

**PURIFICATION OF *STAPHYLOCOCCUS AUREUS* TYPE 5 AND TYPE 8
CAPSULAR SACCHARIDES**

This application claims the benefit of U.S. Provisional Application Serial No. 61/256,905, filed on 30th October 2009, which is incorporated by reference herein in its entirety.

5 TECHNICAL FIELD

This invention is in the field of purifying bacterial capsular polysaccharides, particularly those of *Staphylococcus aureus* type 5 and type 8, and particularly for use in the preparation of vaccines.

BACKGROUND ART

10 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. 96].

15 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
20 references 1 to 9.

The starting point for polysaccharide-based vaccines is the polysaccharide itself, and this is generally purified from the target bacterium. Fattom *et al.* have developed a complex process for purification of the type 5 and type 8 capsular polysaccharides that is described in detail in reference 1, and involves the following key steps after bacterial culture: suspension of bacterial cells in buffer, treatment with
25 lysostaphin, treatment with DNase and RNase, centrifugation, dialysis against buffer, treatment with protease, further dialysis, filtration, addition of ethanol to 25% with calcium chloride to precipitate contaminants; further addition of ethanol to 75% to precipitate the polysaccharide; collection and drying of the precipitate; anion exchange chromatography; dialysis; lyophilisation; size exclusion chromatography; dialysis and final lyophilisation.

30 The Fattom process involves the use of lysostaphin to lyse the bacterial cell walls and thereby release capsular polysaccharide. However, this step is time-consuming and makes the process difficult to scale-up to an industrial setting. It also increases the overall cost and complexity of the process. Other

researchers have attempted to omit this step and develop a simpler, more efficient method of purifying the polysaccharide. For example, reference [10] describes an alternative process that involves autoclaving *S.aureus* cells, ultrafiltration of the polysaccharide-containing supernatant, concentration, lyophilisation, treatment with sodium metaperiodate, further ultrafiltration, diafiltration, high performance size exclusion liquid chromatography, dialysis and freeze-drying. The authors suggest that this method provides a good yield and is suitable for large scale production of polysaccharide. In this method, the lysostaphin treatment is replaced by autoclaving to release capsular polysaccharide. The method was further developed in reference [11]. An important step in these alternative methods is the treatment with sodium metaperiodate. This step is carried out to remove teichoic acid contamination of the capsular polysaccharide. However, once again this step increases the duration, complexity and overall cost of the process. Reference [12] describes a similar process that again involves autoclaving to release capsular polysaccharide and treatment with sodium metaperiodate to remove teichoic acid. In contrast, most other groups use processes that retain lysostaphin treatment (see, for example, references 13, 14, 15, 16, 17 and 18), sometimes including treatment with sodium metaperiodate (*e.g.* in references 13 and 14).

The above methods are complex and may leave contamination in the resultant polysaccharide. There is thus a need for further and improved processes for purifying *S.aureus* type 5 and type 8 capsular polysaccharides, and particularly for less complex processes that result in less contamination.

DISCLOSURE OF THE INVENTION

The invention is based on a purification process in which the polysaccharide is initially released from the bacterial cells by treatment with an acid. This step removes the need for lysostaphin treatment and can be used as an alternative to autoclaving, as in the above processes. The inventors have found that the process results in a purified polysaccharide with low teichoic acid contamination. This means that it is not necessary to treat the polysaccharide with sodium metaperiodate. The purified polysaccharide also has low peptidoglycan contamination, making it particularly suitable for medical uses. The inventors' process can be quick and simple because laborious steps in previous processes are not necessary.

The invention provides a method for releasing capsular polysaccharide from *S.aureus* type 5 or type 8 cells, comprising the step of treating the cells with acid. The invention further provides a process for purifying capsular polysaccharide from *S.aureus* type 5 or type 8 cells comprising this method. Other processing steps may be included in the process, such as enzymatic treatment, *e.g.* to remove nucleic acid, protein and/or peptidoglycan contaminants; diafiltration, *e.g.* to remove low molecular weight contaminants; anion exchange chromatography, *e.g.* to remove residual protein; and concentration.

Accordingly, the invention provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, comprising the step of releasing the polysaccharide from *S.aureus* type 5 or type 8 cells by treating the cells with acid. Similarly, the invention provides, in a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, the improvement consisting of the use of acid treatment of *S.aureus* type 5 or type 8 cells to release the polysaccharide from the cells. Release by acid treatment removes the need for lysostaphin treatment or autoclaving to release the polysaccharide.

The invention also provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the process does not involve a step of lysostaphin treatment. Similarly, the invention provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the process does not involve a step of sodium metaperiodate treatment. Typically, the process does not involve one or both of these steps.

The invention also provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the process provides a composition comprising the polysaccharide and a level of peptidoglycan contamination that is less than 5% (e.g. $\leq 4\%$, $\leq 3\%$, $\leq 2\%$, $\leq 1\%$, etc.) by weight peptidoglycan relative to the total weight of the polysaccharide. Typically, the composition comprises less than 4%, particularly less than 3%, by weight peptidoglycan. The inventors have found that levels of about 2% or even about 1% can be obtained using the methods of the invention. The inventors have found that compositions with this level of peptidoglycan are useful in vaccine manufacture. In contrast, reference 17 teaches that levels above 5% should be used for this purpose. The level of peptidoglycan contamination may be measured using the methods described herein.

Similarly, the invention provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the process provides a composition comprising the polysaccharide and a level of protein contamination that is less than 5% (e.g. $\leq 4\%$, $\leq 3\%$, $\leq 2\%$, $\leq 1\%$, $\leq 0.5\%$, etc.) by weight protein relative to the total weight of the polysaccharide. Typically, the composition comprises less than 3%, particularly about 2.4%, by weight protein. The level of protein contamination may be measured using a MicroBCA assay (Pierce).

The invention also provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the process provides a composition comprising the polysaccharide and a level of nucleic acid contamination that is less than 1% (e.g. $\leq 0.75\%$, $\leq 0.50\%$, $\leq 0.25\%$, $\leq 0.10\%$, $\leq 0.01\%$, etc.) by weight nucleic acid relative to the total weight of the polysaccharide. Typically, the composition comprises less than 0.25%, particularly about 0.09%, by weight nucleic acid. The level of nucleic acid contamination may be measured by absorption at 260nm in a spectrophotometer.

The invention also provides a process for purifying *S.aureus* type 5 or type 8 capsular polysaccharide, wherein (a) the level of peptidoglycan acid contamination is less than 5% (as described above); (b) the level of protein contamination is less than 5% (as described above); (c) the level of nucleic acid contamination that is less than 1% (as described above).

- 5 The invention also provides a composition comprising a *S.aureus* type 5 or type 8 capsular polysaccharide, obtainable by any of the processes of the invention.

In particular, the invention provides a composition comprising *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the composition comprises a level of peptidoglycan contamination that is less than 5% (e.g. $\leq 4\%$, $\leq 3\%$, $\leq 2\%$, $\leq 1\%$, etc.) by weight peptidoglycan relative to the total weight of the polysaccharide. Typically, the composition comprises less than 3%, particularly less than 2%, by weight peptidoglycan. Compositions with levels of about 2% or even about 1% are specifically provided by the invention.

Similarly, the invention provides a composition comprising *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the composition comprises a level of protein contamination that is less than 5% (e.g. $\leq 4\%$, $\leq 3\%$, $\leq 2\%$, $\leq 1\%$, $\leq 0.5\%$, etc.) by weight protein relative to the total weight of the polysaccharide. Typically, the composition comprises less than 3%, particularly about 2.4%, by weight protein.

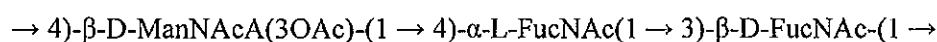
The invention also provides a composition comprising *S.aureus* type 5 or type 8 capsular polysaccharide, wherein the composition comprises a level of nucleic acid contamination that is less than 1% (e.g. $\leq 0.75\%$, $\leq 0.50\%$, $\leq 0.25\%$, $\leq 0.10\%$, $\leq 0.01\%$, etc.) by weight nucleic acid relative to the total weight of the polysaccharide. Typically, the composition comprises less than 0.25%, particularly about 0.09%, by weight nucleic acid.

The invention also provides a composition comprising *S.aureus* type 5 or type 8 capsular polysaccharide, wherein a) a level of peptidoglycan acid contamination is less than 5% (as described above); (b) the level of protein contamination is less than 5% (as described above); (c) the level of nucleic acid contamination that is less than 1% (as described above).

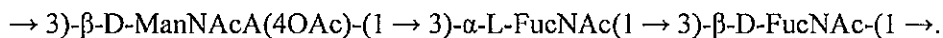
The capsular polysaccharide

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 19 and 20 as:

30 Type 5

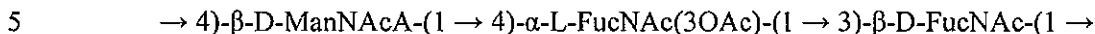


Type 8

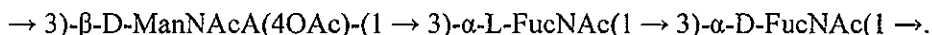


Recent NMR spectroscopy data [21] has led to a revision of these structures to:

Type 5



Type 8



After release from the *S.aureus* type 5 or type 8 cells, 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 other processing steps, but typically occurs before any conjugation step. 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 polysaccharides are 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 22, 23, 24 or 25). A further method is described in reference 26. 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 [27] 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.

Starting material

The process of the invention starts with *S.aureus* type 5 or type 8 cells. Typically, the cells are grown by fermentation prior to release of capsular polysaccharide. Suitable methods of cultivating *S.aureus* type 5 or type 8 cells are well known to the skilled person and are disclosed, for example, in references 1 to 21 and the references cited therein. After cell growth, the cells are usually deactivated. A suitable method for deactivation is treatment with phenol:ethanol, *e.g.* as described in reference 1.

The cells may be centrifuged prior to release of capsular polysaccharide. The process may therefore start with the cells in the form of a wet cell paste. Typically, however, the cells are resuspended in an aqueous medium that is suitable for the next step in the process, *e.g.* in a buffer or in distilled water. The cells may be washed with this medium prior to re-suspension. In another embodiment, the cells may be treated in suspension in their original culture medium. Alternatively, the cells are treated in a dried form.

Acid treatment

In the method of the invention, *S.aureus* type 5 or type 8 cells are treated with acid. This step results in release of capsular polysaccharide from the cells. In contrast, previous methods have used lysostaphin treatment or autoclaving to release the polysaccharide. The acid treatment of the invention is preferably carried out using a mild acid, *e.g.* acetic acid, to minimise damage to the polysaccharide. The skilled person would be capable of identifying suitable acids and conditions (*e.g.* of concentration, temperature and/or time) for release of the polysaccharide. For example, the inventors have found that treatment of

cells suspended at about 0.5mg/ml in distilled water with 1% acetic acid (v/v) at 100°C for 2 hours is suitable. Treatment with other acids, *e.g.* trifluoroacetic or other organic acids, may also be suitable.

The efficacy of different acid treatments may be tested using routine methods. For example, after acid treatment, the cells may be isolated and treated using known methods of *S.aureus* type 5 or type 8 capsular polysaccharide release (*e.g.* the lysostaphin-based method of reference 1) to see if additional capsular polysaccharide can be released. If additional capsular polysaccharide is released, then the acid treatment conditions may be altered so that a greater proportion of the capsular saccharide is released during acid treatment. In this way, it is possible to optimise the acid treatment conditions so that an optimal amount of capsular saccharide is released. For example, the inventors have found that after treatment of cells suspended at about 0.5mg/ml in distilled water with 1% acetic acid (v/v) at 100°C for 2 hours, very little additional capsular saccharide is releasable from the cells by subsequent lysostaphin treatment.

The inventors have found that after acid treatment, the degree of O-acetylation of the type 5 capsular polysaccharide may be between 60-100%. In particular, the degree of O-acetylation may be between the 65-95%, particularly 70-90%. Typically, the degree of O-acetylation is between 75-85%, *e.g.* about 80%. Similar values may be obtained for the type 8 capsular saccharide. If desired, the degree of O-acetylation of the capsular saccharide may then be altered by further processing steps as discussed above.

After acid treatment, the reaction mixture is typically neutralised. This may be achieved by the addition of a base, *e.g.* NaOH. The cells may be centrifuged and the polysaccharide-containing supernatant collected for storage and/or additional processing.

Enzymatic treatment

The polysaccharide obtained after acid treatment may be impure and contaminated with bacterial nucleic acids and proteins. These contaminants may be removed by enzymatic treatment. For example, RNA may be removed by treatment with RNase, DNA with DNase and protein with protease (*e.g.* pronase). The skilled person would be capable of identifying suitable enzymes and conditions for removal of the contaminants. For example, the inventors have found that treatment of polysaccharide-containing supernatant with 50µg/ml each of DNase and RNase at 37°C for 6-8 hours is suitable. Other suitable conditions are disclosed in the literature, *e.g.* in reference 1.

The polysaccharide obtained after acid treatment may also or alternatively be contaminated with peptidoglycan. This contaminant may also be removed by enzymatic treatment. The inventors have found that treatment with mutanolysin is effective at removing peptidoglycan contamination. The

skilled person would be capable of identifying suitable conditions for removal of the peptidoglycan with mutanolysin. For example, the inventors have found that treatment of polysaccharide-containing supernatant with 180U/ml each of mutanolysin at 37°C for 16 hours is suitable. After treatment, the suspension may be clarified by centrifugation and the polysaccharide-containing supernatant collected for storage and/or additional processing.

Diafiltration

The process of the invention may involve a step of diafiltration. This step is typically performed after the acid treatment and/or enzymatic treatment discussed above. The inventors have found that a diafiltration step, particularly by tangential flow filtration, is particularly effective for removing impurities from the polysaccharide. The impurities are typically low molecular weight contaminants like teichoic and/or peptidoglycan fragments. The tangential flow filtration is suitably carried out against 1M NaCl (*e.g.* against about 10 volumes) and then NaPi 10mM pH 7.2 buffer (*e.g.* against another 10 volumes). The filtration membrane should thus be one that allows passage of small molecular weight contaminants while retaining the capsular polysaccharide. A cut-off in the range 10kDa-30kDa is typical. The inventors have found that tangential flow filtration using a 30kDa cut-off membrane is particularly suitable for large-scale processes.

At least 5 cycles of tangential flow diafiltration are usually performed *e.g.* 6, 7, 8, 9, 10, 11 or more.

The polysaccharide-containing retentate from the diafiltration is collected for storage and/or additional processing.

Anion exchange chromatography

The polysaccharide may be further purified by a step of anion exchange chromatography. The inventors have found that anion exchange chromatography is particularly effective at removing residual protein and nucleic acid contamination, while maintaining a good yield of the polysaccharide.

The anion exchange chromatography step may be performed after the acid treatment, enzymatic treatment and/or diafiltration steps discussed above.

The anion exchange chromatography may be carried out using any suitable anionic exchange matrix. Commonly used anion exchange matrices are resins such as Q-resins (based on quaternary amines) and DEAE resins (based on diethylaminoethane). The inventors have found that DEAE-resins (*e.g.* a DEAE-Sepharose™ Fast Flow resin (GE Healthcare)) are particularly suitable, although other resins may be used.

Appropriate starting buffers and mobile phase buffers for the anion exchange chromatography can also be determined by routine experiments without undue burden. Typical buffers for use in anion exchange chromatography include N-methyl piperazine, piperazine, L-histidine, bis-Tris, bis-Tris propane, triethanolamine, Tris, N-methyl-diethanolamine, diethanolamine, 1,3-diaminopropane, ethanolamine, piperidine, sodium chloride and phosphate buffers. The inventors have found that phosphate buffers, e.g. a sodium phosphate buffer, are suitable as the starting buffer for the anion exchange chromatography. The buffer may be at any suitable concentration. For example, 10 mM sodium phosphate has been found to be suitable. Material bound to the anionic exchange resin may be eluted with a suitable buffer. The inventors have found that a gradient of NaCl 1M is suitable.

- 10 Eluate fractions containing polysaccharide may be determined by measuring UV absorption at 215 nm. Fractions containing polysaccharide, usually combined together, are collected for storage and/or additional processing.

The anion exchange chromatography step may be repeated, e.g. 1, 2, 3, 4 or 5 times. Typically the anion exchange chromatography step is carried out once.

15 ***Gel filtration***

The process of the invention may involve one or more step(s) of gel filtration. This gel filtration is used to select polysaccharide molecules of a particular length and to further reduce contamination, particularly by proteins. However, the inventors have found that contrary to previous methods like those of references 1 to 9, a gel filtration step is not required to obtain polysaccharide of high purity.

- 20 Accordingly, this step may be omitted from the processes of the invention. The omission of this step is advantageous because it simplifies the process and reduces the overall cost.

When present, the gel filtration step(s) may be performed after the acid treatment, enzymatic treatment, diafiltration and/or anion exchange chromatography steps discussed above. Typically, any gel filtration step(s) are carried out after the anion exchange chromatography step discussed above.

- 25 The gel filtration step(s) may be carried out using any suitable gel filtration matrix. Commonly used gel filtration matrices are based on dextran gels, agarose gels, polyacrylamide gels, polyacryloylmorpholine gels, and polystyrene gels *etc.* Cross-linked dextran gels and mixed polyacrylamide/agarose gels may also be used. The inventors have found that dextran gels (e.g. a Sephacryl™ S300 gel (GE Healthcare)) are particularly suitable, although other gels may be used.

- 30 Appropriate mobile phase buffers for the gel filtration can be determined by routine experiments without undue burden. Typical buffers for use in gel filtration include N-methyl piperazine, piperazine, L-histidine, bis-Tris, bis-Tris propane, triethanolamine, Tris, N-methyl-diethanolamine, diethanolamine,

1,3-diaminopropane, ethanolamine, piperidine, sodium chloride and phosphate buffers. For example, sodium chloride buffers may be suitable. The buffer may be at any suitable concentration. For example, 50 mM sodium chloride may be used for the mobile phase.

Eluate fractions containing polysaccharide may be determined by measuring UV absorption at 215 nm.

- 5 Fractions containing polysaccharide, usually combined together, are collected for storage and/or additional processing.

Concentration

- 10 In addition to, or instead of, the one or more step(s) of gel filtration, the process of the invention may involve one or more steps of concentrating the polysaccharide. This concentration is useful for obtaining a sample of the correct concentration for any subsequent conjugation of the polysaccharide to a carrier molecule, as described below. However, the inventors have found that this concentration step is not required to obtain polysaccharide of high purity. Accordingly, this step may be omitted from the processes of the invention.

- 15 When present, the concentration step(s) may be performed after the acid treatment, enzymatic treatment, diafiltration, anion exchange chromatography and/or gel filtration steps discussed above. Typically, any concentration step(s) are carried out after the anion exchange chromatography step discussed above. If used in addition to the gel filtration step(s) discussed above, the concentration step(s) may be carried out before or after the gel filtration step(s) discussed above. However, typically, concentration step(s) are used instead of gel filtration step(s).

- 20 The concentration step(s) may be carried out by any suitable method. For example, the inventors have found that the concentration step(s) may be diafiltration step(s) as described above, for example tangential flow filtration using a 30kDa cut-off membrane. For example, a Hydrosart™ (Sartorius) 30kDa cut-off membrane (with a 200 cm² membrane area) may be used.

The concentrated polysaccharide sample is collected for storage and/or additional processing.

25 *Further treatment of the capsular polysaccharide*

After purification, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (e.g. for human vaccine production).

- 30 The molecular mass of the purified *S.aureus* type 5 or type 8 capsular polysaccharide can be measured by gel filtration relative to pullulan standards, such as those available from Polymer Standard Service [28]. Typically, the purified polysaccharide is a mixture of polysaccharides with masses within a range

of values. For the type 5 capsular polysaccharide, the molecular mass of the purified polysaccharide typically is between 2-3500 kDa, *e.g.* between 10-2000 kDa, particularly between 20-1000 kDa and more particularly between 100-600 kDa. Similarly, for the type 8 capsular polysaccharide, the molecular mass of the purified polysaccharide may be between 2-3500 kDa, *e.g.* between 10-2000 kDa, particularly
5 between 20-1000 kDa and more particularly between 100-600 kDa.

The purified polysaccharide may be depolymerised to form an oligosaccharide. Oligosaccharides may be preferred for use in vaccines. Depolymerisation to oligosaccharide may occur before or after any of the steps mentioned above. Typically, depolymerisation takes place after the anion exchange chromatography described above. If the polysaccharide is concentrated after this chromatography, then
10 depolymerisation typically takes place after this concentration. Where the composition of the invention includes a depolymerised polysaccharide, it is preferred that depolymerisation precedes any conjugation

Full-length polysaccharides may be depolymerised to give shorter fragments for use in the invention by various methods. Preferably, the method described in reference 29 is used. Alternatively, other methods for depolymerisation of the polysaccharide may be used. For example, the polysaccharide may be
15 heated or subjected to microfluidisation [30] or sonic radiation [3]. Alternatively, depolymerisation by oxidation-reduction [31] or ozonolysis [32] may be used.

Oligosaccharides can be identified by chromatography, *e.g.* size exclusion chromatography. The products may be sized in order to remove short-length oligosaccharides. This can be achieved in various ways, such as gel filtration. Specific molecular masses can be measured by gel filtration relative to
20 pullulan standards, such as those available from Polymer Standard Service [33].

If N-acetyl groups in the native capsular polysaccharide have been de-N-acetylated then the processes of the invention may include a step of re-N-acetylation. Controlled re-N-acetylation can conveniently be performed using a reagent such as acetic anhydride (CH₃CO)₂O *e.g.* in 5% ammonium bicarbonate [34].

Further rounds of filtration, *e.g.* sterile filtration, can also be performed.

25 These additional steps can generally be performed at room temperature.

Storage

The *S.aureus* type 5 or type 8 capsular polysaccharide preparation may be lyophilised, *e.g.* by freeze-drying under vacuum, or frozen in solution (*e.g.* as the eluate from the final concentration step, if included) for storage at any stage during the purification process. Accordingly, it is not necessary for the
30 preparation to be transferred immediately from one step of the process to another. For example, if the polysaccharide preparation is to be purified by diafiltration, then it may be lyophilised or frozen in

solution prior to this purification. Similarly, the polysaccharide may be lyophilised or frozen in solution prior to the anion exchange chromatography step. If the polysaccharide preparation is to be purified by gel filtration, then it may be lyophilised or frozen in solution prior to this step. Similarly, if the polysaccharide preparation is to be concentrated, then it may be lyophilised or frozen in solution prior to this step. The lyophilised preparation is reconstituted in an appropriate solution prior to further treatment. Similarly, the frozen solution is defrosted prior to further treatment.

The purified polysaccharide obtained by the process of the invention may be processed for storage in any suitable way. For example, the polysaccharide may be lyophilised as described above. Alternatively, the polysaccharide may be stored in aqueous solution, typically at low temperature, *e.g.* at -20°C. Conveniently, the polysaccharide may be stored as the eluate from the anion exchange chromatography, gel filtration or concentration steps.

Conjugation

The final purified capsular polysaccharide of the invention can be used as an antigen without further modification *e.g.* for use in *in vitro* diagnostic assays, for use in immunisation, *etc.*

For immunisation purposes, however, it is preferred to conjugate the polysaccharide to a carrier molecule, such as a protein. In general, covalent conjugation of polysaccharides to carriers enhances the immunogenicity of polysaccharides 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. 35] and is a well known technique [*e.g.* reviewed in refs. 36 to 44]. Thus the processes of the invention may include the further step of conjugating the purified polysaccharide to a carrier molecule.

Conjugation of *S.aureus* type 5 and type 8 capsular polysaccharides has been widely reported *e.g.* see references 1 to 9. The typical process used in the literature for conjugation 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 *Pseudomonas aeruginosa* endotoxin A (ETA), typically via a linker [2]. Conjugate vaccines prepared in this manner have been shown to be safe and immunogenic in humans [5]. Other researchers have carried out conjugation of purified type 5 and type 8 capsular polysaccharides by reductive amination [45 and 12]; glutaraldehyde coupling [45]; or reaction of hydroxyl groups on the polysaccharides with cyanating agents like CDAP [46] or cyanuric trichloride [11]. Preferably, the process described in reference 29 is used.

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 [47] 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]).
5 *S.aureus* α -haemolysin (α -toxin) ([45] and [48]), ovalbumin [11] and human serum albumin [12] 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 [49], synthetic peptides [50,51], heat shock proteins [52,53], pertussis proteins [54,55], cytokines [56], lymphokines [56], hormones [56], growth factors [56], human serum albumin (typically recombinant),
10 artificial proteins comprising multiple human CD4⁺ T cell epitopes from various pathogen-derived antigens [57] such as N19 [58], protein D from *H.influenzae* [59-61], pneumococcal surface protein PspA [62], pneumolysin [63] or its non-toxic derivatives [64], iron-uptake proteins [65], toxin A or B from *C.difficile* [66], a GBS protein [67], a GAS protein [68] etc.

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

Attachment to the carrier is preferably via a -NH₂ group e.g. in the side chain of a lysine residue in a carrier protein, or of an arginine residue. Attachment may also be via a -SH group e.g. in the side chain of a cysteine residue.

It is possible to use more than one carrier protein e.g. to reduce the risk of carrier suppression. Thus
20 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.

25 A single carrier protein might carry more than one polysaccharide antigen [69,70]. 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.

30 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 used. 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 used in the literature tend to have higher ratios, *e.g.* between 0.73 and 1.08 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.

- 5 Conjugates may be used in conjunction with free carrier [71]. 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
10 methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 72 & 73, *etc.*].

Combinations of conjugates and other antigens

Polysaccharides prepared by the methods of the invention (in particular after conjugation as described above) can be mixed *e.g.* with each other and/or with other antigens. Thus the processes of the invention
15 may include the further step of mixing the polysaccharide with one or more further antigens. The invention therefore provides a composition comprising a polysaccharide prepared by the method of the invention and one or more further antigens. The composition is typically an immunogenic composition.

The further antigen(s) may comprise further polysaccharides prepared by the method of the invention, and so the invention provides a composition comprising more than one polysaccharide of the invention.
20 In particular, the present invention provides a composition comprising a type 5 capsular polysaccharide of the invention and a type 8 capsular polysaccharide of the invention. Alternatively, the further antigen(s) may be type 5 or type 8 capsular polysaccharides prepared by methods other than those of the invention, *e.g.* the methods of references 1 to 18 above. Accordingly, the invention provides a composition comprising a type 5 capsular polysaccharide and a type 8 capsular polysaccharide, wherein
25 one of the polysaccharides (the type 5 polysaccharide or the type 8 polysaccharide) is a polysaccharide of the invention and the other polysaccharide is not a polysaccharide of the invention.

Where multiple different *S.aureus* conjugates are mixed then these may include different types of conjugate from the same *S.aureus* serotype and/or conjugates from different *S.aureus* serotypes. For example, the conjugates may be from *S.aureus* type 5 and type 8. The composition will be produced by
30 preparing separate conjugates (*e.g.* a different conjugate for each serotype) and then combining the conjugates.

The further antigen(s) may comprise other *S.aureus* antigens, including the saccharide and protein antigens 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,
5 viral or parasitic antigens. These may be selected from the following:

- a protein antigen from *N.meningitidis* serogroup B, such as those in refs. 74 to 80, with protein ‘287’ (see below) and derivatives (e.g. ‘ΔG287’) being particularly preferred.
- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in refs. 81, 82, 83, 84 etc.
- 10 – a saccharide antigen from *N.meningitidis* serogroup A, C, W135 and/or Y, such as the oligosaccharide disclosed in ref. 85 from serogroup C or the oligosaccharides of ref. 86.
- a saccharide antigen from *Streptococcus pneumoniae* [e.g. refs. 87-89; chapters 22 & 23 of ref. 96].
- an antigen from hepatitis A virus, such as inactivated virus [e.g. 90, 91; chapter 15 of ref. 96].
- 15 – an antigen from hepatitis B virus, such as the surface and/or core antigens [e.g. 91,92; chapter 16 of ref. 96].
- an antigen from hepatitis C virus [e.g. 93].
- 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
20 agglutinogens 2 and 3 [e.g. refs. 94 & 95; chapter 21 of ref. 96].
- a diphtheria antigen, such as a diphtheria toxoid [e.g. chapter 13 of ref. 96].
- a tetanus antigen, such as a tetanus toxoid [e.g. chapter 27 of ref. 96].
- a saccharide antigen from *Haemophilus influenzae* B [e.g. chapter 14 of ref. 96]
- an antigen from *N.gonorrhoeae* [e.g. 74, 75, 76].
- 25 – an antigen from *Chlamydia pneumoniae* [e.g. 97, 98, 99, 100, 101, 102, 103].
- an antigen from *Chlamydia trachomatis* [e.g. 104].
- an antigen from *Porphyromonas gingivalis* [e.g. 105].
- polio antigen(s) [e.g. 106, 107; chapter 24 of ref. 96] such as IPV.
- rabies antigen(s) [e.g. 108] such as lyophilised inactivated virus [e.g. 109, RabAvert™].
- 30 – measles, mumps and/or rubella antigens [e.g. chapters 19, 20 and 26 of ref. 96].
- influenza antigen(s) [e.g. chapters 17 & 18 of ref. 96], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [e.g. 110].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [e.g. 111, 112, 113].

- an antigen from *Streptococcus agalactiae* (group B streptococcus) [e.g. 68, 114-116].
- 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. 117, 118 and 119.

Where a saccharide or carbohydrate antigen is used, it is preferably 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 [95]).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred 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* polysaccharides of the invention can be combined with one or more antigens from the following non-*S.aureus* pathogens: *Streptococcus 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* polysaccharides 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. 120 to 128]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (preferably 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.

5 ***Pharmaceutical compositions and methods***

The invention provides processes for preparing pharmaceutical compositions, comprising the steps of mixing (a) a polysaccharide of the invention (optionally in the form of a conjugate) with (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, 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 129.

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.

The pharmaceutical compositions may be packaged into vials or into 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 polysaccharides of the invention are 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 process for reconstituting such a lyophilised vaccine, comprising the step of mixing the lyophilised material with an aqueous composition of the invention. The reconstituted material can be used for injection.

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 conjugates 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 [130,131]. For example, PNAG may be isolated from *S.aureus* strain MN8m [132]. The saccharide antigen may be a polysaccharide having the size that arises during purification of the exopolysaccharide from bacteria, or it may be a 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 133, 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 134. 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 [135,136]. 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 137-144]: AhpC, AhpF, Autolysin amidase, Autolysin glucosaminidase, Collagen binding protein CAN, EblB, 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 comprise one of the following combinations of
5 *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.
- 10 (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
15 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.
- 20 (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.
- 25 (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
30 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 145.

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).

- 5 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 ('ClfA₄₀₋₅₅₉'). This fragment omits the long repetitive region towards the C-terminal of SEQ ID NO: 1.

clfB

- 20 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 ('ClfB₄₅₋₅₅₂'). 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: ClfB₄₅₋₃₆₀ (also known as CLfB-N12; SEQ ID NO: 5); ClfB₂₁₂₋₅₄₂ (also known as CLfB-N23; SEQ ID NO: 6); and ClfB₃₆₀₋₅₄₂ (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 ('SdrE₅₃₋₆₃₂'). 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 ('SdrC₅₁₋₅₁₈'). 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 ('Emp₃₅₋₃₄₀', 'Emp₂₇₋₃₃₄', 'Emp₃₅₋₃₃₄' and 'Emp₂₇₋₁₄₇', 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 ('SdrD₅₃₋₅₉₂'). 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 SdrD₃₉₄₋₅₉₂ (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 [146]. These domains display ~80% identity at the amino acid level, are 56 to 61 residues in length, and are organized as tandem repeats [147]. SpA is synthesized as a precursor protein with an N-terminal signal peptide and a C-terminal sorting signal [148,149]. Cell wall-anchored spa is displayed in great abundance on the staphylococcal surface [150,151]. 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) [152,153], the VH3 heavy chain (Fab) of IgM (*i.e.* the B cell receptor) [154], the von Willebrand factor at its A1 domain [155] and/or the TNF- α receptor I (TNFRI) [156], 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 157 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 ('Spa₃₇₋₃₂₅'). 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 157, 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. 157, 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 [158]. 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 159. 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 165.

Reference 160 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
 5 (residues 27-76 of SEQ ID NO: 28) [161]. 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
 10 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:
 15 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 162 and 163.

SEQ ID NOs: 33, 34 & 35 are three useful fragments of SEQ ID NO: 28 ('Hla₂₇₋₇₆', 'Hla₂₇₋₈₉' and 'Hla₂₇₋₇₉', respectively). SEQ ID NOs: 36, 37 and 38 are the corresponding fragments from SEQ ID NO: 29.

20 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%,
 25 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
 30 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.

5 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; 'IsdA₄₀₋₁₈₄') 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 164 and 165.

IsdA does not adsorb well to aluminium hydroxide adjuvants, so IsdA present in a composition may be 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 [166], 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 165 and 167 *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 be 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.

- 10 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 $\text{NH}_2\text{-A-}\{-\text{X-L-}\}_n\text{-B-COOH}$, 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.

- 25 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 $X_2 \dots X_n$ will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

- 30 For each n instances of $\{-\text{X-L-}\}$, linker amino acid sequence -L- may be present or absent. For instance, when $n=2$ the hybrid may be $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-L}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-L}_2\text{-COOH}$, 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
 5 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
 10 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 X₁ lacks its own N-terminus methionine, -A- is preferably an
 15 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
 20 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
 25 ('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 168-175, *etc.*

30 "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. 176. 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. 177.

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 [178,179] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [180], matrix-based approaches [181], MAPITOPE [182], TEPITOPE [183,184], neural networks [185], OptiMer & EpiMer [186, 187], ADEPT [188], Tsites [189], hydrophilicity [190], antigenic index [191] or the methods disclosed in references 192-196, *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. The 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.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 illustrates a process for purifying *S.aureus* type 5 and type 8 capsular polysaccharides based on the method of reference 13.

Figure 2 shows a DEAE Sepharose chromatogram of capsular polysaccharide and a ¹H NMR spectrum of capsular polysaccharide-containing fractions (fractions 68-80) prepared according to the method of Figure 1.

Figure 3 shows a S300 Sephacryl chromatogram of capsular polysaccharide and a ¹H NMR spectrum of capsular polysaccharide-containing fractions (fractions 22-44) prepared according to the method of Figure 1.

Figure 4 illustrates an exemplary process of the invention for purifying *S.aureus* type 5 and type 8 capsular polysaccharides.

Figure 5 shows a DEAE Sepharose chromatogram of capsular polysaccharide prepared according to a method of the invention.

Figure 6 shows a ¹H NMR spectrum for purified *S.aureus* type 5 capsular polysaccharide.

Figure 7 shows the chemical structure of the peptidoglycan of *S.aureus* based on references 197, 198, 199 and 200. The repeat unit is highlighted.

MODES FOR CARRYING OUT THE INVENTION

A. Purification of S.aureus type 5 capsular polysaccharide (comparative example)

S.aureus type 5 capsular polysaccharide was purified according to the scheme illustrated in Figure 1, based on the method of reference 13. The conditions and rationale for the various steps of this method are described in Table 1:

Step	Conditions	Rationale
Bacterial growth on plates		
Bacterial pellet centrifugation		Harvest of cells
Reaction with Lysostaphin	100 µg/ml of Lysostaphin overnight at 37°C	Cell wall lysis and release of capsular polysaccharide
Reaction with DNase/RNase	50 µg/ml of DNase and RNase at 37°C for 6-8hrs	Nucleic acid hydrolysis
Reaction with NaIO ₄	0.05M NaIO ₄ for 5hrs at RT in the dark	Teichoic acid hydrolysis
Diafiltration 30kDa	Washing with NaCl 1M and H ₂ O	Low molecular weight species removal
Anion exchange chromatography (DEAE SepharoseFF resin)	NaCl 1M gradient	Separation according to charge (protein removal)
Gel filtration (Sephacryl S300)	NaPi 10 mM pH 7.2 and NaCl 10 mM	Separation according to molecular weight

Table 1

Bacterial pellet centrifugation and enzymatic reactions (Lysostaphin and RNase/DNase)

5 *S.aureus* was grown in solid medium to provide a bacterial suspension of 600-800 ml. The wet cell pellet, harvested by centrifugation at 8000 rpm, had a mass of around 30-50 g. The harvested pellet was washed three times with 50mM Tris-2mM MgSO₄ pH7.5 and then suspended at 0.25-0.5g per ml in 50mM Tris-2mM MgSO₄ pH7.5 and treated with 0.1-0.13mg/ml of lysostaphin (Sigma-Aldrich). The reaction mixture was incubated at 37°C for 16hrs (ON) with mild stirring. 0.05 mg/ml of DNase/RNase (Sigma-Aldrich) was added to the suspension and incubated for 5-7hrs at 37°C. The suspension was then

10 clarified by centrifugation.

Reaction with NaIO₄

The material was incubated with 50mM NaIO₄ (Sigma-Aldrich) in the dark for 5-7hrs. NaIO₄ was then removed by the addition of excess glycerol for 30 minutes with stirring in the light.

30 kDa tangential flow filtration

15 Tangential flow filtration was carried out as indicated in Table 2:

Membrane type	Sartorius Hydrosart™ 30kDa
Surface area	0.1m ²
P _{in} /P _{out}	0.4/0.0 bar
Permeate flow rate	80 ml/min
Diafiltration volumes	10 volumes of NaCl 1M followed by 10 volumes of distilled water
Product recovery	Retentate volume + two washings with distilled water equal to the dead volume of the system (with completely open retentate and closed permeate)

Table 2

The tangential flow filtration was performed in a Sartorius™ holder for 0.1m² cassettes using a WatsonMarlon™ peristaltic pump. Afterwards, the membrane was washed with NaOH 1M and stored in NaOH 0.1M at +2-8°C.

DEAE Sepharose Fast Flow chromatography

Residual protein, nucleic acid and other impurities were removed by anion exchange chromatography carried out in accordance with Table 3:

Resin	DEAE Sepharose™ Fast Flow resin (G&E Healthcare)
Column dimension	Ø = 5cm; h = 7.5cm; V = 150ml
Equilibration	10mM NaPi buffer pH7.2 q.b. to reach 1.8-2.0mS/cm eluate conductivity
Load	Retentate from 30K UF buffered to 10mM NaPi buffer pH7.2
Elution	20 column volumes of 10mM NaPi buffer pH7.2
Stripping	20 column volumes of NaCl 1M

Table 3

- 10 The chromatography was performed using an Akta™ system (G&E Healthcare) and the capsular polysaccharide was detected by measuring UV absorption at 215nm. The capsular polysaccharide solution was first added to 100mM NaPi buffer pH7.2 to obtain a final buffer concentration of 10mM NaPi pH7.2. The DEAE resin was pre-equilibrated with 100mM NaPi buffer pH7.2 to pH7.2 and then equilibrated with 10mM NaPi buffer pH7.2 to achieve the indicated conductivity (10mM NaPi buffer
- 15 pH7.2 conductivity). The resultant fractions were analyzed by NMR and those containing capsular polysaccharide pooled together (Figure 2).

S300 Sephacryl chromatography

The polysaccharide was further purified by gel-filtration chromatography carried out in accordance with Table 4:

Resin	S300 Sephacryl™ resin (G&E Healthcare)
Column dimension	Ø = 2.6cm; h = 95cm; V = 500ml
Equilibration	50mMNaCl buffer q.b. to reach 6.3-6.5mS/cm eluate conductivity
Load	12-14ml
Elution	50mMNaCl buffer

Table 4

The chromatography was performed on an Akta™ system (G&E Healthcare) and the capsular polysaccharide was detected by measuring UV absorption at 215nm. The resultant fractions were analyzed by NMR and those containing capsular polysaccharide pooled together (Figure 3).

5 B. Purification of *S.aureus* type 5 and type 8 capsular polysaccharides (example)

S.aureus type 5 and type 8 capsular polysaccharides were purified according to the scheme illustrated in Figure 4. The conditions and rationale for the various steps of this method are described in Table 5:

Step	Conditions	Rationale
Bacterial growth on plates		
Bacterial pellet centrifugation		Harvest of cells
Reaction with AcOH1%	2hrs at 100°C	Cell wall lysis and release of capsular polysaccharide
Reaction with mutanolysin	180U/ml of mutanolysin at 37°C over-night	Further removal of peptidoglycan
Reaction with DNase/RNase	50 µg/ml of DNase and RNase at 37°C for 6-8hrs	Nucleic acid hydrolysis
Diafiltration 30kDa	Washing with NaCl 1M and H ₂ O	Low molecular weight species removal
Anion exchange chromatography (DEAE SepharoseFF resin)	NaCl 1M gradient	Separation according to charge (protein removal)

Table 5

10 Bacterial pellet centrifugation and acid and enzymatic reactions (acetic acid, RNase/DNase and mutanolysin)

S.aureus was grown in solid medium to provide a bacterial suspension of 600-800 ml. The wet cell pellet, harvested by centrifugation at 8000 rpm, had a mass of around 30-50 g. The harvested pellet was washed three times with 50mM Tris-2mM MgSO₄ pH7.5 and then suspended at 0.5-0.6g per ml in distilled water and stirred vigorously while the temperature was raised to 100°C. Acetic acid was then added to a final concentration of 1% and the mixture kept at 100°C for 2hrs. The mixture was neutralised with NaOH 1M and centrifuged at 8000 rpm.

The supernatant was decanted from the pellet and combined with 0.05 mg/ml of DNase/RNase (Sigma-Aldrich). The mixture was then incubated for 5-7hrs at 37°C and afterwards clarified by centrifugation.

180U/ml of mutanolysin (Sigma-Aldrich) was then added to the suspension and the mixture incubated over-night (for 16hrs) at 37°C with mild stirring. The suspension was then clarified again by centrifugation

30 kDa tangential flow filtration

- 5 Tangential flow filtration was carried out as indicated in Table 6:

Membrane type	Sartorius Hydrosart™ 30kDa
Surface area	0.2m ²
P _{in} /P _{out}	0.7/0.0 bar
Permeate flow rate	11 ml/min
Diafiltration volumes	10 volumes of NaCl 1M followed by 10 volumes of NaPi 10mM pH 7.2 buffer
Product recovery	Retentate volume + two washings with distilled water equal to the dead volume of the system (with completely open retentate and closed permeate)

Table 6

- The tangential flow filtration was performed in a Sartorius™ holder for 0.2m² cassettes using a WatsonMarlon™ peristaltic pump. Afterwards, the membrane was washed with NaOH 1M and stored in NaOH 0.1M at +2-8°C.
- 10

DEAE Sepharose Fast Flow chromatography

Residual protein, nucleic acid and other impurities were removed by anion exchange chromatography carried out in accordance with Table 7:

Resin	DEAE Sepharose™ Fast Flow resin (G&E Healthcare)
Column dimension	Ø = 5cm; h = 7.5cm; V = 150ml
Equilibration	10mM NaPi buffer pH7.2 q.b. to reach 1.8-2.0mS/cm eluate conductivity
Load	Retentate from 30K UF
Elution	20 column volumes of 10mM NaPi buffer pH7.2
Stripping	20 column volumes of NaCl 1M

Table 7

- 15 The chromatography was performed using an Akta™ system (G&E Healthcare) and the capsular polysaccharide was detected by measuring UV absorption at 215nm. The capsular polysaccharide solution was first added to 100mM NaPi buffer pH7.2 to obtain a final buffer concentration of 10mM NaPi pH7.2. The DEAE resin was pre-equilibrated with 100mM NaPi buffer pH7.2 to pH7.2 and then equilibrated with 10mM NaPi buffer pH7.2 to achieve the indicated conductivity (10mM NaPi buffer

pH7.2 conductivity). The resultant fractions were analyzed by NMR and those containing capsular polysaccharide pooled together (Figure 5).

30 kDa tangential flow filtration

5 Tangential flow filtration was carried out to remove NaCl left over from the anion exchange chromatography and to concentrate the purified polysaccharides. The filtration was carried out as indicated in Table 8:

Membrane type	Sartorius Hydrosart™ 30kDa
Surface area	0.2m ²
P _{in} /P _{out}	0.7/0.0 bar
Permeate flow rate	11 ml/min
Diafiltration volumes	10 volumes of distilled water
Product recovery	Retentate volume + two washings with distilled water equal to the dead volume of the system (with completely open retentate and closed permeate)

Table 8

10 The tangential flow filtration was performed in a Sartorius™ holder for 0.2m² cassettes using a WatsonMarlon™ peristaltic pump. Afterwards, the membrane was washed with NaOH 1M and stored in NaOH 0.1M at +2-8°C. The purified polysaccharide was analysed by NMR (e.g. Figure 6 for the type 5 capsular polysaccharide).

C. Determination of peptidoglycan contamination in purified polysaccharide

15 The peptidoglycan (Figure 7) content of purified type 5 polysaccharide obtained according to the methods in sections A and B above was determined by amino acid analysis using HPAEC-PAD according to the Dionex AAA-Direct™ system (AminoPac™ PA10 AAA-Direct™, Dionex) in accordance with the manufacturer's instructions. Briefly, 20μL of 100μM norleucine was added to 200μL of polysaccharide at 250μg/mL in water in a 400°C treated glass tube and dried using a Speedvac system. The norleucine serves as an internal standard. Samples were hydrolyzed *in vacuo* using the
20 vapor of boiling hydrochloric acid/phenol in order to yield free amino acids from residual protein and peptidoglycan contamination. Separation of free amino acids was performed on an AminoPac™ PA10 column (2x250mm) equipped with an AminoPac™ PA10 guard column (2x50mm) using a gradient condition for amino acids and carbohydrates according to the manufacturer's recommendations. These gradient conditions are summarized in Table 9:

Time (min)	%E1	%E2	%E3	Curve	Comments
Initiation	84	16	0		Autosampler fills the sample loop
0.0	84	16	0		Valve from Load to Inject
2.0	84	16	0		Begin hydroxide gradient
12.1	68	32	0	8	
16.0	68	32	0		Begin acetate gradient
24.0	36	24	40	8	
40.0	36	24	40		
40.1	20	80	0	5	Column wash with hydroxide
42.1	20	80	0		
42.2	84	16	0	5	Equilibrate to starting conditions
65.0	84	16	0		

Eluent E1: Deionized Water; Eluent E2: 0.250 M Sodium Hydroxide; Eluent E3: 1.0 M Sodium Acetate and Flow=0.25mL/min

Table 9

Detection was performed using a AAA-Direct waveform potential (Table 10).

Time (sec)	Potential (V) vs. Ag/AgCl	Potential (V) vs. pH	Integration
0.000	-0.20	+0.13	
0.040	-0.20	+0.13	
0.050	0.00	+0.33	
0.210	0.00	+0.33	Begin
0.220	+0.22	+0.55	
0.460	+0.22	+0.55	
0.470	0.00	+0.33	
0.560	0.00	+0.33	End
0.570	-0.20	-1.67	
0.580	-0.20	-1.67	
0.590	+0.60	+0.93	
0.600	-0.20	+0.13	

Table 10

- 5 The quantification was performed using a non-hydrolyzed 17 amino acid standard solution (Fluka P/N 09428) in the range 2.5-50µM. Standard samples were analyzed with and without norleucine, at the same sample concentration. The ratio of the norleucine peak area in the sample divided by the average norleucine peak area in the standards was used as a correction factor for possible amino acid loss in the hydrolysis step. A BSA sample was used as control sample.

10 *Peptidoglycan content estimation*

Peptidoglycan content was estimated using two different methods. The first method (method 1) was based on the method used in reference 17, which involves a summation of the lysine, alanine, glycine and glutamate content. In the second method (method 2), a conversion factor is calculated for each amino acid according to the following formula:

$$\frac{(\text{molecular mass of amino acid}) \times (\text{number of residues in the peptidoglycan structure})}{(\text{molecular mass of the repeating unit of peptidoglycan})}$$

The molecular mass of the repeating unit of peptidoglycan is 1233.27 Da (Figure 7). The peptidoglycan content was then calculated as the average peptidoglycan concentration obtained by calculating the ratio of the amino acid concentration and the conversion factor.

The peptidoglycan content of the purified type 5 capsular polysaccharide after anionic exchange chromatography is given in Table 11:

Measurement method	Details of calculation	% Peptidoglycan	
		Measurement 1	Measurement 2
1	Calculated according to reference 17 as sum of Lys-Ala-Gly-Glx concentration	2.04	0.74
1	Calculated according to reference 17 as sum of all amino acids detectable except for Lys-Ala-Gly-Glx	0.48	0.85
2	Calculated using Ala and Gly concentration divided by PG conversion factor (Ala=0.2167, Gly=0.3043)	0.88	0.81

Table 11

The method of the invention provides a very low content of peptidoglycan in the purified polysaccharide.

D. Conjugation and immunogenicity of purified polysaccharides

Purified type 5 polysaccharides obtained from the methods in sections A and B above were conjugated to CRM197 according to the method of reference 29. Total saccharide in the conjugate was determined by HPAEC-PAD analysis and protein content by MicroBCA assay (Table 12).

Purification method	Lot	Protein (µg/ml)	Saccharide (µg/ml)	Saccharide/protein (w/w)
A	1	51.52	1.72	0.03
A	2	161.80	17.10	0.11
A	3	34.42	4.22	0.12
B	4	444.0	139.0	0.31
B	5	40.56	12.70	0.31

TABLE 12

The conjugates prepared using polysaccharides purified by the method of the invention (lots 4 and 5) had higher polysaccharide:protein ratios.

The immunogenicity of lot 5 was tested in a mouse lethal model of *S.aureus* infection. Briefly, CD1 mice were immunised by intraperitoneal injection with a 2 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 by intraperitoneal injection of a bacterial suspension of 5×10^8 CFU type 5 *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.

Group 1 – PBS plus alum

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

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

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

Survival data is presented in Table 13:

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
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 13

The conjugates prepared using polysaccharides purified by the method of the invention gave a high level of survival. Survival was enhanced by addition of *S.aureus* protein antigens.

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 method for releasing capsular polysaccharide from *S.aureus* type 5 or type 8 cells, comprising the step of treating the cells with acid to release capsular polysaccharide, wherein the cells are not autoclaved prior to treating with acid.
2. The method according to claim 1, wherein the cells are *S.aureus* type 5 cells.
3. The method according to claim 1, wherein the cells are *S.aureus* type 8 cells.
4. The method according to any one of claims 1 to 3, wherein the cells are in the form of a wet cell paste or are suspended in an aqueous medium.
5. The method according to any one of claims 1 to 4 wherein the acid treatment is carried out using acetic acid.
6. The method according to any one of claims 1 to 5, wherein the acid treatment results in the capsular polysaccharide having a degree of O-acetylation between 60-100%.
7. The method according to any one of claims 1 to 6, wherein the method further comprises a step of neutralisation.
8. The method according to any one of claims 1 to 7, wherein the method further comprises a step of centrifugation of the cells and collection of the polysaccharide-containing supernatant.
9. A process for purifying capsular polysaccharide from *S.aureus* type 5 or type 8 cells comprising the method according to any one of claims 1 to 8.
10. The process according to claim 9, wherein the process further comprises a step of treatment of the capsular polysaccharide with DNase and/or RNase.
11. The process according to claim 9 or 10, wherein the process further comprises a step of treatment of the capsular polysaccharide with mutanolysin.
12. The process according to any one of claims 9 to 11, wherein the process further comprises a step of diafiltration.
13. The process according to claim 12, wherein the diafiltration is tangential flow filtration.
14. The process according to any one of claims 9 to 13, wherein the process further comprises a step of anion exchange chromatography.
15. The process according to any one of claims 9 to 14, wherein the process further comprises a step of gel filtration.

16. The process according to any one of claims 9 to 15, wherein the process further comprises a step of concentration of the polysaccharide.
17. The process according to any one of claims 9 to 16, wherein the molecular mass of the purified polysaccharide is between 2-3500 kDa.
18. The process according to any one of claims 9 to 17, wherein the process further comprises a step of depolymerisation of the purified polysaccharide to form an oligosaccharide.
19. The process according to any one of claims 9 to 18, wherein the process further comprises a step of sterile filtration.
20. The process according to any one of claims 9 to 19, wherein the process provides a composition comprising the polysaccharide and a level of peptidoglycan contamination that is less than 5% by weight peptidoglycan relative to the total weight of the polysaccharide.
21. The process according to claim 20, wherein the level of peptidoglycan contamination is about 2%.
22. The process according to any one of claims 9 to 21, wherein the process provides a composition comprising the polysaccharide and a level of protein contamination that is less than 5% by weight protein relative to the total weight of the polysaccharide.
23. The process according to any one of claims 9 to 22, wherein the process provides a composition comprising the polysaccharide and a level of nucleic acid contamination that is less than 1% by weight nucleic acid relative to the total weight of the polysaccharide.
24. The process according to any one of claims 9 to 23, wherein the process further comprises a step of conjugation to a carrier molecule.
25. A composition comprising a *S.aureus* type 5 or type 8 capsular polysaccharide when obtained by the process according to any one of claims 9 to 23 or a conjugate when obtained by the process according to claim 24.
26. The composition according to claim 25 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 *sta011* antigen; a *isdA* antigen; a *isdB* antigen; and a *sta073* antigen.
27. The composition according to claim 26, 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 *sta011* antigen; and a *sta073* antigen.

28. The composition according to claim 27, 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 *sta011* antigen;
- (3) a *esxA* antigen, a *esxB* antigen and a *sta011* antigen;
- (4) a *esxA* antigen, a *esxB* antigen, a *Hla* antigen, a *sta006* antigen and a *sta011* antigen;
- (5) a *esxA* antigen, a *esxB* antigen and a *Hla* antigen;
- (6) a *Hla* antigen, a *sta006* antigen and a *sta011* 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 *sta011* antigen and a *sta073* antigen; and
- (10) a *sta006* antigen and a *sta011* antigen.