fattom *et al.*, and is described in documents such as references 1 to 9. The Fattom process for type 5 and type 8 polysaccharide conjugation typically involves thiolation of a purified polysaccharide using cystamine. The reaction relies on the presence of carboxylate groups in the capsular polysaccharide. These groups react with cystamine in the presence of a carbodiimide, e.g. EDAC. The derivatised polysaccharide is then conjugated to a carrier protein such as the *Pseudomononas aeruginosa* endotoxin A (ETA), typically via a linker [2]. Other researchers have carried out conjugation of purified type 5 and type 8 capsular polysaccharides by reductive amination [10 and 11]; glutaradehyde coupling [10]; or reaction of hydroxyl groups on the polysaccharides with cyanylating agents like CDAP [12] or cyanuric trichloride [13].

Although conjugate vaccines prepared by the Fattom process have been shown to be safe and immunogenic in humans [5], there remains a need for further and better ways of preparing conjugates of *S.aureus* type 5 or type 8 capsular polysaccharides.

DISCLOSURE OF THE INVENTION

The invention is based on a conjugation method that can be used in place of the conjugation methods disclosed in the prior art. Unlike these methods, the method of the invention does not involve conjugation via hydroxyl or carboxylate groups in the polysaccharide. The method therefore leaves

these groups in a form that is closer than the prior art to the form seen in the native polysaccharide. Instead of using these groups, the method involves the generation of reactive aldehyde groups in the polysaccharide for use in conjugation. The resultant conjugates may have different, preferably improved, immunological properties compared to the conjugates of the prior art.

The invention therefore provides alternative or improved methods for conjugating *S.aureus* type 5 or type 8 capsular polysaccharide to a carrier protein and conjugates obtained therefrom. The invention also provides intermediates that are useful in the methods of the invention and methods for preparing these intermediates.

In a first aspect, the invention provides a process for preparing a conjugate of a *S.aureus* type 5 or type 8 capsular polysaccharide and a carrier molecule, comprising the steps of: (a) depolymerising the capsular polysaccharide, to give a polysaccharide fragment; (b) oxidising the fragment in order to introduce an aldehyde group into at least one saccharide residue in the fragment, to give an oxidised saccharide residue; and (c) coupling the oxidised saccharide residue to a carrier molecule via the aldehyde group, thereby giving the conjugate. The coupling in step (c) may be direct, or it may be via a linker molecule. The invention also provides a conjugate obtained or obtainable by this process.

The capsular polysaccharide

The invention is based on the capsular polysaccharides of *S.aureus* type 5 and type 8. The structures of type 5 and type 8 capsular polysaccharides were described in references 14 and 15 as:

Type 5

→ 4)-β-D-ManNAcA(3OAc)-(1 → 4)-α-L-FucNAc(1 → 3)-β-D-FucNAc-(1 →

Type 8

→ 3)-β-D-ManNAcA(4OAc)-(1 → 3)-α-L-FucNAc(1 → 3)-β-D-FucNAc-(1 →

Recent NMR spectroscopy data [16] has led to a revision of these structures to:

Type 5

→ 4)-β-D-ManNAcA-(1 → 4)-α-L-FucNAc(3OAc)-(1 → 3)-β-D-FucNAc-(1 →

Type 8

→ 3)-β-D-ManNAcA(4OAc)-(1 → 3)-α-L-FucNAc(1 → 3)-α-D-FucNAc(1 →

The polysaccharide may be chemically modified relative to the capsular polysaccharide as found in nature.

For example, the polysaccharide may be de-O-acetylated (partially or fully), de-N-acetylated (partially or fully), N-propionated (partially or fully), *etc.* De-acetylation may occur before, during or after conjugation, but typically occurs before conjugation. Depending on the particular

polysaccharide, de-acetylation may or may not affect immunogenicity *e.g.* the NeisVac-C™ vaccine uses a de-O-acetylated polysaccharide, whereas Menjugate™ is acetylated, but both vaccines are effective. The effect of de-acetylation *etc.* can be assessed by routine assays. For example, the relevance of O-acetylation on *S.aureus* type 5 or type 8 capsular polysaccharides is discussed in reference 6. The native polysaccharides are said in this document to have 75% O-acetylation. These polysaccharides induced antibodies to both the polysaccharide backbone and O-acetyl groups. Polysaccharides with 0% O-acetylation still elicited antibodies to the polysaccharide backbone. Both types of antibody were opsonic against *S.aureus* strains that varied in their O-acetyl content. Accordingly, the type 5 or type 8 capsular polysaccharides used in the present invention may have between 0 and 100% O-acetylation. For example, the degree of O-acetylation of the type 5 capsular polysaccharide may be 10-100%, 10-100%, 20-100%, 30-100%, 40-100%, 50-100%, 60-100%, 70-100%, 80-100%, 90-100%, 50- 90%, 60-90%, 70-90% or 80-90%. Alternatively, 0% O-acetylated type 5 capsular polysaccharide may be used. Similarly, the degree of O-acetylation of the type 8 capsular polysaccharide may be 10-100%, 10-100%, 20-100%, 30-100%, 40-100%, 50-100%, 60-100%, 70-100%, 80-100%, 90-100%, 50- 90%, 60-90%, 70-90% or 80-90%. Alternatively, 0% O-acetylated type 8 capsular polysaccharide may be used. In one embodiment, the degree of O-acetylation of the type 5 and type 8 capsular polysaccharides may be 10-100%, 20-100%, 30-100%, 40-100%, 50-100%, 60-100%, 70-100%, 80-100%, 90-100%, 50-90%, 60-90%, 70-90% or 80-90%. In other embodiments, 0% O-acetylated type 5 and type 8 capsular polysaccharides are used. The degree of N-acetylation of the type 5 capsular polysaccharide used in the invention may be 0-100%, 50-100%, 75-100%, 80-100%, 90-100%, or 95-100%. Typically, the degree of N-acetylation of the type 5 capsular polysaccharide is 100%. Similarly, the degree of N-acetylation of the type 8 capsular polysaccharide used in the invention may be 0-100%, 50-100%, 75-100%, 80-100%, 90-100%, or 95-100%. Typically, the degree of N-acetylation of the type 8 capsular polysaccharide is 100%. In one embodiment, the degree of N-acetylation of the type 5 and type 8 capsular polysaccharides may be 0-100%, 50-100%, 75-100%, 80-100%, 90-100%, or 95-100%. Typically, the degree of N-acetylation of the type 5 and type 8 capsular polysaccharide is 100%.

The degree of O-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (*e.g.* as described in references 17, 18, 19 or 20). A further method is described in reference 21. Similar methods may be used to determine the degree of N-acetylation of the polysaccharide. O-acetyl groups may be removed by hydrolysis, for example by treatment with a base such as anhydrous hydrazine [22] or NaOH [6]. Similar methods may be used to remove N-acetyl groups. To maintain high levels of O-acetylation on type 5 and/or 8 capsular polysaccharides, treatments that lead to hydrolysis of the O-acetyl groups are minimised, *e.g.* treatments at extremes of pH.

Capsular polysaccharides can be purified by known techniques, as described in the references herein. A typical process involves phenol-ethanol inactivation of *S.aureus* cells, centrifugation, lysostaphin

treatment, RNase/DNase treatment, centrifugation, dialysis, protease treatment, further dialysis, filtration, precipitation with ethanol/$CaCl_2$, dialysis, freeze-drying, anion exchange chromatography, dialysis, freeze-drying, size exclusion chromatography, dialysis and freeze-drying [1]. An alternative process involves autoclaving *S.aureus* cells, ultrafiltration of the polysaccharide-containing supernatant, concentration, lyophilisation, treatment with sodium metaperiodate to remove teichoic acid, further ultrafiltration, diafiltration, high performance size exclusion liquid chromatography, dialysis and freeze-drying [23]. Preferably, the purification process described in reference 24 is used.

The invention is not limited to polysaccharides purified from natural sources, however, and the polysaccharides may be obtained by other methods, such as total or partial synthesis.

The carrier molecule

The invention involves the use of carrier molecules, which are typically proteins. In general, covalent conjugation of saccharides to carriers enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens, thus allowing priming for immunological memory. Conjugation is particularly useful for paediatric vaccines [*e.g.* ref. 25] and is a well known technique [*e.g.* reviewed in refs. 26 to 34].

Preferred carrier proteins are bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants thereof. The inventors have found that the CRM197 diphtheria toxin mutant [35] is suitable. *Pseudomonas aeruginosa* exotoxin A (ETA) and its non-toxic mutant recombinant exoprotein A (rEPA) have been used as carrier proteins for *S.aureus* type 5 or type 8 capsular polysaccharides ([1] and [2]). *S.aureus* α-haemolysin (α-toxin) ([10] and [36]), ovalbumin [13] and human serum albumin [11] have also been used. These carriers may be used in the present invention.

Other suitable carrier proteins include the *N.meningitidis* outer membrane protein complex [37], synthetic peptides [38,39], heat shock proteins [40,41], pertussis proteins [42,43], cytokines [44], lymphokines [44], hormones [44], growth factors [44], human serum albumin (typically recombinant), artificial proteins comprising multiple human $CD4^+$ T cell epitopes from various pathogen-derived antigens [45] such as N19 [46], protein D from *H.influenzae* [47-49], pneumococcal surface protein PspA [50], pneumolysin [51] or its non-toxic derivatives [52], iron-uptake proteins [53], toxin A or B from *C.difficile* [54], a GBS protein [55], a GAS protein [56] *etc.*

Other suitable carrier proteins include *S.aureus* protein antigens, for example the *S.aureus* protein antigens set out below.

It is possible to use more than one carrier protein *e.g.* to reduce the risk of carrier suppression. Thus different carrier proteins can be used for the type 5 and type 8 capsular polysaccharides, *e.g.* type 5 polysaccharide might be conjugated to CRM197 while type 8 polysaccharide might be conjugated to rEPA. It is also possible to use more than one carrier protein for a particular polysaccharide antigen

e.g. type 5 polysaccharide might be in two groups, with one group conjugated to *CRM197* and the other conjugated to rEPA. Typically, however, the same carrier protein is used for all polysaccharides.

A single carrier protein might carry more than one polysaccharide antigen [57,58]. For example, a single carrier protein might have conjugated to it type 5 and type 8 capsular polysaccharides. To achieve this goal, different polysaccharides can be mixed prior to the conjugation process. Typically, however, there are separate conjugates for each polysaccharide, with the different polysaccharides being mixed after conjugation. The separate conjugates may be based on the same carrier.

Depolymerisation

In step (a) of the process of the invention, the capsular polysaccharide is depolymerised to give a polysaccharide fragment. Depolymerisation of the type 8 capsular polysaccharide by sonication prior to conjugation has been reported [3]. The authors concluded that low molecular weight type 8 was not immunogenic. Although these authors therefore favoured high molecular weight polysaccharides, the present invention surprisingly makes use of polysaccharide fragments with a lower molecular weight than native capsular polysaccharides.

Full-length polysaccharides may be depolymerised to give shorter fragments for use in the invention by various methods. The inventors have found that methods that result in cleavage of (1 → 3) glycosidic linkages between the α-L-FucNAc(3OAc) and β-D-FucNAc residues in the type 5 capsular polysaccharide are particularly suitable. When these methods are applied to the type 5 capsular polysaccharide, they result in a polysaccharide fragment having a β-D-FucNAc-(1 → moiety at its non-reducing terminus. This moiety includes two vicinal hydroxyl groups. Similarly, when these methods are applied to the type 8 capsular polysaccharide, they are thought to result in a polysaccharide fragment having an α-D-FucNAc-(1 → moiety at is non-reducing terminus, which moiety also includes two vicinal hydroxyl groups. The vicinal hydroxyl groups in the type 5 or type 8 polysaccharide fragment provide a handle for subsequent conjugation of the fragment to a carrier molecule, as described below.

Accordingly, in a further aspect the invention provides a process for treating a *S.aureus* type 5 capsular polysaccharide comprising the step of depolymerising the capsular polysaccharide, to give a polysaccharide fragment having a β-D-FucNAc-(1 → moiety at its non-reducing terminus. In a related aspect, the invention provides a process for treating a *S.aureus* type 8 capsular polysaccharide comprising the step of depolymerising the capsular polysaccharide, to give a polysaccharide fragment having an α-D-FucNAc-(1 → moiety at its non-reducing terminus. The capsular polysaccharide may be a *S.aureus* type 5 or type 8 capsular polysaccharide as described in "*The capsular polysaccharide*" above. The invention also provides a polysaccharide fragment obtained or obtainable by either of these processes.

The inventors have found that the depolymerisation may be carried out by acid hydrolysis. For acid hydrolysis, it is preferred to use a mild acid, *e.g.* acetic acid, to avoid side-reactions at other groups within the polysaccharide. The skilled person would be capable of identifying suitable acids and conditions (*e.g.* of concentration, temperature and/or time) for hydrolysis. For example, the inventors have found that treatment of polysaccharide at 2mg/ml with 2% acetic acid (v/v) at 90°C for 3 hours is suitable. The inventors have also found that treatment at 2mg/ml with 5% acetic acid at 90°C for 30 minutes, 5 or 6 hours is suitable. Treatment with other acids, *e.g.* trifluoroacetic or other organic acids, may also be suitable. In particular, the inventors have found that depolymerisation efficiency may be increased, particularly for type 8 capsular polysaccharide, by using hydrochloric acid. For example, the inventors have found that treatment of polysaccharide with 2M hydrochloric acid at 100°C for 30 minutes is suitable. The inventors have also found that treatment with 2M hydrochloric acid at 100°C for 1, 1.5, 2 or 2.5 hours is suitable. Such treatment with hydrochloric acid may result in de-O-acetylation of the polysaccharide, *e.g.* as described below.

Other methods for depolymerisation of the polysaccharide may be suitable. These methods include heating, microfluidisation [59], sonic radiation [3], oxidation-reduction [60] or ozonolysis [61].

Polysaccharide fragments can be identified by chromatography, *e.g.* size exclusion chromatography. Specific molecular masses can be measured by gel filtration relative to pullulan standards, such as those available from Polymer Standard Service [62]. Typically, the fragment of the invention is a mixture of fragments with masses within a range of values. For the depolymerised type 5 capsular polysaccharide, the molecular mass of the fragment typically varies between 1-500 kDa, *e.g.* between 5 and 100 kDa, particularly between 10 and 50 kDa and more particularly between 20 and 30 kDa. Similarly, for the depolymerised type 8 capsular polysaccharide, the molecular mass of the fragment may vary between 1-500 kDa, *e.g.* between 5 and 100 kDa, particularly between 10 and 50 kDa and more particularly between 20 and 30 kDa. In some embodiments, low molecular weight type 5 and/or type 8 polysaccharide fragments are selected for use in the invention. For example, gel filtration fractions corresponding to low molecular weight fragments may be selected and pooled. The low molecular weight polysaccharide fragments typically have a molecular mass that varies between 5 and 20 kDa.

The depolymerisation may result in a change to the degree of O-acetylation of the capsular polysaccharide. For the example, the inventors have found that acid hydrolysis may result in a decrease in the degree of O-acetylation. In some embodiments, the degree of O-acetylation of the fragment may be 10-90%, 20-70%, 30-50%, particularly 35-45%. In other embodiments, the degree of O-acetylation of the fragment may be 0-10%, 0-5%, 0-2%, particularly 0%.

Introduction of an aldehyde group

In step (b) of the process, the fragment is oxidised in order to introduce an aldehyde group into at least one saccharide residue in the fragment. This step may involve the introduction of more than one aldehyde group into the saccharide residue. In particular, two aldehyde groups may be

introduced. For example, when the depolymerisation in step (a) results in a type 5 polysaccharide fragment having a β-D-FucNAc-(1 → moiety at its non-reducing terminus, the two vicinal hydroxyl groups in this moiety may be oxidised in order to introduce two aldehyde groups into the moiety. In this way, the β-D-FucNAc-(1 → moiety may be the saccharide residue of step (b). Similarly, when the depolymerisation results in a type 8 polysaccharide fragment having an α-D-FucNAc-(1 → moiety at is non-reducing terminus, the two vicinal hydroxyl groups in this moiety may be oxidised to introduce two aldehyde groups. In this way, the α-D-FucNAc-(1 → moiety may be the saccharide residue of step (b).

Accordingly, in a further aspect the invention provides a process for providing a *S.aureus* type 5 capsular polysaccharide derivative comprising the step of oxidising a *S.aureus* type 5 capsular polysaccharide having a β-D-FucNAc-(1 → moiety at its non-reducing terminus to convert two vicinal hydroxyl groups in the β-D-FucNAc-(1 → moiety into two aldehyde groups. In a related aspect, the invention provides a process for providing a *S.aureus* type 8 capsular polysaccharide derivative comprising the step of oxidising a *S.aureus* type 8 capsular polysaccharide having an α-D-FucNAc-(1 → moiety at is non-reducing terminus to convert two vicinal hydroxyl groups in the α-D-FucNAc-(1 → moiety into two aldehyde groups. The capsular polysaccharide may be a polysaccharide fragment as described in "*Depolymerisation*" above. The invention also provides a *S.aureus* capsular polysaccharide derivative obtained or obtainable by either of these processes.

Typical reactions to produce aldehydes include the use of periodate salts, and particularly meta-periodates (*e.g.* sodium or potassium meta-periodate *e.g.* $NaIO_4$), to oxidise vicinal hydroxyl groups [63]. The skilled person would be capable of identifying suitable conditions for oxidation. For example, the inventors have found that treatment of polysaccharide at 2mg/ml with $NaIO_4$ at a 1:1 ratio (w/w) at room temperature for 1-2 hours in the dark is suitable. The inventors have also found that treatment of polysaccharide at 2mg/ml with 93mM $NaIO_4$ at room temperature for 8 hours in the dark is suitable. Other oxidation conditions can be used, *e.g.* use of osmium tetroxide, *etc.*

Coupling to a carrier molecule

The coupling of the oxidised saccharide residue to the carrier molecule in step (c) of the process may be direct or via a linker. Any suitable conjugation reaction can be used, with any suitable linker if desired.

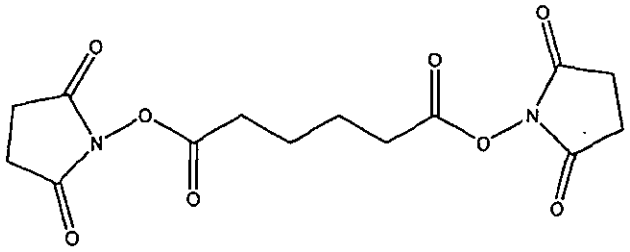
When the oxidation in step (b) results in a type 5 polysaccharide fragment having a β-D-FucNAc-(1 → moiety at its non-reducing terminus in which two aldehyde groups have been introduced into the moiety, the coupling in step (c) may be via one of these aldehyde groups. In this way, the oxidised β-D-FucNAc-(1 → moiety may be the oxidised saccharide residue of step (c). Similarly, when the oxidation results in a type 8 polysaccharide fragment having an α-D-FucNAc-(1 → moiety at is non-reducing terminus in which two aldehyde groups have been introduced into the moiety, the coupling in step (c) may be via one of these aldehyde groups. In this way, the oxidised α-D-FucNAc-(1 → moiety may be the oxidised saccharide residue of step (c).

Accordingly, in a further aspect the invention provides a process for providing a conjugated *S.aureus* type 5 capsular polysaccharide comprising the step of coupling to a carrier molecule a *S.aureus* type 5 capsular polysaccharide having a β-D-FucNAc-(1 → moiety at its non-reducing terminus that has been oxidised to convert two vicinal hydroxyl groups into two aldehyde groups, wherein the coupling is via one of the aldehyde groups. In a related aspect, the invention provides a process for providing a conjugated *S.aureus* type 8 capsular polysaccharide comprising the step of coupling to a carrier molecule a *S.aureus* type 8 capsular polysaccharide having an α-D-FucNAc-(1 → moiety at is non-reducing terminus that has been oxidised to convert two vicinal hydroxyl groups into two aldehyde groups, wherein the coupling is via one of the aldehyde groups. The capsular polysaccharide may be a capsular polysaccharide as described in "*Introduction of an aldehyde group*" above. The carrier molecule may be a carrier as described in "*The carrier molecule*" above. The invention also provides a conjugated capsular polysaccharide obtained or obtainable by either of these processes.

Attachment of the oxidised saccharide residue or linker to the carrier is typically via an amine ($-NH_2$) group *e.g.* in the side chain of a lysine or residue in a carrier protein, or of an arginine residue. Attachment to the carrier may also be via a sulphydryl (-SH) group *e.g.* in the side chain of a cysteine residue. The inventors have found that direct coupling may be conveniently achieved by reacting an aldehyde group in the oxidised saccharide residue with an amine group in the carrier by reductive amination. Direct coupling of this nature is therefore preferred in the present invention. In contrast, reference 2 suggests that linkers may be advantageous in *S.aureus* type 5 and 8 conjugates. If desired, coupling via a linker may be used in the present invention, *e.g.* by reacting an aldehyde group in the oxidised saccharide residue with an amine group in the linker by reductive amination, or by converting the aldehyde group into an amine group by reductive amination to provide an amine group for attachment of the linker.

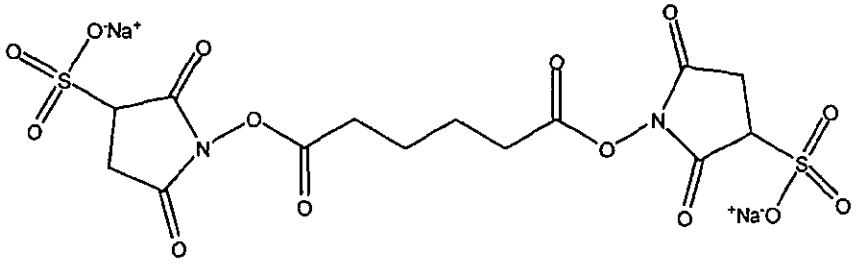
Reductive amination is a standard technique in organic chemistry, and has been used extensively in the production of conjugates of capsular polysaccharides for vaccine use, including *S.aureus* capsular polysaccharides [10]. In one embodiment, an aldehyde group in the oxidised saccharide residue reacts with an amine group in the carrier or linker. This can conveniently be achieved by combining the polysaccharide with the carrier or linker in the presence of an appropriate reducing agent (*e.g.* cyanoborohydrides, such as sodium cyanoborohydride $NaBH_3CN$; borane-pyridine; sodium triacetoxyborohydride; borohydride exchange resin; *etc.*). In another embodiment, an aldehyde group is converted into an amine group by reductive amination to provide an amine group for attachment of the linker. The reductive amination involves either ammonia or a primary amine (NH_2R). This can conveniently be achieved by using an ammonium salt (*e.g.* ammonium chloride) in combination with an appropriate reducing agent (*e.g. as listed above*). The skilled person would be capable of identifying suitable conditions for reductive amination. For example, the inventors have found that treatment of polysaccharide at 10mg/ml with carrier protein at a 4:1 polysaccharide:protein ratio (w/w) and $NaBH_3CN$ at a 2:1 polysaccharide:$NaBH_3CN$ ratio is suitable.

Coupling via a linker group may be made using any known procedure, *e.g.* the reductive amination procedures described above. In one embodiment, a bifunctional linker may be used to provide a first group for coupling to the aldehyde group in the oxidised saccharide residue and a second group for coupling to the carrier. For example, a bifunctional linker of the formula X_1-L-X_2 may be used, where X_1 can react with the aldehyde; X_2 can react with the carrier; and L is a linking moiety in the linker. A typical X_1 group is an amine group. Typical L groups are straight chain alkyls with 1 to 10 carbon atoms (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}) *e.g.* -$(CH_2)_4$- or -$(CH_2)_3$-. In another embodiment, a bifunctional linker may be used to provide a first group for coupling to an amine group derived from the aldehyde group in the oxidised saccharide residue (*e.g.* by reductive amination as described above) and a second group for coupling to the carrier (typically for coupling to an amine in the carrier). For example, a homobifunctional linker of the formula X-L-X may be used, where the two X groups are the same as each other and can react with the amines; and where L is a linking moiety in the linker. A typical X group is N-oxysuccinimide. L typically has formula -L'-L^2-L'-, where L' is carbonyl. Typical L^2 groups are straight chain alkyls with 1 to 10 carbon atoms (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}) *e.g.* -$(CH_2)_4$-. A typical linker is thus adipic acid N-hydroxysuccinimide diester (SIDEA):



Other X groups are those which form esters when combined with HO-L-OH, such as norborane, p-nitrobenzoic acid, and sulfo-N-hydroxysuccinimide. Further bifunctional linkers reactive with amines for use with the invention include acryloyl halides (*e.g.* chloride) [65], haloacylhalides [66], disuccinimidyl glutarate, disuccinimidyl suberate, ethylene glycol bis[succinimidylsuccinate], *etc.*

The linker will generally be added in molar excess to the polysaccharide. The linker/polysaccharide reaction will generally take place in an aprotic solvent (*e.g.* DMSO, ethanol acetate, *etc.*), as the linkers are typically insoluble in water. Where water-soluble linkers are used, however, then a wider range of solvents is available, including protic solvents such as water. Suitable linkers include sulphonated forms, such as sulphonated SIDEA:



When a linker is used, the conjugate will comprise a linker moiety. This moiety originates neither in the polysaccharide nor the carrier, but is a third molecule used during conjugate preparation, and can readily be distinguished from both the polysaccharide and carrier protein in a final conjugate product. The linker moiety may include atoms such as carbon, hydrogen, oxygen and/or nitrogen. Linkers that comprise carbon and hydrogen are typical, and linkers that further comprise oxygen and/or nitrogen are also typically used. Linkers that include nitrogen atoms may include a carbon atom bonded to a nitrogen atom, which in turn is bonded to a second carbon atom (–C–N–C–). Linkers that include an oxygen atom typically include it as part of a carbonyl group. Linker moieties with a molecular weight of between 30-500 Da are typical. Linkers containing two carbonyl groups are also typical.

A particularly useful linker moiety is $-NH-C(O)-(CH_2)_n-C(O)-$, wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. The value of n is typically 4. The terminal –NH– in this linker is usually attached to a carbon atom from the polysaccharide moiety. The terminal –C(O)– is usually attached to a nitrogen atom in an amino acid side chain in the carrier. A preferred linker moiety can conveniently be introduced by a process involving: reductive amination of the aldehyde in the oxidised saccharide residue; reaction of the resulting $-NH_2$ group with a bifunctional linker that is a diester (*e.g.* a disuccinimidyl ester) of a dioic acid (*e.g.* of adipic acid, $HOOC-(CH_2)_4-COOH$); and reductive amination of the product (see Figure 6 [64]).

Other chemistries that can be used to attach a linker to a $-NH_2$ group in the polysaccharide, include:

- acryloylation (*e.g.* by reaction with acryloyl chloride), followed by Michael-type addition to either the ε-NH_2 of an amino acid side chain or to a –SH of a cysteine side chain [65]. The resulting linker is $-NH-C(O)-(CH_2)_2-$ (propionamido).
- reaction with a haloacylhalide, followed by reaction with the ε-NH_2 of an amino acid side chain or to a -SH of a cysteine side chain [66]. The linker is $-NH-C(O)-CH_2-$.

Conjugates with a polysaccharide:protein ratio (w/w) of between 1:20 (*i.e.* excess protein) and 20 :1 (*i.e.* excess polysaccharide) are typically produced by the method of the invention. Ratios of 1:10 to 1:1 are preferred, particularly ratios between 1:5 and 1:2 and, most preferably, about 1:3. In contrast, type 5 and type 8 capsular polysaccharide conjugates made by processes of the prior art tend to have higher ratios, *e.g.* between 0.73:1 and 1.08:1 in references 1, 2 and 3. In particular embodiments of the invention, the polysaccharide:protein ratio (w/w) for type 5 capsular polysaccharide conjugate is between 1:10 and 1:2; and/or the polysaccharide:protein ratio (w/w) for type 8 capsular polysaccharide conjugate is between 1:5 and 7:10.

Compositions may include a small amount of free carrier [67]. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% by weight.

After conjugation, free and conjugated polysaccharides can be separated. There are many suitable methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 68 & 69, *etc.*].

Combinations of conjugates and other antigens

As well as providing individual conjugates as described above, the invention provides a composition comprising a conjugate of the invention and one or more further antigens. The composition is typically an immunogenic composition.

The further antigen(s) may comprise further conjugates of the invention, and so the invention provides a composition comprising more than one conjugate of the invention. In particular, the present invention provides a composition comprising a type 5 capsular polysaccharide conjugate of the invention and a type 8 capsular polysaccharide conjugate of the invention. Alternatively, the further antigen(s) may be type 5 or type 8 capsular polysaccharide conjugates prepared by methods other than those of the invention, *e.g.* the methods of references 1 to 13 above. The further antigen(s) may similarly be type 5 or type 8 capsular polysaccharide conjugates prepared by the methods of references 59, 70, 71, 72, 73, and 74, and particularly the exemplified methods in those documents. Accordingly, the invention provides a composition comprising a type 5 capsular polysaccharide conjugate and a type 8 capsular polysaccharide conjugate, wherein one of the conjugates (the type 5 conjugate or the type 8 conjugate) is a conjugate of the invention and the other conjugate is not a conjugate of the invention.

The further antigen(s) may comprise other *S.aureus* antigens, including protein and saccharide antigens, as set out below.

The further antigen(s) may comprise antigens from non-*S.aureus* pathogens. Thus the compositions of the invention may further comprise one or more non-*S.aureus* antigens, including additional bacterial, viral or parasitic antigens. These may be selected from the following:

- a protein antigen from *N.meningitidis* serogroup B, such as those in refs. 75 to 81, with protein '287' (see below) and derivatives (*e.g.* 'ΔG287') being particularly useful.
- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in refs. 82, 83, 84, 85 *etc.*
- a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. 86 from serogroup C or the oligosaccharides of ref. 87.
- a saccharide antigen from *Streptococcus pneumoniae* [*e.g.* refs. 88-90; chapters 22 & 23 of ref. 97].
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 91, 92; chapter 15 of ref. 97].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 92,93; chpater 16 of ref. 97].
- an antigen from hepatitis C virus [*e.g.* 94].

- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 95 & 96; chapter 21 of ref. 97].
- a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 13 of ref. 97].
- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 27 of ref. 97].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* chapter 14 of ref. 97]
- an antigen from *N.gonorrhoeae* [*e.g.* 75, 76, 77].
- an antigen from *Chlamydia pneumoniae* [*e.g.* 98, 99, 100, 101, 102, 103, 104].
- an antigen from *Chlamydia trachomatis* [*e.g.* 105].
- an antigen from *Porphyromonas gingivalis* [*e.g.* 106].
- polio antigen(s) [*e.g.* 107, 108; chapter 24 of ref. 97] such as IPV.
- rabies antigen(s) [*e.g.* 109] such as lyophilised inactivated virus [*e.g.*110, RabAvert™].
- measles, mumps and/or rubella antigens [*e.g.* chapters 19, 20 and 26 of ref. 97].
- influenza antigen(s) [*e.g.* chapters 17 & 18 of ref. 97], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 111].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 112, 113, 114].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.*56, 115-117].
- an antigen from *S.epidermidis* [*e.g.* type I, II and/or III capsular polysaccharide obtainable from strains ATCC-31432, SE-360 and SE-10 as described in refs. 118, 119 and 120.

Where a saccharide or carbohydrate antigen is used, it is typically conjugated to a carrier in order to enhance immunogenicity. Conjugation of *H.influenzae* B, meningococcal and pneumococcal saccharide antigens is well known.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [96]).

Where a diphtheria antigen is included in the composition it is typical also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is typical also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is typical also to include diphtheria and tetanus antigens.

Antigens may be adsorbed to an aluminium salt.

One type of preferred composition includes further antigens that affect the immunocompromised, and so the *S.aureus* conjugates of the invention can be combined with one or more antigens from the following non-*S.aureus* pathogens: *Steptococcus agalactiae*, *Staphylococcus epidermis*, influenza virus, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Listeria monocytogenes*, *Neisseria meningitidis*, and parainfluenza virus.

Another type of preferred composition includes further antigens from bacteria associated with nosocomial infections, and so the *S.aureus* conjugates of the invention can be combined with one or more antigens from the following non-*S.aureus* pathogens: *Clostridium difficile; Pseudomonas aeruginosa; Candida albicans;* and extraintestinal pathogenic *Escherichia coli.*

Antigens in the composition will typically be present at a concentration of at least 1μg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

As an alternative to using proteins antigens in the composition of the invention, nucleic acid encoding the antigen may be used [*e.g.* refs. 121 to 129]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (usually DNA *e.g.* in the form of a plasmid) that encodes the protein.

In practical terms, there may be an upper limit to the number of antigens included in compositions of the invention. The number of antigens (including *S.aureus* antigens) in a composition of the invention may be less than 20, less than 19, less than 18, less than 17, less than 16, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, or less than 3. The number of *S.aureus* antigens in a composition of the invention may be less than 6, less than 5, or less than 4.

Pharmaceutical compositions and methods

The invention provides a pharmaceutical composition comprising (a) a conjugate of the invention and (b) a pharmaceutically acceptable carrier. Typical 'pharmaceutically acceptable carriers' include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, sucrose [130], trehalose [131], lactose, and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. Sterile pyrogen-free, phosphate-buffered physiologic saline is a typical carrier. A thorough discussion of pharmaceutically acceptable excipients is available in reference 132.

Compositions of the invention may be in aqueous form (*i.e.* solutions or suspensions) or in a dried form (*e.g.* lyophilised). If a dried vaccine is used then it will be reconstituted into a liquid medium prior to injection. Lyophilisation of conjugate vaccines is known in the art *e.g.* the Menjugate™ product is presented in lyophilised form, whereas NeisVac-C™ and Meningitec™ are presented in aqueous form. To stabilise conjugates during lyophilisation, it may be typical to include a sugar alcohol (*e.g.* mannitol) or a disaccharide (*e.g.* sucrose or trehalose) *e.g.* at between 1mg/ml and 30mg/ml (*e.g.* about 25 mg/ml) in the composition.

Compositions may be presented in vials, or they may be presented in ready-filled syringes. The syringes may be supplied with or without needles. A syringe will include a single dose of the composition, whereas a vial may include a single dose or multiple doses.

Aqueous compositions of the invention are also suitable for reconstituting other vaccines from a lyophilised form. Where a composition of the invention is to be used for such extemporaneous reconstitution, the invention provides a kit, which may comprise two vials, or may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

Compositions of the invention may be packaged in unit dose form or in multiple dose form. For multiple dose forms, vials are preferred to pre-filled syringes. Effective dosage volumes can be routinely established, but a typical human dose of the composition has a volume of 0.5ml *e.g.* for intramuscular injection.

The pH of the composition is typically between 6 and 8, *e.g.* about 7. Stable pH may be maintained by the use of a buffer. If a composition comprises an aluminium hydroxide salt, it is typical to use a histidine buffer [133]. The composition may be sterile and/or pyrogen-free. Compositions of the invention may be isotonic with respect to humans.

Compositions of the invention are immunogenic, and are more preferably vaccine compositions. Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic. Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Within each dose, the quantity of an individual saccharide antigen will generally be between 1-50 μg (measured as mass of saccharide) *e.g.* about 1μg, about 2.5μg, about 4μg, about 5μg, or about 10μg.

S.aureus affects various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration *e.g.* as spray, drops, gel or powder [*e.g.* refs 134 & 135]. Success with nasal administration of pneumococcal

saccharides [136,137],Hib saccharides [138], MenC saccharides [139], and mixtures of Hib and MenC saccharide conjugates [140] has been reported.

Compositions of the invention may include an antimicrobial, particularly when packaged in multiple dose format.

Compositions of the invention may comprise detergent *e.g.* a Tween (polysorbate), such as Tween 80. Detergents are generally present at low levels *e.g.* <0.01%.

Compositions of the invention may include sodium salts (*e.g.* sodium chloride) to give tonicity. A concentration of 10±2mg/ml NaCl is typical.

Compositions of the invention will generally include a buffer. A phosphate buffer is typical.

Compositions of the invention will generally be administered in conjunction with other immunoregulatory agents. In particular, compositions will usually include one or more adjuvants. Such adjuvants include, but are not limited to:

A. Mineral-containing compositions

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts. The invention includes mineral salts such as hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates), sulphates, *etc.* [*e.g.* see chapters 8 & 9 of ref. 141], or mixtures of different mineral compounds (*e.g.* a mixture of a phosphate and a hydroxide adjuvant, optionally with an excess of the phosphate), with the compounds taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*), and with adsorption to the salt(s) being typical. The mineral containing compositions may also be formulated as a particle of metal salt [142].

Aluminum salts may be included in vaccines of the invention such that the dose of Al^{3+} is between 0.2 and 1.0 mg per dose.

A typical aluminium phosphate adjuvant is amorphous aluminium hydroxyphosphate with PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al^{3+}/ml. Adsorption with a low dose of aluminium phosphate may be used *e.g.* between 50 and 100μg Al^{3+} per conjugate per dose. Where an aluminium phosphate it used and it is desired not to adsorb an antigen to the adjuvant, this is favoured by including free phosphate ions in solution (*e.g.* by the use of a phosphate buffer).

B. Oil Emulsions

Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [Chapter 10 of ref. 141; see also refs. 143-145]. MF59 is used as the adjuvant in the FLUAD™ influenza virus trivalent subunit vaccine.

Particularly useful adjuvants for use in the compositions are submicron oil-in-water emulsions. Preferred submicron oil-in-water emulsions for use herein are squalene/water emulsions optionally

containing varying amounts of MTP-PE, such as a submicron oil-in-water emulsion containing 4-5% w/v squalene, 0.25-1.0% w/v Tween 80 (polyoxyelthylenesorbitan monooleate), and/or 0.25-1.0% Span 85 (sorbitan trioleate), and, optionally, N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphophoryloxy)-ethylamine (MTP-PE). Submicron oil-in-water emulsions, methods of making the same and immunostimulating agents, such as muramyl peptides, for use in the compositions, are described in detail in references 143 & 146-147.

Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used as adjuvants in the invention.

C. Saponin formulations [chapter 22 of ref. 141]

Saponin formulations may also be used as adjuvants in the invention. Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponins isolated from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsaprilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs.

Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 148. Saponin formulations may also comprise a sterol, such as cholesterol [149].

Combinations of saponins and cholesterols can be used to form unique particles called immunostimulating complexs (ISCOMs) [chapter 23 of ref. 141]. ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA and QHC. ISCOMs are further described in refs. 149-151. Optionally, the ISCOMS may be devoid of additional detergent(s) [152].

A review of the development of saponin based adjuvants can be found in refs. 153 & 154.

D. Virosomes and virus-like particles

Virosomes and virus-like particles (VLPs) can also be used as adjuvants in the invention. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Qß-phage (such as coat proteins), GA-

phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1). VLPs are discussed further in refs. 155-160. Virosomes are discussed further in, for example, ref. 161

E. Bacterial or microbial derivatives

Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylating toxins and detoxified derivatives thereof.

Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 162. Such "small particles" of 3dMPL are small enough to be sterile filtered through a 0.22μm membrane [162]. Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [163,164].

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in refs. 165 & 166.

Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. References 167, 168 and 169 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 170-175.

The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [176]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 177-179. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, refs. 176 & 180-182.

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E.coli* (*E.coli* heat labile enterotoxin "LT"), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 183 and as parenteral adjuvants in ref. 184. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and

detoxified derivaties thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in refs. 185-192. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in ref. 193, specifically incorporated herein by reference in its entirety.

F. Human immunomodulators

Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [194], *etc.*) [195], interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, and tumor necrosis factor.

G. Bioadhesives and Mucoadhesives

Bioadhesives and mucoadhesives may also be used as adjuvants in the invention. Suitable bioadhesives include esterified hyaluronic acid microspheres [196] or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention [197].

H. Microparticles

Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

I. Liposomes (Chapters 13 & 14 of ref. 141)

Examples of liposome formulations suitable for use as adjuvants are described in refs. 198-200.

J. Polyoxyethylene ether and polyoxyethylene ester formulations

Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters [201]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [202] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [203]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether, polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

K. Polyphosphazene (PCPP)

PCPP formulations are described, for example, in refs. 204 and 205.

L. Muramyl peptides

Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

M. Imidazoquinolone Compounds.

Examples of imidazoquinolone compounds suitable for use adjuvants in the invention include Imiquamod and its homologues (*e.g.* "Resiquimod 3M"), described further in refs. 206 and 207.

N. Thiosemicarbazone Compounds.

Examples of thiosemicarbazone compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in ref. 208. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

O. Tryptanthrin Compounds.

Examples of tryptanthrin compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in ref. 209. The tryptanthrin compounds are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

The invention may also comprise combinations of aspects of one or more of the adjuvants identified above. For example, the following combinations may be used as adjuvant compositions in the invention: (1) a saponin and an oil-in-water emulsion [210]; (2) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) [211]; (3) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol; (4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [212]; (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [213]; (6) SAF, containing 10% squalane, 0.4% Tween 80™, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. (7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and (8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dMPL).

Other substances that act as immunostimulating agents are disclosed in chapter 7 of ref. 141.

The use of aluminium salt adjuvants is particularly useful, and antigens are generally adsorbed to such salts. The Menjugate™ and NeisVac™ conjugates use a hydroxide adjuvant, whereas Meningitec™ uses a phosphate adjuvant. It is possible in compositions of the invention to adsorb some antigens to an aluminium hydroxide but to have other antigens in association with an

aluminium phosphate. Typically, however, only a single salt is used, *e.g.* a hydroxide or a phosphate, but not both. Not all conjugates need to be adsorbed *i.e.* some or all can be free in solution.

Methods of treatment

The invention also provides a method for raising an immune response in a mammal, comprising administering a pharmaceutical composition of the invention to the mammal. The immune response is preferably protective and preferably involves antibodies. The method may raise a booster response.

The mammal is preferably a human. Where the vaccine is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably an adult. A vaccine intended for children may also be administered to adults *e.g.* to assess safety, dosage, immunogenicity, *etc.* A preferred class of humans for treatment are patients at risk of nosocomial infection, particularly those with end-stage renal disease and/or on haemodialysis. Other patients at risk of nosocomial infection are also preferred, *e.g.* immunodeficient patients or those who have undergone surgery, especially cardiac surgery, or trauma. Another preferred class of humans for treatment are patients at risk of bacteremia. A further preferred class are patients suffering from or previously exposed to influenza virus, as *S.aureus* has been linked with post-infection pneumonia in these patients.

The invention also provides a composition of the invention for use as a medicament. The medicament is preferably able to raise an immune response in a mammal (*i.e.* it is an immunogenic composition) and is more preferably a vaccine.

The invention also provides the use of a conjugate of the invention in the manufacture of a medicament for raising an immune response in a mammal.

These uses and methods are preferably for the prevention and/or treatment of a disease caused by *S.aureus, e.g.* skin infections, such as impetigo, boils, cellulitis folliculitis, styes, furuncles, carbuncles, scalded skin syndrome and abscesses, septic arthritis, pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis, toxic shock syndrome (TSS), septicaemia and nosocomial infections.

One way of checking efficacy of therapeutic treatment involves monitoring *S.aureus* infection after administration of the composition of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses against the *S.aureus* antigens after administration of the composition.

Preferred compositions of the invention can confer an antibody titre in a patient that is superior to the criterion for seroprotection for each antigenic component for an acceptable percentage of human subjects. Antigens with an associated antibody titre above which a host is considered to be seroconverted against the antigen are well known, and such titres are published by organisations such

as WHO. Preferably more than 80% of a statistically significant sample of subjects is seroconverted, more preferably more than 90%, still more preferably more than 93% and most preferably 96-100%.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular administration to the thigh or the upper arm is preferred. Injection may be via a needle (*e.g.* a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is 0.5 ml.

The invention may be used to elicit systemic and/or mucosal immunity.

Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. A primary dose schedule may be followed by a booster dose schedule. Suitable timing between priming doses (*e.g.* between 4-16 weeks), and between priming and boosting, can be routinely determined.

S.aureus antigens

As mentioned above, one or more further *S.aureus* antigens can be included in compositions of the invention. The antigens may be protein or saccharide antigens. *S.aureus* protein antigens may be used as carrier proteins for conjugates of the invention, carrier proteins for other conjugates, or as unconjugated protein antigens. *S.aureus* saccharide antigens may be used as the saccharides for other conjugate or as unconjugated saccharide antigens.

Suitable *S.aureus* saccharide antigens include the exopolysaccharide of *S.aureus*, which is a poly-N-acetylglucosamine (PNAG). This polysaccharide is present in both *S.aureus* and *S.epidermidis* and can be isolated from either source [214,215]. For example, PNAG may be isolated from *S.aureus* strain MN8m [216]. The saccharide antigen may be a polysaccharide having the size that arises during purification of the exopolysaccharide from bacteria, or it may be an polysaccharide achieved by fragmentation of such a polysaccharide *e.g.* size can vary from over 400kDa to between 75 and 400kDa, or between 10 and 75kDa, or up to 30 repeat units. The saccharide antigen can have various degrees of N-acetylation and, as described in reference 217, the PNAG may be less than 40% N-acetylated (*e.g.* less than 35, 30, 20, 15, 10 or 5% N-acetylated; deacetylated PNAG is also known as dPNAG). Deacetylated epitopes of PNAG can elicit antibodies that are capable of mediating opsonic killing. The preparation of dPNAG is described in reference 218. The PNAG may or may not be O-succinylated *e.g.* it may be O-succinylated on fewer less than 25, 20, 15, 10, 5, 2, 1 or 0.1% of residues. The PNAG may be conjugated to a carrier molecule as described above or alternatively unconjugated.

Another suitable *S.aureus* saccharide antigen is the type 336 antigen, which is a β-linked hexosamine with no O-acetylation [219,220]. The type 336 antigen is cross-reactive with antibodies raised

against the 336 strain (ATCC 55804). The type 336 antigen may be conjugated to a carrier molecule as described above or alternatively unconjugated.

Suitable *S.aureus* protein antigens include the following *S.aureus* antigens (or antigens comprising immunogenic fragment(s) thereof) [*e.g.* see references 221-228]: AhpC, AhpF, Autolysin amidase, Autolysin glucosaminidase, Collagen binding protein CAN, EbhB, GehD lipase, Heparin binding protein HBP (17kDa), Laminin receptor, MAP, MntC (also known as SitC), MRPII, Npase, ORF0594, ORF0657n, ORF0826, PBP4, RAP (RNA III activating protein), Sai-1, SasK, SBI, SdrG, SdrH, SSP-1, SSP-2 and Vitronectin-binding protein.

Further suitable *S.aureus* protein antigens include a clfA antigen; a clfB antigen; a sdrE2 antigen; a sdrC antigen; a sasF antigen, a emp antigen; a sdrD antigen; a spa antigen; a esaC antigen; a esxA antigen; a esxB antigen; a sta006 antigen; a isdC antigen; a Hla antigen; a sta011 antigen; a isdA antigen; a isdB antigen; and a sta073 antigen, as described below. One or more (*i.e.* 1, 2, 3, 4, 5, 6 or more) of these antigens may be present in a composition of the invention. Of these antigens, the use of one or more (*i.e.* 1, 2, 3, 4, 5, 6 or more) of a esxA antigen; a esxB antigen; a sta006 antigen; a Hla antigen; a sta011 antigen; and/or a sta073 antigen is specifically envisaged.

For example, a composition of the invention may further comprise one of the following combinations of *S.aureus* protein antigens:

(1) A esxA antigen, a esxB antigen, a sta006 antigen and a Hla antigen. The esxA and esxB antigens can usefully be combined as a hybrid polypeptide, as discussed below, *e.g.* a EsxAB hybrid with a esxB antigen downstream of a esxA antigen. The Hla antigen may be a detoxified mutant *e.g.* including a H35L mutation.

(2) A esxA antigen, a esxB antigen, a sta006 antigen and a sta011 antigen. The esxA and esxB antigens may be combined as a hybrid polypeptide, as discussed below, *e.g.* an EsxAB hybrid.

(3) A esxA antigen, a esxB antigen and a sta011 antigen. The esxA and esxB antigens can usefully be combined as a hybrid polypeptide, as discussed below, *e.g.* a EsxAB hybrid.

(4) A esxA antigen, a esxB antigen, a Hla antigen, a sta006 antigen and a sta011 antigen. The esxA and esxB antigens may be combined as a hybrid polypeptide, as discussed below, *e.g.* an EsxAB hybrid. The Hla antigen may be a detoxified mutant *e.g.* including a H35L mutation.

(5) A esxA antigen, a esxB antigen and a Hla antigen. The esxA and esxB antigens can usefully be combined as a hybrid polypeptide, as discussed below, *e.g.* a EsxAB hybrid. The Hla antigen may be a detoxified mutant *e.g.* including a H35L mutation.

(6) A Hla antigen, a sta006 antigen and a sta011 antigen. The Hla antigen may be a detoxified mutant *e.g.* including a H35L mutation.

(7) A esxA antigen and a esxB antigen. The esxA and esxB antigens can usefully be combined as a hybrid polypeptide, as discussed below, *e.g.* an EsxAB hybrid.

(8) A esxA antigen, a esxB antigen and a sta006 antigen. The esxA and esxB antigens can usefully be combined as a hybrid polypeptide, as discussed below, *e.g.* a EsxAB hybrid.

(9) A esxA antigen, a esxB antigen, a sta011 antigen and a sta073 antigen. The esxA and esxB antigens may be combined as a hybrid polypeptide, as discussed below, *e.g.* an EsxAB hybrid.

(10) A sta006 antigen and a sta011 antigen.

Further *Staphylococcus aureus* antigens are disclosed in reference 229.

clfA

The 'clfA' antigen is annotated as 'clumping factor A'. In the NCTC 8325 strain clfA is SAOUHSC_00812 and has amino acid sequence SEQ ID NO: 1 (GI:88194572). In the Newman strain it is nwmn_0756 (GI:151220968).

Useful clfA antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 1 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These clfA proteins include variants of SEQ ID NO: 1. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 1. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 1 while retaining at least one epitope of SEQ ID NO: 1. The final 368 C-terminal amino acids of SEQ ID NO: 1 can usefully be omitted. The first 39 N-terminal amino acids of SEQ ID NO: 1 can usefully be omitted. Other fragments omit one or more protein domains.

SEQ ID NO: 2 is a useful fragment of SEQ ID NO: 1 ('$ClfA_{40-559}$'). This fragments omits the long repetitive region towards the C-terminal of SEQ ID NO: 1.

clfB

The 'clfB' antigen is annotated as 'clumping factor B'. In the NCTC 8325 strain clfB is SAOUHSC_02963 and has amino acid sequence SEQ ID NO: 3 (GI:88196585). In the Newman strain it is nwmn_2529 (GI:151222741).

Useful clfB antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 3 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of

SEQ ID NO: 3, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These clfB proteins include variants of SEQ ID NO: 3. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 3. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 3 while retaining at least one epitope of SEQ ID NO: 3. The final 40 C-terminal amino acids of SEQ ID NO: 3 can usefully be omitted. The first 44 N-terminal amino acids of SEQ ID NO: 3 can usefully be omitted. Other fragments omit one or more protein domains. ClfB is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 4 is a useful fragment of SEQ ID NO: 3 ('$ClfB_{45-552}$'). This fragment includes the most exposed domain of ClfB and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins. Other useful fragments, based on a 3-domain model of ClfB, include: $ClfB_{45-360}$ (also known as CLfB-N12; SEQ ID NO: 5); $ClfB_{212-542}$ (also known as CLfB-N23; SEQ ID NO: 6); and $ClfB_{360-542}$ (also known as CLfB-N3; SEQ ID NO: 7).

sdrE2

The 'sdrE2' antigen is annotated as 'Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE'. In the Newman strain sdrE2 is NWMN_0525 and has amino acid sequence SEQ ID NO: 8 (GI:151220737).

Useful sdrE2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 8 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 8; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 8, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sdrE2 proteins include variants of SEQ ID NO: 8. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 8. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 8 while retaining at least one epitope of SEQ ID NO: 8. The final 38 C-terminal amino acids of SEQ ID NO: 8 can usefully be omitted. The first 52 N-terminal amino acids of SEQ ID NO: 8 can usefully be omitted. Other fragments omit one or more protein domains. SdrE2 is naturally a long protein and so the use of fragments is very helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 9 is a useful fragment of SEQ ID NO: 8 ('$SdrE_{53-632}$'). This fragment includes the most exposed domain of SdrE2 and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

sdrC

The 'sdrC' antigen is annotated as 'sdrC protein'. In the NCTC 8325 strain sdrC is SAOUHSC_00544 and has amino acid sequence SEQ ID NO: 10 (GI:88194324).

Useful sdrC antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 10 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 10; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 10, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sdrC proteins include variants of SEQ ID NO: 10. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 10. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 10 while retaining at least one epitope of SEQ ID NO: 10. The final 38 C-terminal amino acids of SEQ ID NO: 10 can usefully be omitted. The first 50 N-terminal amino acids of SEQ ID NO: 10 can usefully be omitted. Other fragments omit one or more protein domains. SdrC is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 11 is a useful fragment of SEQ ID NO: 10 ('$SdrC5_{1\text{-}518}$'). This fragment includes the most exposed domain of SdrC and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

sasF

The 'sasF' antigen is annotated as 'sasF protein'. In the NCTC 8325 strain sasF is SAOUHSC_02982 and has amino acid sequence SEQ ID NO: 12 (GI:88196601).

Useful sasF antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 12 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 12; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 12, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sasF proteins include variants of SEQ ID NO: 12. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 12. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 12 while retaining at least one epitope of SEQ ID NO: 12. The final 39 C-terminal amino acids of SEQ ID NO: 12 can usefully be omitted. The first 37 N-terminal amino acids of SEQ ID NO: 12 can usefully be omitted. Other fragments omit one or more protein domains.

emp

The 'emp' antigen is annotated as 'extracellular matrix and plasma binding protein'. In the NCTC 8325 strain emp is SAOUHSC_00816 and has amino acid sequence SEQ ID NO: 13 (GI:88194575). In the Newman strain it is nwmn_0758 (GI:151220970).

Useful emp antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 13 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 13; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 13, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These emp proteins include variants of SEQ ID NO: 13. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 13. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 13 while retaining at least one epitope of SEQ ID NO: 13. The first 26 N-terminal amino acids of SEQ ID NO: 13 can usefully be omitted. Other fragments omit one or more protein domains.

SEQ ID NOs: 14, 15, 16 and 17 are useful fragments of SEQ ID NO: 13 ('Emp_{35-340}', 'Emp_{27-334}', 'Emp_{35-334}' and 'Emp_{27-147}', respectively).

sdrD

The 'sdrD' antigen is annotated as 'sdrD protein'. In the NCTC 8325 strain sdrD is SAOUHSC_00545 and has amino acid sequence SEQ ID NO: 18 (GI:88194325).

Useful sdrD antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 18 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 18; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 18, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sdrD proteins include variants of SEQ ID NO: 18. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 18. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 18 while retaining at least one epitope of SEQ ID NO: 18. The final 38 C-terminal amino acids of SEQ ID NO: 18 can usefully be omitted. The first 52 N-terminal amino acids of SEQ ID NO: 18 can usefully be omitted. Other fragments omit one or more protein domains. SdrD is naturally a long protein and so the use of fragments is very helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 19 is a useful fragment of SEQ ID NO: 18 ('$SdrD_{53-592}$'). This fragment includes the most exposed domain of SdrD and is more easily used at an industrial scale. It also reduces the

antigen's similarity with human proteins. A nother useful fragment, with the same C-terminus residue, is $SdrD_{394-592}$ (also known as SdrD-N3; SEQ ID NO: 20).

spa

The 'spa' antigen is annotated as 'protein A' or 'SpA'. In the NCTC 8325 strain spa is SAOUHSC_00069 and has amino acid sequence SEQ ID NO: 21 (GI:88193885). In the Newman strain it is nwmn_0055 (GI:151220267). All *S.aureus* strains express the structural gene for spa, a well characterized virulence factor whose cell wall-anchored surface protein product has five highly homologous immunoglobulin binding domains designated E, D, A, B, and C [230]. These domains display ~80% identity at the amino acid level, are 56 to 61 residues in length, and are organized as tandem repeats [231]. SpA is synthesized as a precursor protein with an N-terminal signal peptide and a C-terminal sorting signal [232,233]. Cell wall-anchored spa is displayed in great abundance on the staphylococcal surface [234,235]. Each of its immunoglobulin binding domains is composed of anti-parallel α-helices that assemble into a three helix bundle and can bind the Fc domain of immunoglobulin G (IgG) [236,237], the VH3 heavy chain (Fab) of IgM (*i.e.* the B cell receptor) [238], the von Willebrand factor at its A1 domain [239] and/or the TNF-α receptor I (TNFRI) [240], which is displayed on surfaces of airway epithelia.

Useful spa antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 21 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 21; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 21, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These spa proteins include variants of SEQ ID NO: 21. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 21. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 21 while retaining at least one epitope of SEQ ID NO: 21. The final 35 C-terminal amino acids of SEQ ID NO: 21 can usefully be omitted. The first 36 N-terminal amino acids of SEQ ID NO: 21 can usefully be omitted. Other fragments omit one or more protein domains. Reference 241 suggests that individual IgG-binding domains might be useful immunogens, alone or in combination.

SEQ ID NO: 22 is a useful fragment of SEQ ID NO: 21 ('Spa_{37-325}'). This fragment contains all the five SpA Ig-binding domains and includes the most exposed domain of SpA. It also reduces the antigen's similarity with human proteins. Other useful fragments may omit 1, 2, 3 or 4 of the natural A, B, C, D and/or E domains. As reported in reference 241, other useful fragments may include only 1, 2, 3 or 4 of the natural A, B, C, D and/or E domains *e.g.* comprise only the SpA(A) domain but not B to E, or comprise only the SpA(D) domain but not A, B, C or E, *etc.* Thus a spa antigen useful with the invention may include 1, 2, 3, 4 or 5 IgG-binding domains, but ideally has 4 or fewer. If an

antigen includes only one type of spa domain (*e.g.* only the Spa(A) or SpA(D) domain), it may include more than one copy of this domain *e.g.* multiple SpA(D) domains in a single polypeptide chain. An individual domain within the antigen may be mutated at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids relative to SEQ ID NO: 21 (*e.g.* see ref. 241, disclosing mutations at residues 3 and/or 24 of domain D, at residue 46 and/or 53 of domain A, *etc.*). Such mutants should not remove the antigen's ability to elicit an antibody that recognises SEQ ID NO: 21, but may remove the antigen's binding to IgG. In certain aspects a spa antigen includes a substitution at (a) one or more amino acid substitution in an IgG Fc binding sub-domain of SpA domain A, B, C, D and/or E that disrupts or decreases binding to IgG Fc, and (b) one or more amino acid substitution in a V_H3 binding sub-domain of SpA domain A, B, C, D, and/or E that disrupts or decreases binding to V_H3. In certain embodiments, a variant SpA comprises at least or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more variant SpA domain D peptides.

esaC

The 'esaC' antigen is annotated as 'esaC'. In the NCTC 8325 strain esaC is SAOUHSC_00264 and has amino acid sequence SEQ ID NO: 23 (GI:88194069).

Useful esaC antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 23 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 23; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 23, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 or more). These esaC proteins include variants of SEQ ID NO: 23. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 23. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 23 while retaining at least one epitope of SEQ ID NO: 23. Other fragments omit one or more protein domains.

esxA

The 'esxA' antigen is annotated as 'protein'. In the NCTC 8325 strain esxA is SAOUHSC_00257 and has amino acid sequence SEQ ID NO: 24 (GI:88194063).

Useful esxA antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 24 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 24; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 24, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 or more). These esxA proteins include variants of SEQ ID NO: 24. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 24. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino

acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 24 while retaining at least one epitope of SEQ ID NO: 24. Other fragments omit one or more protein domains.

esxB

The 'esxB' antigen is annotated as 'esxB'. In the NCTC 8325 strain esxB is SAOUHSC_00265 and has amino acid sequence SEQ ID NO: 25 (GI:88194070).

Useful esxB antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 25 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 25; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 25, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 or more). These esxB proteins include variants of SEQ ID NO: 25. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 25. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 25 while retaining at least one epitope of SEQ ID NO: 25. Other fragments omit one or more protein domains.

sta006

The 'sta006' antigen is annotated as 'ferrichrome-binding protein', and has also been referred to as 'FhuD2' in the literature [242]. In the NCTC 8325 strain sta006 is SAOUHSC_02554 and has amino acid sequence SEQ ID NO: 26 (GI:88196199). In the Newman strain it is nwmn_2185 (GI:151222397).

Useful sta006 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 26 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 26; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 26, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sta006 proteins include variants of SEQ ID NO: 26. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 26. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 26 while retaining at least one epitope of SEQ ID NO: 26. The first 17 N-terminal amino acids of SEQ ID NO: 26 can usefully be omitted. Other fragments omit one or more protein domains. Mutant forms of sta006 are reported in reference 243. A sta006 antigen may be lipidated *e.g.* with an acylated N-terminus cysteine.

isdC

The 'isdC' antigen is annotated as 'protein'. In the NCTC 8325 strain isdC is SAOUHSC_01082 and has amino acid sequence SEQ ID NO: 27 (GI:88194830).

Useful isdC antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 27 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 27; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 27, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200 or more). These isdC proteins include variants of SEQ ID NO: 27. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 27. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 27 while retaining at least one epitope of SEQ ID NO: 27. The final 39 C-terminal amino acids of SEQ ID NO: 27 can usefully be omitted. The first 28 N-terminal amino acids of SEQ ID NO: 27 can usefully be omitted. Other fragments omit one or more protein domains. Useful fragments of IsdB are disclosed in reference 249.

Reference 244 discloses antigens which usefully include epitopes from both IsdB and IsdH.

Hla

The 'Hla' antigen is the 'alpha-hemolysin precursor' also known as 'alpha toxin' or simply 'hemolysin'. In the NCTC 8325 strain Hla is SAOUHSC_01121 and has amino acid sequence SEQ ID NO: 28 (GI:88194865). In the Newman strain it is nwmn_1073 (GI:151221285). Hla is an important virulence determinant produced by most strains of *S.aureus*, having pore-forming and haemolytic activity. Anti-Hla antibodies can neutralise the detrimental effects of the toxin in animal models, and Hla is particularly useful for protecting against pneumonia.

Useful Hla antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 28 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 28; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 28, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These Hla proteins include variants of SEQ ID NO: 28. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 28. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 28 while retaining at least one epitope of SEQ ID NO: 28. The first 26 N-terminal amino acids of SEQ ID NO: 28 can usefully be omitted. Truncation at the C-terminus can also be used *e.g.* leaving only 50 amino acids (residues 27-76 of SEQ ID NO: 28) [245]. Other fragments omit one or more protein domains.

Hla's toxicity can be avoided in compositions of the invention by chemical inactivation (*e.g.* using formaldehyde, glutaraldehyde or other cross-linking reagents). Instead, however, it is preferred to use mutant forms of Hla which remove its toxic activity while retaining its immunogenicity. Such detoxified mutants are already known in the art. One useful Hla antigen has a mutation at residue 61 of SEQ ID NO: 28, which is residue 35 of the mature antigen (*i.e.* after omitting the first 26 N-terminal amino acids). Thus residue 61 may not be histidine, and may instead be *e.g.* Ile, Val or preferably Leu. A His-Arg mutation at this position can also be used. For example, SEQ ID NO: 29 is the mature mutant Hla-H35L sequence and a useful Hla antigen comprises SEQ ID NO: 29. Another useful mutation replaces a long loop with a short sequence *e.g.* to replace the 39mer at residues 136-174 of SEQ ID NO: 28 with a tetramer such as PSGS (SEQ ID NO: 30), as in SEQ ID NO: 31 (which also includes the H35L mutation) and SEQ ID NO: 32 (which does not include the H35L mutation).

Further useful Hla antigens are disclosed in references 246 and 247.

SEQ ID NOs: 33, 34 & 35 are three useful fragments of SEQ ID NO: 28 ('Hla_{27-76}', 'Hla_{27-89}' and 'Hla_{27-79}', respectively). SEQ ID NOs: 36, 37 and 38 are the corresponding fragments from SEQ ID NO: 29.

sta011

The 'sta011' antigen is annotated as 'lipoprotein'. In the NCTC 8325 strain sta011 is SAOUHSC_00052 and has amino acid sequence SEQ ID NO: 39 (GI:88193872).

Useful sta011 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 39 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 39; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 39, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sta011 proteins include variants of SEQ ID NO: 39. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 39. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 39 while retaining at least one epitope of SEQ ID NO: 39. The first 23 N-terminal amino acids of SEQ ID NO: 39 can usefully be omitted. Other fragments omit one or more protein domains. A sta006 antigen may be lipidated *e.g.* with an acylated N-terminus cysteine.

Variant forms of SEQ ID NO: 39 which may be used for preparing sta011 antigens include, but are not limited to, SEQ ID NOs: 40, 41 and 42 with various Ile/Val/Leu substitutions.

isdA

The 'isdA' antigen is annotated as 'IsdA protein'. In the NCTC 8325 strain isdA is SAOUHSC_01081 and has amino acid sequence SEQ ID NO: 43 (GI:88194829). In the Newman strain it is nwmn_1041 (GI:151221253).

Useful isdA antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 43 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 43; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 43, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These isdA proteins include variants of SEQ ID NO: 43. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 43. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 43 while retaining at least one epitope of SEQ ID NO: 43. The final 38 C-terminal amino acids of SEQ ID NO: 43 can usefully be omitted. The first 46 N-terminal amino acids of SEQ ID NO: 43 can usefully be omitted. Truncation to exclude the C-terminal 38mer of SEQ ID NO: 43 (beginning with the LPKTG motif) is also useful. Other fragments omit one or more protein domains.

SEQ ID NO: 44 is a useful fragment of SEQ ID NO: 43 (amino acids 40-184 of SEQ ID NO: 43; '$IsdA_{40-184}$') which includes the natural protein's heme binding site and includes the antigen's most exposed domain. It also reduces the antigen's similarity with human proteins. Other useful fragments are disclosed in references 248 and 249.

IsdA does not adsorb well to aluminium hydroxide adjuvants, so IsdA present in a composition may me unadsorbed or may be adsorbed to an alternative adjuvant *e.g.* to an aluminium phosphate.

isdB

The 'isdB' antigen is annotated as 'neurofilament protein isdB'. In the NCTC 8325 strain isdB is SAOUHSC_01079 and has amino acid sequence SEQ ID NO: 45 (GI:88194828). IsdB has been proposed for use as a vaccine antigen on its own [250], but this may not prevent pneumonia.

Useful isdB antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 45 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 45; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 45, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These isdB proteins include variants of SEQ ID NO: 45. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 45. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ

ID NO: 45 while retaining at least one epitope of SEQ ID NO: 45. The final 36 C-terminal amino acids of SEQ ID NO: 45 can usefully be omitted. The first 40 N-terminal amino acids of SEQ ID NO: 45 can usefully be omitted. Other fragments omit one or more protein domains. Useful fragments of IsdB are disclosed in references 249 and 251 *e.g.* lacking 37 internal amino acids of SEQ ID NO: 45.

In some embodiments, compositions of the invention do not include an isdB antigen.

sta073

The 'sta073' antigen is annotated as 'bifunctional autolysin precursor'. In the NCTC 8325 strain sta073 is SAOUHSC_00994 and has amino acid sequence SEQ ID NO: 46 (GI:88194750). In the Newman strain it is nwmn_0922 (GI:151221134). Proteomic analysis has revealed that this protein is secreted or surface-exposed.

Useful sta073 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 46 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 46; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 46, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These sta073 proteins include variants of SEQ ID NO: 46. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 46. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 46 while retaining at least one epitope of SEQ ID NO: 46. The first 24 N-terminal amino acids of SEQ ID NO: 46 can usefully be omitted. Other fragments omit one or more protein domains.

Sta073 does not adsorb well to aluminium hydroxide adjuvants, so Sta073 present in a composition may me unadsorbed or may be adsorbed to an alternative adjuvant *e.g.* to an aluminium phosphate.

Hybrid polypeptides

S.aureus protein antigens used in the invention may be present in the composition as individual separate polypeptides. Where more than one antigen is used, however, they do not have to be present as separate polypeptides. Instead, at least two (*e.g.* 2, 3, 4, 5, or more) antigens can be expressed as a single polypeptide chain (a 'hybrid' polypeptide). Hybrid polypeptides offer two main advantages: first, a polypeptide that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem; second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful.

The hybrid polypeptide may comprise two or more polypeptide sequences from each of the antigens listed above, or two or more variants of the same antigen in the cases in which the sequence has partial variability across strains.

Hybrids consisting of amino acid sequences from two, three, four, five, six, seven, eight, nine, or ten antigens are useful. In particular, hybrids consisting of amino acid sequences from two, three, four, or five antigens are preferred, such as two or three antigens.

Different hybrid polypeptides may be mixed together in a single formulation. Hybrids may be combined with non-hybrid antigens selected from the first, second or third antigen groups. Within such combinations, an antigen may be present in more than one hybrid polypeptide and/or as a non-hybrid polypeptide. It is preferred, however, that an antigen is present either as a hybrid or as a non-hybrid, but not as both.

Hybrid polypeptides can be represented by the formula NH_2-A-{-X-L-}$_n$-B-COOH, wherein: X is an amino acid sequence of a *S.aureus* antigen, as described above; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; *n* is an integer of 2 or more (*e.g.* 2, 3, 4, 5, 6, *etc.*). Usually *n* is 2 or 3.

If a -X- moiety has a leader peptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein *i.e.* the leader peptide of X_1 will be retained, but the leader peptides of X_2 ... X_n will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

For each *n* instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when *n*=2 the hybrid may be NH_2-X_1-L_1-X_2-L_2-COOH, NH_2-X_1-X_2-COOH, NH_2-X_1-L_1-X_2-COOH, NH_2-X_1-X_2-L_2-COOH, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* comprising Gly_n where n = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO: 47) or GSGSGGGG (SEQ ID NO: 48), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(Gly)_4$ tetrapeptide being a typical poly-glycine linker. Other suitable linkers, particularly for use as the final L_n are ASGGGS (SEQ ID NO: 49 *e.g.* encoded by SEQ ID NO: 50) or a Leu-Glu dipeptide.

-A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid

sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A- is preferably an oligopeptide (*e.g.* with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine *e.g.* Met-Ala-Ser, or a single Met residue.

-B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more, such as SEQ ID NO: 51), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

One hybrid polypeptide of the invention may include both EsxA and EsxB antigens. These may be in either order, N- to C- terminus. SEQ ID NOs: 52 ('EsxAB'; encoded by SEQ ID NO: 53) and 54 ('EsxBA') are examples of such hybrids, both having hexapeptide linkers ASGGGS (SEQ ID NO: 49).

General

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, references 252-259, *etc.*

"GI" numbering is used above. A GI number, or "GenInfo Identifier", is a series of digits assigned consecutively to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence is updated (*e.g.* for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 260. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 261.

Where the invention concerns an "epitope", this epitope may be a B-cell epitope and/or a T-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [262,263] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [264], matrix-based approaches [265], MAPITOPE [266], TEPITOPE [267,268], neural networks [269], OptiMer & EpiMer [270, 271], ADEPT [272], Tsites [273], hydrophilicity [274], antigenic index [275] or the methods disclosed in references 276-280, *etc.*). Epitopes are the parts of an antigen that are

recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as "antigenic determinants".

Where an antigen "domain" is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value *x* means, for example, $x \pm 10\%$.

The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

Where the invention provides a process involving multiple sequential steps, the invention can also provide a process involving less than the total number of steps. For example, the invention provides a process comprising the steps of: (a) depolymerising a *S.aureus* type 5 or type 8 capsular polysaccharide, to give a polysaccharide fragment; and (b) oxidising the fragment in order to introduce an aldehyde group into at least one saccharide residue in the fragment, to give an oxidised saccharide residue. The further step (c) need not be performed in order to fall within the scope of the invention, as the product of steps (a) and (b) has utility as an intermediate in conjugate preparation, and may be used, stored, exported, *etc.* for separate and later use. This later use might involve carrying out step (c). Alternatively, the product of steps (a) and (b) might be coupled to a carrier molecule in a different way, for example via a hydroxyl or carboxyl group that is retained in the oxidised saccharide residue.

Similarly, where a starting polysaccharide material is already partially processed then the invention encompasses processes involving only the later steps of a method. For example, the invention encompasses a process comprising a step of coupling an oxidised saccharide residue in a type 5 or type 8 capsular polysaccharide to a carrier molecule via an aldehyde group, in which the starting material for the process is a type 5 or type 8 capsular polysaccharide that was previously depolymerised to give a polysaccharide fragment and then oxidised to introduce an the aldehyde group into the saccharide residue.

These different steps can be performed at very different times by different people in different places (*e.g.* in different countries).

It will be appreciated that sugar rings can exist in open and closed form and that, whilst closed forms are shown in structural formulae herein, open forms are also encompassed by the invention. Similarly, it will be appreciated that sugars can exist in pyranose and furanose forms and that, whilst pyranose forms are shown in structural formulae herein, furanose forms are also encompassed. Different anomeric forms of sugars are also encompassed.

A primary amine can be represented by formula NH_2R. The R group will typically be electron donating, and includes C_{1-8}hydrocarbyl, particularly C_{1-8}alkyl, especially methyl. R is often $-CH_3$, $-C_2H_5$ or $-C_3H_7$. The hydrocarbyl may be substituted with one or more groups, such as: halogen (*e.g.* Cl, Br, F, I), trihalomethyl, $-NO_2$, -CN, $-N^+(C_{1-6}alkyl)_2O^-$, $-SO_3H$, $-SOC_{1-6}alkyl$, $-SO_2C_{1-6}alkyl$, $-SO_3C_{1-6}alkyl$, $-OC(=O)OC_{1-6}alkyl$, $-C(=O)H$, $-C(=O)C_{1-6}alkyl$, $-OC(=O)C_{1-6}alkyl$, $-N(C_{1-6}alkyl)_2$, $C_{1-6}alkyl$, $-N(C_{1-6}alkyl)_2$, $-C(=O)N(C_{1-6}alkyl)_2$, $-N(C_{1-6}alkyl)C(=O)O(C_{1-6}alkyl)$, $-N(C_{1-6}alkyl)C(=O)N(C_{1-6}alkyl)_2$, $-CO_2H$, $-OC(=O)N(C_{1-6}alkyl)_2$, $-N(C_{1-6}alkyl)C(=O)C_{1-6}alkyl$, $-N(C_{1-6}alkyl)C(=S)C_{1-6}alkyl$, $-N(C_{1-6}alkyl)SO_2N(C_{1-6}alkyl)_2$, $-CO_2C_{1-6}alkyl$, $-SO_2N(C_{1-6}alkyl)_2$, $-C(=O)NH_2$, $-C(=S)N(C_{1-6}alkyl)_2$, $-N(C_{1-6}alkyl)SO_2C_{1-6}alkyl$, $-N(C_{1-6}alkyl)C(=S)N(C_{1-6}alkyl)_2$, $-NH-C_{1-6}alkyl$, $-S-C_{1-6}alkyl$ or $-O-C_{1-6}alkyl$. The term 'hydrocarbyl' includes linear, branched or cyclic monovalent groups consisting of carbon and hydrogen. Hydrocarbyl groups thus include alkyl, alkenyl and alkynyl groups, cycloalkyl (including polycycloalkyl), cycloalkenyl and aryl groups and combinations thereof, *e.g.* alkylcycloalkyl, alkylpolycycloalkyl, alkylaryl, alkenylaryl, cycloalkylaryl, cycloalkenylaryl, cycloalkylalkyl, polycycloalkylalkyl, arylalkyl, arylalkenyl, arylcycloalkyl and arylcycloalkenyl groups. Typical hydrocarbyl are C_{1-14} hydrocarbyl, more particularly C_{1-8} hydrocarbyl.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a scheme for making an *S.aureus* type 5 capsular polysaccharide-CRM197 conjugate using an adipic acid dihydrazine linker and carbodiimide chemistry.

Figure 2a shows an SDS-PAGE analysis of the *S.aureus* type 5 capsular polysaccharide-CRM197 conjugate made using an adipic acid dihydrazine linker and carbodiimide chemistry. Figure 2b shows an S300 Sephacryl™ chromatogram of the *S.aureus* type 5 capsular polysaccharide-CRM197 conjugate made using an adipic acid dihydrazine linker and carbodiimide chemistry.

Figure 3 shows an S300 Sephacryl™ chromatogram of depolymerised type 5 capsular polysaccharide.

Figure 4A compares 1D ^1H signals in the ^1H anomeric region for depolymerised and native type 5 capsular polysaccharide. Some notable differences are marked. Figures 4B and 4C compare the anomeric and Methyl-Fucose regions respectively of 2D (^1H, ^1H) scalar coupling spectra for these polysaccharides. Some notable differences are marked.

Figure 5a shows an SDS-PAGE analysis of a *S.aureus* type 5 capsular polysaccharide-CRM197 conjugate made using a method of the invention. Figure 5b shows an S300 Sephacryl™ chromatogram of a *S.aureus* type 5 capsular polysaccharide-CRM197 conjugate made using a method of the invention.

Figure 6 shows an S300 Sephacryl™ chromatogram of *S.aureus* type 5 capsular polysaccharides depolymerised under various conditions.

Figure 7 shows SDS-PAGE analyses of *S.aureus* type 5 capsular polysaccharide-CRM197 conjugates made using methods of the invention.

Figure 8 shows the IgG response to various antigens in a mouse kidney abscess model of *S.aureus* infection.

Figure 9 compares IgG and IgM responses to various antigens in the mouse kidney abscess model.

Figure 10 shows protective responses to various antigens in the mouse kidney abscess model.

Figure 11 compares the IgG response to different conjugates in the mouse kidney abscess model.

Figure 12 compares protective responses to different conjugates in the mouse kidney abscess model.

Figures 13A and 13B compare protective responses to further conjugates in the mouse kidney abscess model.

Figure 14 compares IgG and IgM responses to further conjugates in the mouse kidney abscess model.

Figure 15 compares protective responses to a further conjugate when adjuvanted with different agents in the mouse kidney abscess model.

Figure 16 compares responses to various antigens in a mouse lethal model of *S.aureus* infection.

Figure 17 shows a SEC-HPLC chromatogram of *S.aureus* type 8 capsular polysaccharide depolymerised with 2M hydrochloric acid.

Figure 18 shows an NMR spectrum of *S.aureus* type 8 capsular polysaccharide depolymerised with 2M hydrochloric acid.

MODES FOR CARRYING OUT THE INVENTION

Conjugate production and characterisation

A purified *S.aureus* type 5 capsular polysaccharide was conjugated to CRM197 using carbodiimide chemistry and an adipic acid dihydrazine linker, similar to the method used in reference 2 (see below). In this method, the capsular polysaccharide is conjugated to derivatised CRM197 using EDC (Figure 1). The reaction involves the carboxyl groups of the capsular polysaccharide. The carbodiimide (EDC) activates the carboxyl groups to bind to the $-NH_2$ group from the derivatised carrier protein (CRMadh), forming an amide linkage. The derivatised CRMadh is prepared using the same carbodiimide chemistry.

CRMadh preparation:

To a solution of CRM197 was added 100mM MES pH6.0 buffer in order to reach a final concentration of 10-12mg/ml. Then 3.5mg/ml of ADH (adipic acid dihydrazide) and 0.15 (EDC/CRM, w/w) was added, and the reaction kept under mild stirring for 1h at RT. The mixture was then dialyzed against first 200mM NaCl, 10mM MES pH7.3 buffer and then against 5mM MES pH7.0 buffer, using a 6-8 kDa membrane (SpectraPor). The product was characterized by MicroBCA, SDS-Page (3-8%), HPLC and MS. The CRMadh was found to be derivatised with 6-8 linker of ADH).

Conjugation reaction:

The conjugation reaction was performed at capsular polysaccharide concentration of 2 mg/mL in 50mM MES buffer pH6.04. The derivatised carrier protein, CRMadh, was added to the solution of capsular polysaccharide to a final concentration of 4.0 mg/ml. The solution was kept at RT for 3h. The polysaccharide:protein ratio in the reaction mixture was 1:2 (weight/weight), the polysaccharide:EDC ratio was 1:6.66 (equivalent/equivalent) and the polysaccharide:SulfoNHS ratio was 1:0.53 (equivalent/equivalent).

After 3h, formation of the conjugate was verified by SDS-PAGE using a NuPAGE® 3-8% Tris-Acetate Gel (Invitrogen) (Figure 2a). After conjugation, the conjugate was purified by gel-filtration chromatography (performed on an Akta™ system (G&E Healthcare) using a S300 Sephacryl™ resin (G&E Healthcare), with a 10mM NaPi, 10mM NaCl, pH7.2 mobile phase buffer). The conjugate was detected at 215nm, 254nm and 280nm (Figure 2b). The conjugate solution was stored at -20°C until further use. Total saccharide in the conjugate was determined by HPAEC-PAD analysis and protein content by MicroBCA assay, as described in reference 281 (Table 1).

Conjugate (lot)	Protein (µg/ml)	Saccharide (µg/ml)	Saccharide/protein (w/w)
1	26.00	12.10	0.47
2	33.90	11.00	0.32
3	62.21	29.40	0.47
4	45.21	9.30	0.21

TABLE 1

Purified *S.aureus* type 5 and type 8 capsular polysaccharides were separately conjugated to CRM197 using a method of the invention (see below).

Depolymerisation

Purified capsular polysaccharide was dissolved in distilled water at 2 mg/mL. Acetic acid was added to a final concentration of 2% (v/v) and the reaction kept at 90°C for 3 hours (or overnight in the case of Lot B). The solution was then neutralized with 1M NaOH and the depolymerised polysaccharide purified on a gel-filtration column (performed on an Akta™ system (G&E Healthcare) using a S300 Sephacryl™ resin (G&E Healthcare), with a 10mM NaPi, 10mM NaCl, pH7.2 mobile phase buffer). The saccharide was detected at 215nm (Figure 3). Pooled fractions were dialyzed against distilled water using a 1kDa membrane (SpectraPor) and lyophilized.

The site of cleavage was verified as being at (1 → 3) glycosidic linkages within the type 5 polysaccharide using ^{1}H NMR. Briefly, samples of native and depolymerised type 5 capsular polysaccharide were freeze-dried to eliminate protonated water solvent and dissolved in deuterium oxide (99.9% deuterium, Sigma-Aldrich). All NMR spectra were recoded at 50°C on a Bruker

Avance III 400 MHz spectrometer using a 5-mm broadband probe and the TopSpin 2.1 software package (Bruker) for data acquisition and processing. 1D ^{1}H spectra were collected using a standard one-pulse experiment over a spectral width of 4,000Hz and collecting 32k data points. The transmitter was set at the residual HDO frequency (4.79 ppm). The spectra were obtained in a quantitative manner using a total recycle time to ensure a full recovering of each signal (5x Longitudinal Relaxation Time T1). Spectra were Fourier transformed after applying a 0.2 Hz line broadening function. 2D (^{1}H, ^{1}H) scalar correlation spectra were recorded by DQF-COSY pulse sequence. 4096 data points were collected in the F2 domain and 256 in the F1 domain.

1D ^{1}H signals for the native polysaccharide were compared with published values and found to be in agreement (Table 2).

Signal	Measured δ (ppm)*	Pubd. δ (ppm)**	Pubd. δ (ppm)***
$H_3^{L\text{-}FucNAc\text{-}OAc}$	4.958	5.005	5.005
$H_1^{L\text{-}FucNAc\text{-}OAc}$	4.929	4.981	4.975
$H_3^{L\text{-}FucNAc\text{-}deOAc}$	4.864	4.935	4.911
$H_1^{ManNAc\text{-}deOAc}$	4.801	4.860	4.847
$H_1^{ManNAc\text{-}OAc}$	4.638	4.698	4.683
$H_2^{ManNAc\text{-}deOAc}$	4.625	4.680	4.670
$H_2^{ManNAc\text{-}deOAc}$	4.584	4.645	4.629
$H_1^{D\text{-}FucNAc\text{-}OAc/deOAc}$	4.405	4.461	4.452
$H_4^{L\text{-}FucNAc\text{-}OAc}$	4.320	4.382	4.367
$H_2^{L\text{-}FucNAc\text{-}OAc}$	4.292	4.368	4.338
$H_5^{L\text{-}FucNAc\text{-}OAc}$	4.121	4.175	4.168
$H_5^{L\text{-}FucNAc\text{-}deOAc}$	4.080	4.142	4.126
$H_2^{L\text{-}FucNAc\text{-}deOAc}$	4.033	4.105	4.077
$H_4^{L\text{-}FucNAc\text{-}deOAc}$	4.005	4.060	4.051
$NAc^{D\text{-}FucNAc\text{-}OAc}$	2.083	2.149	2.131
$NAc^{D\text{-}FucNAc\text{-}deOAc}$	2.067	2.126	2.115
$OAc^{L\text{-}FucNAc\text{-}OAc}$	2.004	2.070	2.051

$NAc^{L\text{-}FucNAc\text{-}deOAc}$	1.995	2..057	2.002
$NAc^{L\text{-}FucNAc\text{-}OAc}$	1.955	2.023	2.043
$NAc^{ManNAc\text{-}deOAc}$	1.948	2.018	1.996
$NAc^{ManNAc\text{-}OAc}$	1.943	2.011	1.992
$H_6{}^{D\text{-}FucNAc\text{-}OAc}$	1.238	1.300	1.287
$H_6{}^{D\text{-}FucNAc\text{-}deOAc}$		1.298	
$H_6{}^{L\text{-}FucNAc\text{-}OAc/deOAc}$	1.183	1.242	1.231

* *HDO signal at 4.484 ppm*

** *Jones C. Carbohydr. Res. 2005, 340(6), 1097-1106 - HDO signal at 4.484 ppm*

*** *Jones C. Carbohydr. Res. 2005, 340(6), 1097-1106 - H1L-FucNAc = 5.005 ppm, therefore HDO signal at 4.532 ppm instead of 4.484 ppm*

TABLE 2

Figure 4A compares 1D ^1H signals in the ^1H anomeric region for the depolymerised and native polysaccharides. Figures 4B and 4C compare the anomeric and Methyl-Fucose regions respectively of 2D (^1H, ^1H) scalar coupling spectra for these polysaccharides. The data show that the acetic acid treatment resulted in cleavage of (1 → 3) glycosidic linkages between the α-L-FucNAc(3OAc) and β-D-FucNAc residues in the type 5 capsular polysaccharide.

Oxidation

The depolymerised capsular polysaccharide was dissolved in distilled water at 2 mg/mL. $NaIO_4$ was added at a polysaccharide:$NaIO_4$ ratio of 1:1 (weight/weight) and the reaction kept at room temperature for 1-2 hours in the dark. The solution was then dialyzed against distilled water using a 1kDa membrane (SpectraPor) and lyophilized once again.

Conjugation

The oxidised capsular polysaccharide was dissolved in a 200mM NaPi, 1M NaCl, pH7.2 buffer at a concentration of 10 mg/mL. CRM197 was added to the solution at a polysaccharide:protein ratio of 4:1 (weight/weight) and $NaBH_3CN$ (Aldrich) added at a sacchaaride:$NaBCNH_3$ ratio of 2:1 (weight/weight). The solution was kept at 37°C for 2 days. SDS-PAGE was used to confirm formation of the conjugate (see Figure 5a for the type 5 conjugate). After conjugation, the conjugate was purified by gel-filtration chromatography (performed on an Akta™ system (G&E Healthcare) using a S300 Sephacryl™ resin (G&E Healthcare), with a 10mM NaPi, 10mM NaCl, pH7.2 mobile phase buffer). The conjugate was detected at 215nm, 254nm and 280nm (see Figure 5b for the type 5 conjugate). The conjugate solution was stored at -20°C until further use. Total saccharide in the

conjugate was determined by HPAEC-PAD analysis and protein content by MicroBCA assay (see Table 3a for the type 5 conjugate and Table 3b for type 8 conjugate).

Conjugate (lot)	Protein (μg/ml)	Saccharide (μg/ml)	Saccharide/protein (w/w)
A	51.52	1.72	0.03
B	161.80	17.10	0.11
C	34.42	4.22	0.12
D	40.56	12.70	0.31
E	196.00	55.17	0.28

TABLE 3a

Conjugate (lot)	Protein (μg/ml)	Saccharide (μg/ml)	Saccharide/protein (w/w)
α	518.00	82.30	0.16
β	11.00	7.94	0.72
γ	23.22	5.57	0.24
δ	22.87	5.08	0.22

TABLE 3b

Purified *S.aureus* type 5 was conjugated to CRM197 using another method of the invention. In this method, the depolymerisation, oxidation and conjugation steps were carried out as described above, except that the conjugation step was carried out with the derivatised carrier protein described above (CRMadh) instead of CRM197. Total saccharide in the conjugate was determined by HPAEC-PAD analysis and protein content by MicroBCA assay (Table 4).

Conjugate (lot)	Protein (μg/ml)	Saccharide (μg/ml)	Saccharide/protein (w/w)
A'	58.25	2.49	0.043

TABLE 4

Alternative depolymerisation methods

In other studies, different conditions were tested for depolymerisation of the purified capsular polysaccharide. The polysaccharide was dissolved in distilled water at 2 mg/mL. Acetic acid was

added to a final concentration of 2% or 5% (v/v) and the reaction kept at 90°C for 30 minutes, 3 hours, 5 hours or 6 hours. The solution was then neutralized and purified on a gel-filtration column as described above. The saccharide was detected at 215nm and pooled (Figure 6).

The pooled fractions were then oxidised and dialyzed against water as described above. The fractions were conjugated to CRM197 or CRMadh as described above and the resultant conjugates purified by gel-filtration chromatography also as described above (Figure 7).

In another study, hydrochloric acid at 0.5M was used instead of acetic acid for type 8 capsular polysaccharide, and the reaction kept at 90°C for 2.5 hours, with the reaction being sampled every 30 minutes. Samples were analysed by NMR and SEC-HPLC. No depolymerisation was observed, and the level of O-acetylation remained almost unchanged. In contrast, when hydrochloric acid at 2M was used, and the reaction kept at 100°C, depolymerisation was observed even after only 30 minutes. The level of O-acetylation gradually fell over the 2.5 hours (Figures 17 and 18 (with acetyl peak circled)).

Immunisation study – abscess model (1)

General assay protocol: Mice were immunized according to the schedule described below and challenged by intravenous injection of a bacterial suspension of *S.aureus*. The culture of *S.aureus* was centrifuged, washed twice and diluted in PBS before challenge. Further dilutions were needed for the desired inoculum, which was experimentally verified by agar plating and colony formation. For organ harvest, mice were euthanized and their kidneys removed and homogenized in 1% Triton X-100. Aliquots were then diluted and plated on agar media for triplicate determination of CFU. For histology, kidney tissue was incubated at room temperature in 10% formalin for 24 hours. Tissues were embedded in paraffin, thin sectioned, hematoxylin/eosin stained and examined by microscopy.

CD1 mice at 3 weeks old were immunised at days 0 and 11 by intraperitoneal injection with a 5 µg dose of antigen in an injection volume of 200 µl. The mice were bled on days 0 and 20 and challenged with *S.aureus* on day 21. Organs were harvested at day 25. Immunisations were carried out in groups of eight mice according to the following scheme:

Group 1 – Alum alone

Group 2 – Type 5 capsular polysaccharide alone

Group 3 – Type 5 capsular polysaccharide plus alum

Group 4 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 1)

Group 5 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 1) plus alum

The conjugate induced a specific IgG response against type 5 polysaccharide. The alum formulation gave an improved response (Figure 8). The conjugate also induced a specific IgM response against

type 5 polysaccharide (Figure 9). The alum conjugate formulation also gave the best protection from kidney infection (Figure 10).

Immunisation study – abscess model (2)

CD1 mice at 3 weeks old were immunised at days 1, 14 and 28 by intraperitoneal injection with a 5 µg dose of antigen in an injection volume of 200 µl. The mice were bled on days 0, 27 and 37 and challenged with *S.aureus* on day 38. Organs were harvested at day 42. Immunisations were carried out in groups of eight mice according to the following scheme:

Group 1 – Alum alone

Group 2 –Type 5 capsular polysaccharide plus alum

Group 3 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 2) plus alum

Group 4 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot A') plus alum

The conjugates induced a specific IgG response against type 5 polysaccharide. The conjugates of the invention (represented by lot A') gave a particularly high titre (Figure 11). The conjugates of the invention gave the best protection from kidney infection (Figure 12).

Immunisation study – abscess model (3)

CD1 mice at 3 weeks old were immunised at days 1, 14 and 28 by intraperitoneal injection with a 5 µg dose (or 0.5 µg dose in the case of lot A) of antigen in an injection volume of 200 µl. The mice were bled on days 0, 27 and 37 and challenged with *S.aureus* (grown in liquid or solid medium) on day 38. Organs were harvested at day 42. Immunisations were carried out in groups of eight mice according to the following scheme:

Group 1 – Alum alone

Group 2 –Type 5 capsular polysaccharide plus alum

Group 3 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 2) plus alum

Group 4 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 3) plus alum

Group 5 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot A') plus alum

Group 6 – Type 5 capsular polysaccharide-CRM conjugate (Lot A) plus alum

Group 7 – Type 5 capsular polysaccharide-CRM conjugate (Lot B) plus alum

The conjugates of the invention (represented by lots A', A and B) gave protection from kidney infection (Figures 13A and 13B). The conjugates of the invention gave high titres of specific IgG antibodies with low tites of IgM antibodies (Figure 14).

Immunisation study – abscess model (4)

CD1 mice at 3 weeks old were immunised at days 1 and 14 by intraperitoneal injection with a 1μg dose of antigen in an injection volume of 200 μl. The mice were bled on days 0, 13 and 27 and challenged with *S.aureus* on day 28. Organs were harvested at day 32. Immunisations were carried out in groups of eight or nine mice according to the following scheme:

Group 1 – Type 8 capsular polysaccharide-CRM conjugate (lot α) plus alum

Group 2 –Type 8 capsular polysaccharide-CRM conjugate (lot α) plus MF59

Group 3 – Alum alone

Group 4 – MF59 alone

The conjugates of the invention gave protection from kidney infection (Figure 15). The alum formulation gave better protection than the MF59 formulation.

Immunisation study – lethal model (1)

General assay protocol: Mice were immunized according to the schedule described below and challenged by intraperitoneal injection of a bacterial suspension of *S.aureus*. Cultures of *S.aureus* were centrifuged, washed twice and diluted in PBS before challenge. Further dilutions were needed for the desired inoculum, which was experimentally verified by agar plating and colony formation. Animals were monitored for 14 days and lethal disease recorded.

CD1 mice were immunised by intraperitoneal injection with a 5 μg dose of antigen in an injection volume of 200 μl. Immunisations were carried out in groups of twelve mice according to the following scheme, prior to challenge with $5x10^8$ CFU type 5 *S.aureus*:

Group 1 – PBS plus alum

Group 2 – Type 5 capsular polysaccharide-CRM conjugate (Lot C) plus alum

Group 3 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 3) plus alum

The conjugates of the invention (represented by lot C) gave higher survival (Figure 16).

Immunisation study – lethal model (2)

CD1 mice were immunised by intraperitoneal injection with a 2 μg (saccharide) and 10μ (protein, where present) doses of antigen in an injection volume of 200 μl. Immunisations were carried out in groups of twelve mice according to the following scheme, prior to challenge with $5x10^8$ CFU type 5 *S.aureus*:

Group 1 – PBS plus alum

Group 2 – Type 5 capsular polysaccharide-CRM conjugate (Lot D) plus alum

Group 3 – Type 5 capsular polysaccharide-CRMadh conjugate (Lot 4) plus alum

Group 4 – Type 5 capsular polysaccharide-CRM conjugate (Lot D) plus EsxAB, Sta006 and Sta011 proteins and alum

Group 5 – Type 5 capsular polysaccharide-CRM conjugate (Lot D) plus HlaH35L, Sta006 and Sta011 proteins and alum

Survival data is presented in Table 5.

Group	Time (days)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100	25	17	17	17	17	17	17	17	17	8	0	0	0
2	100	50	50	50	50	50	50	50	50	42	42	42	42	42
3	100	50	42	42	42	42	42	42	42	33	33	33	33	33
4	100	67	67	67	67	67	67	67	67	67	67	67	67	67
5	100	100	100	100	100	100	83	83	75	75	75	75	75	75

TABLE 5

The conjugates of the invention (represented by lot D) gave higher survival. Survival was enhanced by addition of *S.aureus* protein antigens.

Immunisation study – lethal model (3)

CD1 mice were immunised by intraperitoneal injection with a 2 μg (type 5 polysaccharide) 1 μg (type 8 polysaccharide, where present) and 10μ (protein, where present) doses of antigen in an injection volume of 200 μl. Immunisations were carried out in groups of twelve mice according to the following scheme, prior to challenge with $5x10^8$ CFU type 5 *S.aureus*:

Group 1 – PBS plus alum

Group 2 – Type 5 capsular polysaccharide-CRM conjugate (lot E) plus EsxAB, Sta006 and Sta011 proteins and alum

Group 3 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot β) plus EsxAB, Sta006 and Sta011 proteins and alum

Group 4 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot β) plus EsxAB, Sta011 and Sta073 proteins and alum

Survival data is presented in Table 7.

Group	Time (days)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100	50	42	42	42	42	42	42	42	42	33	33	33	33
2	100	42	42	42	42	42	42	42	42	42	33	33	33	33
3	100	75	75	75	75	75	75	75	75	75	58	50	50	50
4	100	92	92	83	83	83	83	83	83	83	75	75	75	75

TABLE 7

Immunisation study – lethal model (4)

CD1 mice were immunised by intraperitoneal injection with a 2 μg (type 5 capsular polysaccharide) 1 μg (type 8 capsular polysaccharide, where present) and 10μ (protein, where present) doses of antigen in an injection volume of 200 μl. Immunisations were carried out in groups of twelve mice according to the following scheme, prior to challenge with $5x10^8$ CFU type 5 *S.aureus*:

Group 1 – PBS plus alum

Group 2 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus EsxAB, Sta006 and Sta011 proteins and alum

Group 3 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus alum

Group 4 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus EsxAB protein and alum

Group 5 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus Sta006 protein and alum

Group 6 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus Sta011 protein and alum

Group 7 – Type 5 capsular polysaccharide-CRM conjugate (lot E) and Type 8 capsular polysaccharide-CRM conjugate (lot γ first dose, lot δ second dose) plus Sta006 and Sta011 proteins and alum

Group 8 – Type 5 capsular polysaccharide-CRM conjugate (lot E) plus HlaH35L, Sta006 and Sta011 proteins and alum

Group 9 – Type 5 capsular polysaccharide-CRM conjugate (lot E) plus HlaH35L protein and alum

Survival data is presented in Table 8.

Group	Time (days)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	100	100	13	13	13	13	13	13	13	13	13	13	13	13
2	100	88	75	75	63	63	63	50	50	50	50	50	50	50
3	100	100	63	63	38	38	38	38	38	38	38	38	38	38
4	100	100	75	75	75	75	75	75	63	50	50	25	25	25
5	100	100	50	50	50	50	50	38	38	38	38	38	38	38
6	100	100	25	25	25	25	25	25	25	25	25	13	13	13
7	100	88	63	63	63	63	63	50	50	50	50	50	50	50
8	100	100	100	100	88	88	88	88	88	75	75	75	75	75
9	100	88	88	63	38	38	38	38	38	13	13	13	13	13

TABLE 8

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for preparing a conjugate of a Staphylococcus aureus type 5 or type 8 capsular polysaccharide and a carrier molecule, said process comprising the steps of:
   (a) depolymerising the capsular polysaccharide to provide a polysaccharide fragment;
   (b) oxidising the polysaccharide fragment in order to introduce an aldehyde group into at least one saccharide residue in the polysaccharide fragment to provide an oxidised saccharide residue; and
   (c) coupling the oxidised saccharide residue to a carrier molecule via the aldehyde group, thereby giving the conjugate.

2. A process for treating a Staphylococcus aureus type 5 capsular polysaccharide, said process comprising the step of depolymerising the capsular polysaccharide to provide a polysaccharide fragment having a β-D-FucNAc-(1→ moiety at its non-reducing terminus.

3. A process for treating a Staphylococcus aureus type 8 capsular polysaccharide, said process comprising the step of depolymerising the capsular polysaccharide, to give a polysaccharide fragment having an α-D-FucNAc-(1→ moiety at is non-reducing terminus.

4. The process according to any one of claims 1 to 3, wherein the depolymerising is carried out by acid hydrolysis using acetic acid.

5. The process according to any one of claims 1 to 4, wherein the polysaccharide fragment has an average molecular mass in a range of 5kDa to 100 kDa.

6. The process according to any one of claims 1 to 5, wherein the degree of O-acetylation of the polysaccharide fragment is 10-90%.

7. The process of claim 1 or any one of claims 4 to 6 when dependent on claim 1, wherein step (a) is performed according to the process of claim 2 or claim 3.

8. A process for providing a Staphylococcus aureus type 5 capsular polysaccharide derivative, said process comprising the step of oxidising a S. aureus type 5 capsular polysaccharide having a β -D-FucNAc-(1→ moiety at its non-reducing terminus to convert two vicinal hydroxyl groups in the β -D-FucNAc-(1→ moiety into two aldehyde groups.

9. A process for providing a Staphylococcus aureus type 8 capsular polysaccharide derivative, said process comprising the step of oxidising a S. aureus type 8 capsular polysaccharide having an α-D-FucNAc-(1→ moiety at its non-reducing terminus to convert two vicinal hydroxyl groups in the α-D-FucNAc-(1→ moiety into two aldehyde groups.

10. The process of claim 7, wherein step (b) is performed according to the process of claim 8 or claim 9.

11. A process for providing a coupled Staphylococcus aureus type 5 capsular polysaccharide, said process comprising the step of coupling to a carrier molecule a S. aureus type 5 capsular polysaccharide having a β-D-FucNAc-l→ moiety at its non-reducing terminus that has been oxidised to convert two vicinal hydroxyl groups into two aldehyde groups, wherein the coupling is via one of the aldehyde groups.

12. A process for providing a coupled Staphylococcus aureus type 8 capsular polysaccharide, said process comprising the step of coupling to a carrier molecule a S. aureus type 8 capsular polysaccharide having an α-D-FucNAc-(l → moiety at its non-reducing terminus that has been oxidised to convert two vicinal hydroxyl groups into two aldehyde groups, wherein the coupling is via one of the aldehyde groups.

13. The process of claim 10, wherein step (a) is performed according to the process of claim 2, and step (b) is performed according to the process of claim 8, such that the conjugate prepared is a conjugate of a S. aureus type 5 capsular polysaccharide and a carrier molecule.

14. The process of claim 10, wherein step (a) is performed according to the process of claim 3, and step (b) is performed according to the process of claim 9, such that the conjugate prepared is a conjugate of a S. aureus type 8 capsular polysaccharide and a carrier molecule.

15. The process according to any one of claims 1, 11 or 12, or any one of claims 4 to 7 or 10, 13 or 14 when dependent on claim 1, wherein the coupling is direct coupling by reacting the aldehyde group with an amine group in the carrier by reductive amination.

16. The process according to any one of claims 1, 11 or 12, or any one of claims 4 to 7 or 10, 13 or 14 when dependent on claim 1, wherein the coupling is via a linker by reacting the aldehyde group with an amine group in the linker by reductive amination.

17. The process of claim 16, wherein the linker is attached to the carrier molecule.

18. The process according to claim 1, 11 or 12, or any one of claims 4 to 7 or 10 or 13 to 17 when dependent on claim 1, 11 or 12, wherein the coupling results in a polysaccharide:protein ratio (w/w) in a range of 1:5 and 1:2.

19. A conjugate when obtained by the process according to claim 1 or any one of claims 4 to 7 or 10 or 13 to 18 when dependent on claim 1, or a polysaccharide fragment when obtained by the process according to claim 2 or 3 or any one of claims 4 to 6 when dependent on claim 2 or 3, or a capsular polysaccharide derivative when obtained by the process according to claim 8 or 9 or a

coupled capsular polysaccharide when obtained by the process of claim 11 or 12 or any one of claims 15 to 18 when dependent on claim 11 or 12.

20. An immunogenic composition comprising a conjugate or coupled polysaccharide according to claim 19.

21. The immunogenic composition of claim 20 further comprising one or more S. aureus protein antigen(s) selected from the group consisting of a clfA antigen; a clfB antigen; a sdrE2 antigen; a sdrC antigen; a sasF antigen; a emp antigen; a sdrD antigen; a spa antigen; a esaC antigen; a esxA antigen; a esxB antigen; a sta006 antigen; a isdC antigen; a Hla antigen; a staOl 1 antigen; a isdA antigen; a isdB antigen; and a sta073 antigen.

22. The immunogenic composition of claim 21, wherein the one or more S. aureus protein antigen(s) are selected from the group consisting of a esxA antigen; a esxB antigen; a sta006 antigen; a Hla antigen; a staOl 1 antigen; and a sta073 antigen.

23. The immunogenic composition of claim 22, wherein the composition comprises S. aureus protein antigens according to one of combinations (1) to (10) below:
    (1) a esxA antigen, a esxB antigen, a sta006 antigen and a Hla antigen;
    (2) a esxA antigen, a esxB antigen, a sta006 antigen and a staOl 1 antigen;
    (3) a esxA antigen, a esxB antigen and a staOl 1 antigen;
    (4) a esxA antigen, a esxB antigen, a Hla antigen, a sta006 antigen and a staOl 1 antigen;
    (5) a esxA antigen, a esxB antigen and a Hla antigen;
    (6) a Hla antigen, a sta006 antigen and a staOl 1 antigen;
    (7) a esxA antigen and a esxB antigen;
    (8) a esxA antigen, a esxB antigen and a sta006 antigen;
    (9) a esxA antigen, a esxB antigen, a staOl 1 antigen and a sta073 antigen; and
    (10) a sta006 antigen and a staOl 1 antigen.