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(54) Title: PURIFICATION OF SECRETED POLYSACCHARIDES FROM S. AGALACTIAE

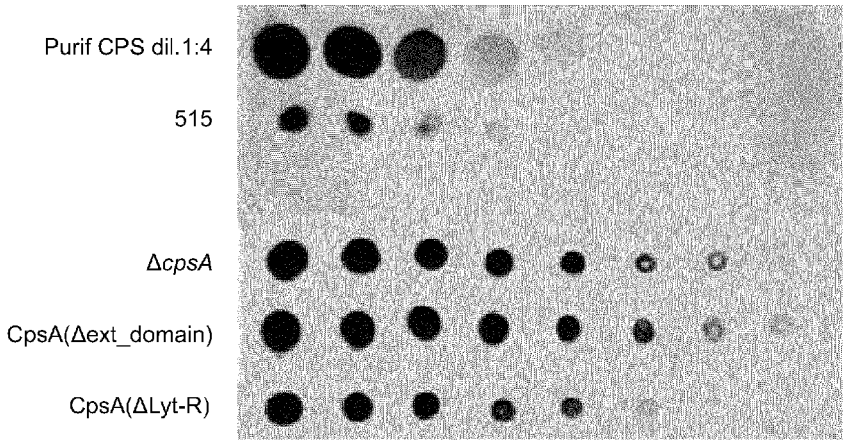


Fig.1

(57) Abstract: The invention relates to bacterial mutants, particularly from *Streptococcus agalactiae,* in which CpsA or CpsD are mutated or deleted and that secrete capsular polysaccharide and methods of purifying the secreted bacterial capsular polysaccharides from culture medium. The extracted polysaccharides are useful for producing vaccines comprising the polysaccharides alone or conjugated to proteins.

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PURIFICATION OF SECRETED POLYSACCHARIDES FROM *S. AGALACTIAE*

FIELD OF THE INVENTION

This invention relates to bacterial mutants, particularly from *Streptococcus agalactiae*, which secrete capsular polysaccharide and methods of purifying the secreted bacterial capsular polysaccharides from culture medium. The extracted polysaccharides are useful for producing vaccines comprising the polysaccharides alone or conjugated to proteins.

BACKGROUND OF THE INVENTION

In the last 25 years, conjugate vaccines, comprising bacterial capsular polysaccharides (cps) conjugated to protein carriers have been developed. Capsular polysaccharides are important immunogens found on the surface of bacteria involved in various bacterial diseases. This feature has led to them being an important component in the design of vaccines. As saccharides are T-independent antigens, generally cps 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.

Therefore, the most effective saccharide vaccines are based on glycoconjugates. Examples include, amongst others, the Haemophilus influenzae type b (Hib) conjugate vaccine, conjugate vaccines against Streptococcus pneumoniae and serogroup C Neisseria meningitidis (MenC). Another bacterium for which conjugate vaccines have been described is Streptococcus agalactiae, also known as 'Group B streptococcus', or simply 'GBS'. The ‘B’ in ‘GBS’ refers to the Lancefield classification which is based on the antigenicity of a carbohydrate which is soluble in dilute acid and called the C carbohydrate. Lancefield identified 13 types of C carbohydrate (designated A to O) that could be serologically differentiated. The organisms that most commonly infect humans are found in groups A, B, D, and G. Within group B, strains of Streptococcus agalactiae are divided into 10 serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) based on the structure of their polysaccharide capsule.

Group B Streptococcus agalactiae causes serious disease, bacteremia and meningitis, in immunocompromised individuals and in neonates. There are two main types of neonatal GBS infection. Early onset disease occurs within 5 days of birth and is manifested by bacteremia (sepsis or infection of the blood) and pneumonia (an infection of the lungs). Late onset disease occurs from the first week of birth up to around three months after birth. Late onset disease is commonly characterized by meningitis (infection of the fluid and lining around the brain) although bacteremia and pneumonia may also occur. GBS colonises the vagina of about 25 percent of young women and is contracted vertically as a baby passes through the birth canal. Approximately 1 percent of infants born via a vaginal birth to colonised mothers will become infected with a mortality rate of between 50-70 percent.

Investigations have been conducted into the development of protein-based and polysaccharide-based vaccines against GBS. Conjugates of each of the capsular polysaccharides vaccines from GBS serotypes Ia, Ib, II, III, and V have been shown to be safe and immunogenic in humans. For example, vaccination of pregnant women with type III cps has been demonstrated to reduce the incidence of the late onset meningitis - infants acquire protective antibodies via placental transfer and are passively immunised.

Large-scale production of capsular polysaccharide vaccines requires adequate supplies of purified capsular polysaccharides. Methods for isolating capsular polysaccharides from bacterial cells exist in the art. For example:

EP0038265 discloses a method of preparing antigenic polysaccharides which comprises phenolizing the fermentation broth to lyse bacteria and release polysaccharide into the fermentation broth.

EP0302887 discloses extraction of GBS type III polysaccharide by the general technique of Jennings et al. (Canadian J. Biochem. 58:112-120 (1980)).

EP1664319 describes a method for producing polysaccharide which comprises: a) using a cationic detergent to precipitate the polysaccharide or part of the contaminants from the supernatant to obtain a first polysaccharide fraction; b) using alcohol to precipitate the polysaccharide from the first polysaccharide fraction to obtain a second polysaccharide fraction; c) subjecting the second

polysaccharide fraction to an alcohol precipitation in the presence of an anionic detergent, whereby the alcohol is present in a concentration which is below the concentration at which the polysaccharide precipitates; d) precipitating the polysaccharide from the soluble fraction using alcohol to obtain a polysaccharide precipitate; e) dissolving the polysaccharide precipitate and subjecting it to concentration and diafiltration.

EP1828230 describes a process for heterologous expression and secretion of complex polysaccharides in non-pathogenic, non-invasive Gram-positive bacteria.

EP1951887 and EP2004223 relate to novel strains of Staphylococcus aureus that produce type 5 capsular polysaccharide at greater levels than wild-type bacteria.

EP1051506 discloses a method for purifying capsular polysaccharides from cellular components of bacteria and culture supernatants. The method utilises alkaline treatment to lyse bacteria but this also causes hydrolysis of the base labile bond that connects the capsular polysaccharide to cellular components it also deacetylates N-acetyl groups.

However, the above methods require many steps of purification, made more complex by attachment of capsular polysaccharide to the cell wall. It is therefore the object of the invention to provide improved methods for producing capsular polysaccharides without the need for bacterial lysis or enzymatic treatment to release polysaccharide thereby simplifying purification and increasing yield.

SUMMARY OF THE INVENTION

The inventors provide a simplified method of production that makes it possible to obtain capsular polysaccharide from the culture medium in significantly greater quantities than was previously possible. By extracting capsular polysaccharide from the culture medium, the method avoids the need for bacterial inactivation and lysis thereby reducing complexity of the method and the length of time required. Advantageously, CpsA and CpsD mutants exhibit reduced virulence such that the risks associated with handling the bacteria are reduced. Similarly, avoiding the use of base extraction results in increases in operator safety whilst

maintaining immunogenicity of extracted polysaccharides because the method avoids deacetylation of N-acetyl groups.

Thus, in a first aspect of the invention there is provided a method for producing a capsular polysaccharide from *Streptococcus agalactiae* comprising: culturing a CpsA or CpsD mutant in a suitable culture medium and recovering the polysaccharide from the culture medium. CpsA and CpsD mutants of the present invention exhibit increased secretion of capsular polysaccharide compared to the wild-type strain.

In a second aspect of the invention, there is provide an isolated polysaccharide from *Streptococcus agalactiae* wherein the polysaccharide has a molecular weight, particularly an average molecular weight, of greater than 800kDa, greater than 900kDa, greater than 1000kDa, greater than 1100kDa, greater than 1200kDa, greater than 1300kDa, greater than 1400kDa, greater than 1500kDa, greater than 1600kDa, about 1700kDa, particularly about 1758kDa or any range there between.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Dot blot with anti Ia capsular polysaccharide monoclonal antibody of secreted capsular polysaccharide. CpsA mutants secreted significantly greater quantities of polysaccharide in comparison with wild-type bacteria (strain 515).

Figure 2: CpsA mutants have significantly less capsular polysaccharide attached to the bacterial cell surface in comparison to wild-type bacteria (strain 515).

Figure 3: CpsA mutants secrete significantly greater amounts of capsular polysaccharide having a broader range of sizes (kDa).

Figure 4: Dot blot with anti Ia capsular polysaccharide monoclonal antibody of secreted capsular polysaccharide. CpsD mutants secreted greater quantities of polysaccharide in comparison with wild-type bacteria (strain 515). Of the exemplified mutants, the K49 and ΔP-tyr secreted greater quantities than both wild-type and ΔCpsD. Thus, preferred mutations occur in the autokinase active site.

Figure 5: CpsD mutants secrete significantly greater amounts of capsular polysaccharide having a greater size than that secreted by wild-type strain 515 (kDa).

DETAILED DESCRIPTION OF THE INVENTION

By introducing mutations into the sequence of the CpsA or CpsD polynucleotide and/or protein, the present inventors have surprisingly discovered that such bacteria produce *greater* quantities of capsular polysaccharide and that the capsular polysaccharide is secreted into the culture medium.

The recombinant *Streptococcus agalactiae* strains secrete an amount of capsular polysaccharide, in mg/l, as determined by the methods described in the Examples, that is greater than that secreted by the wild-type strain cultured under the same culturing conditions. Particularly such mutants exhibit an increase in the level or amount of capsular polysaccharide secreted into the culture medium. Particularly the amount or level is at least 10 percent, at least 20 percent, at least 30 percent, at least 40 percent, at least 50 percent, at least 60 percent, at least 70 percent at least 80 percent, at least 90 percent, at least 100 percent or greater than a wild-type cell of the same serotype. Yet more particularly such mutants exhibit a decreased amount of capsular polysaccharide attached to the cell wall. Particularly an amount or level of less than 10 percent, less than 20 percent, less than 30 percent, less than 40 percent, less than 50 percent, less than 60 percent, less than 70 percent or less than 80 percent that of a wild-type cell of the same serotype.

In the context of the present invention, the term “capsular polysaccharide” is intended to mean the capsular polysaccharides of *Streptococcus agalactiae* In GBS, one of the most important virulence factors is the capsular polysaccharide. To date, ten capsular capsule polysaccharide serotypes have been found: Ia, Ib, II, III, IV, V, VI, VII, VIII and IX.

Capsular saccharides

The capsular saccharide of *Streptococcus agalactiae* is covalently linked to the peptidoglycan backbone of GBS, and is distinct from the group B antigen, which

is another saccharide that is attached to the peptidoglycan backbone. The GBS capsular saccharides are chemically related, but are very different from an antigenic standpoint. All GBS capsular polysaccharides share the following trisaccharide core:

B-D-GlcpNAc(1→3)β-D-Galp(1→4)β-D-Glcp

The various GBS serotypes differ by the way in which this core is modified. The difference between serotypes Ia and III, for instance, arises from the use of either the GlcNAc (Ia) or the Gal (III) in this core for linking consecutive trisaccharide cores. Serotypes Ia and Ib both have a [α D NeupNAc(2→3)β D Galp (1→] disaccharide linked to the GlcNAc in the core, but the linkage is either 1→4 (Ia) or 1→3 (Ib). GBS-related diseases arise primarily from serotypes Ia, Ib, II, III, IV, V, VI, VII, and VIII, with over 85% being caused by five serotypes: Ia, Ib, II, III & V. The invention preferably relates to a saccharide from one or more of these five serotypes, particularly from one or more of serotypes II and V. The capsular saccharides generally include: (a) a terminal N-acetyl-neuraminic acid (NeuNAc) residue (commonly referred to as sialic acid), which in all cases is linked 2→3 to a galactose residue; and (b) a N-acetyl-glucosamine residue (GlcNAc) within the trisaccharide core.

When the GBS saccharide has been purified by base extraction, then O-acetylation is typically lost. Particularly, capsular polysaccharides extracted by the methods of the invention are fully O-acetylated and/or N-acetylated are not de- acetylated (partially or fully). The effect of de-acetylation etc. can be assessed by routine assays.

Particularly the degree of sialic acid oxidation of the GBS capsular polysaccharide is not less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and particularly the N-acetyl-neuraminic acid (NeuNAc or sialic acid) content of the GBS serotype V capsular polysaccharide is greater than 50%, greater than 60%, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, when compared to native GBS serotype polysaccharide wherein the NeuNAc content is considered to be about 100%. Particularly, the GBS polysaccharide is a fully sialylated or “native

polysaccharide". For example, with a sialic acid content of about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, about 90% (or any range between these values) when compared to native GBS polysaccharide.

The saccharide purified according to the invention may be shorter or longer than the GBS polysaccharide found in nature or isolated from a wild-type bacterium. Longer polysaccharides may be depolymerised to give shorter fragments e.g. by hydrolysis in mild acid, by heating, by sizing chromatography, etc.

CpsA

An example of a full-length CpsA amino acid sequence includes Uniprot accession number Q9RPC7 having the following amino acid sequence (SEQ ID NO: 2):

MSNHSRRQQKKHSHTPLRVINLFLLVIFILLSVVSLFLMYRHHFLAFRHLNVIYGV
VIVLIILASLFLCIKNKARIFTTIILVLASIFVATTLYGFKSTIDLTNNLNKTASYSEIEM
SVIVPKDSKITNIEAVSKLAAPVKNDTSNITDLIEHIKSEKGISITPQKTDSYQDAYN
RIKNGDSQAMVLNNAYVSLIELSTPDFKSQIKTIYTYKIKKKINRKNTNHKEGVFNI
YISGIDTFGSISTVSRSDVNIIMTVNTNTHKVLLTTTPRDAYVKIPDGGGNQYDKL
THAGLYGVETSMKTLENLYDINLDYYARINFSSFLKLIDLLGGVTVYNDQAFTSK
HGNFDFPVGQVTLNSEQALGFVRERYSLQGGDNDRGRNQEKVIAAIINKLASSQ
SVTKLNSITSQLQTSVQTNMTIDNINDLINNQLSTGQRFTVESQALTGHGSTGEL
PSYAMPGAQLYMMSIDQSSLSNAKSKIKNTMEE-

In some embodiments, the present invention is based on mutating the CpsA gene by in-frame deletion or other mutation such as substitutions. An in-frame deletion of the CpsA gene can include any truncation of any part of the CpsA gene. In-frame deletions according to the present invention include deletions which remove a segment of the protein coding sequence, yet retain the proper reading frame after the deletion. Some embodiments of the present invention can include deletions that are "clean deletions" i.e., they contain no exogenous DNA sequences inserted into the gene or an in-frame deletion of the CpsA gene may include removal of anywhere from 1 to 485 amino acids. Thus, the inventors have identified residues within SEQ ID NO: 2 that can be modified to alter activity of CpsA such that attachment of capsular polysaccharide to the cell wall is reduced

whilst secretion or release of capsular polysaccharide into the external environment, for example, into the culture medium is increased. Moreover, mutation of these residues can be combined with other modifications such as deletions.

CpsA mutants comprise a mutation in the polynucleotide sequence (SEQ ID NO:1) that encodes the CpsA polypeptide sequence set forth in SEQ ID NO: 2, wherein the mutation results in increased secretion of capsular polysaccharide into the culture medium. Particularly the mutation is selected from the group consisting of an in-frame deletion, a point mutation such as a substitution, a deletion, and an insertion.

In certain embodiments, the mutation results in deletion of the polynucleotide sequence that encodes the entire CpsA polypeptide. In other embodiments, the mutation comprises a deletion, wherein the corresponding protein of the deletion mutation lacks at least 2 amino acids. More particularly the mutation comprises a deletion of the part of the polynucleotide sequence that encodes the LytR and/or PFAM region of the CpsA polypeptide. The LytR domain may comprise amino acids 236 to 458 of SEQ ID NO:2, particularly amino acids 248 to 395 of SEQ ID NO:2, particularly the LytR domain may consist of SEQ ID NO:40. The PFAM region or domain of the CpsA polypeptide may comprise or consist of amino acids 72 to 187 of SEQ ID NO:2, particularly the PFAM domain may comprise or consist of SEQ ID NO: 41. In certain embodiments the CpsA gene comprises a point mutation, particularly a point mutation of one or more of the following residues numbered according to CpsA protein of SEQ ID NO: 2 that causes loss or reduction of CpsA activity and an increase in secretion of capsular polysaccharide: D238, R248, D250, K263, R271, R366, R368, R271, D375, R378, Q382, T437 and E439. Yet more particularly the CpsA gene comprises one or more point mutations from the group consisting of D238, R248, R271 and R366 numbered according to CpsA protein of SEQ ID NO: 2. Not wishing to be bound by theory, the Inventors believe that these residues, which are generally conserved, may be of relevance to substrate binding and/or recognition.

Capsular polysaccharides produced by wild-type *Streptococcus agalactiae* have a molecular weight of about 367kDa. Capsular polysaccharides secreted by CpsA mutants described herein have a molecular weight of greater than 800kDa.

Therefore, invention may also provide purified capsular polysaccharide having a molecular weight, particularly an average molecular weight, of greater than 800kDa, greater than 900kDa, greater than 1000kDa, greater than 1100kDa, greater than 1200kDa, greater than 1300kDa, greater than 1400kDa, greater than 1500kDa, greater than 1600kDa, about 1700kDa, particularly about 1758kDa or any range there between.

Analysis of CpsA mutants has revealed that some capsular polysaccharide is attached to the bacterial cell wall, i.e. it is not all secreted. Analysis of such capsular polysaccharide indicates that it has a molecular weight of about 210kDa. Thus, in certain embodiments capsular polysaccharide may be extracted from the cell wall of CpsA mutants using methods of the prior art to obtain capsular polysaccharide having a molecular weight of less than 300kDa, less than 250kDa, particularly about 210kDa.

CpsD

An example of a full-length CpsD amino acid sequence includes Uniprot accession number K0JNC2 having the following amino acid sequence (SEQ ID NO: 11):

MTRLEIVDSKLRQAKKTEEYFNAIRTNIQFSGKENKILAITSVREGEGKSTTSTSL
ALSLAQAGFKTLLIDADTRNSVMSGTFKATGTIKGLTNYLSGNADLGDIICETNVP
RLMVVPSGKVPPNPTALLQNAYFNKMIEAIKNIFDYIIIDTPPIGLVVDAAIISNACD
GFILVTQAGRIKRNYVEKAKEQMEQSGSKFLGIILNKVSESVATYGDYGDYGNY
GKRDRKRK

In some embodiments, the present invention is based on mutating the CpsD gene by in-frame deletion or other mutation such as substitutions. An in-frame deletion of the CpsD gene can include any truncation of any part of the CpsD gene. In-frame deletions according to the present invention include deletions which remove a segment of the protein coding sequence, yet retain the proper reading frame after the deletion. Some embodiments of the present invention can include deletions that are “clean deletions” i.e., they contain no exogenous DNA sequences inserted into the gene or an in-frame deletion of the CpsD gene may include removal of anywhere from 1 to 232 amino acids. Thus, the inventors have identified residues within SEQ ID NO: 10 that can be modified to alter activity of

CpsD such that attachment of capsular polysaccharide to the cell wall is reduced whilst secretion or release of capsular polysaccharide into the external environment, for example, into the culture medium is increased. Moreover, mutation of these residues can be combined with other modifications such as deletions.

CpsD mutants comprise a mutation in the polynucleotide sequence (SEQ ID NO:10) that encodes the CpsD polypeptide sequence set forth in SEQ ID NO: 11, wherein the mutation results in increased secretion of capsular polysaccharide into the culture medium. Particularly the mutation is selected from the group consisting of an in-frame deletion, a point mutation such as a substitution, a deletion, and an insertion.

In certain embodiments, the mutation results in deletion of the polynucleotide sequence that encodes the entire CpsD polypeptide. In other embodiments, the mutation comprises a deletion, wherein the corresponding protein of the deletion mutation lacks at least 2 amino acids. More particularly the mutation comprises a deletion of the part of the polynucleotide sequence that encodes the phosphoacceptor site (P-tyr region) of the CpsD polypeptide. Other mutations include a deletion of the region from the C-terminal Tyr to Phe. In certain embodiments the CpsD gene comprises one or more point mutations selected from the group consisting of K49, S50, D73 and P154, for example, by way of non-limiting example, K49M and/or S50A. Particularly, mutations in the autokinase active site, particularly a point mutation at position K49 numbered according to CpsD protein of SEQ ID NO: 11, that cause loss or reduction of CpsD activity and an increase in secretion of capsular polysaccharide are useful.

Capsular polysaccharides produced by wild-type *Streptococcus agalactiae* have a molecular weight of about 367kDa. Capsular polysaccharides secreted by CpsD mutants described herein have a molecular weight of greater than 800kDa. Therefore, invention may also provide purified capsular polysaccharide having a molecular weight, particularly an average molecular weight, of greater than 800kDa, greater than 900kDa, greater than 1000kDa, greater than 2000kDa, greater than 3000kDa, greater than 4000kDa, greater than 5000kDa, greater than 6000kDa, greater than 7000kDa, greater than 8000kDa, greater than 9000kDa or any range there between.

Variants

Variants of CpsA and CpsD from other strains of Streptococcus agalactiae are known and can be easily identified by, for example, use of BLAST searches of the above sequence. Variants of CpsA include by way of non-limiting example, Uniprot accession numbers: M1Y5W8, S9KSV2, S9JM66 and S8YXF5, also referred to as LytR family transcriptional regulator, regulatory protein CpsX, capsular polysaccharide biosynthesis protein CpsX and the like. Variants of CpsD include by way of non-limiting example, Uniprot accession numbers: V6H970, S9NRN3, S9PP30, S9E9Q3, also referred to as Tyrosine protein kinase and capsular polysaccharide transporter and the like.

Thus, the invention is also applicable to allelic variants of the disclosed CpsA and CpsD proteins from *Streptococcus agalactiae*. In some embodiments, the degree of sequence identity is greater than 80%, 90%, 95%, 96%, 97%, 97.5%, 98%, 98.5%, 99%, 99.5% or more. These polypeptides include homologs, orthologs, allelic variants and functional mutants. Typically, 50% identity or more between two polypeptides is considered to be an indication of functional equivalence.

Purification

Starting material: CpsA and CpsD mutants secrete and release large amounts of capsular polysaccharide into the culture medium during bacterial growth, and so the starting material for purification is generally the supernatant from a centrifuged bacterial culture. By use of CpsA or CpsD mutants, it is not necessary to treat the capsulated bacteria themselves to release capsular saccharide. Advantageously, since the method of the invention does not require the use of base reagents such as NaOH, the saccharide produced by methods of the invention is not partially or fully de-N-acetylated and the methods of the invention do not comprise or require a step of N-reacetylation.

Alcoholic precipitation and cation exchange: The GBS capsular saccharide obtained after culture will generally be impure and may be contaminated with bacterial nucleic acids and proteins. The process of the invention utilises alcoholic precipitation. Since base extraction is not used, materials will not need to be neutralised prior to the precipitation, again decreasing the length of time taken to purify the capsular polysaccharide.

The alcohol used to precipitate contaminating nucleic acids and/or proteins is preferably a lower alcohol, such as methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols, etc. The selection of an appropriate alcohol can be tested empirically, without undue burden, but alcohols such as ethanol and isopropanol (propan-2-ol) are preferred, rather than alcohols such as phenol.

The alcohol is preferably added to the polysaccharide suspension to give a final alcohol concentration of between 10 percent and 50 percent (e.g. around 30 percent). The most useful concentrations are those which achieve adequate precipitation of contaminants without also precipitating the polysaccharide. The optimum final alcohol concentration may depend on the GBS serotype from which the polysaccharide is obtained, and can be determined by routine experiments without undue burden. Precipitation of polysaccharides as ethanol concentrations>50 percent has been observed.

The alcohol may be added in pure form or may be added in a form diluted with a miscible solvent (e.g. water). Preferred solvent mixtures are ethanol:water mixtures, with a preferred ratio of between around 70:30 and around 95:5 (e.g. 75:25, 80:20, 85:15, 90:10).

The saccharide is also treated with an aqueous metal cation. Monovalent and divalent metal cations are preferred, and divalent cations are particularly preferred, such as Mg++, Mn++, Ca++, etc, as they are more efficient at complex formation. Calcium ions are particularly useful, and so the alcohol mixture preferably includes soluble calcium ions. These may be added to a saccharide/ alcohol mixture in the form of calcium salts, either added as a solid or in an aqueous form. The calcium ions are preferably provided by the use of calcium chloride.

The calcium ions are preferably present at a final concentration of between 10 and 500 mM e.g. about 0.1 M. The optimum final Ca++ concentration may

depend on the GBS serotype from which the polysaccharide is obtained, and can be determined by routine experiments without undue burden.

The alcohol and the cation play different roles (the alcohol is used to precipitate contaminants, whereas the cation stabilises and complexes the saccharide in soluble form) but produce a combined effect. Although the aim is to prepare a mixture of the saccharide, the alcohol and the cation, these three components need not be mixed together simultaneously. Thus the alcohol and cation can be used sequentially or simultaneously. Sequential treatment is preferred, and a particularly preferred process involves addition of the cation to the saccharide followed by addition of the alcohol to the cation/saccharide mixture, although the alcohol can be used before the cation if desired.

After alcoholic precipitation of contaminating proteins and/or nucleic acids, the GBS capsular polysaccharide is left in solution. The precipitated material can be separated from the polysaccharide by any suitable means, such as by centrifugation. The supernatant can be subjected to microfiltration, and in particular to dead-end filtration (perpendicular filtration) in order to remove particles that may clog filters in later steps (e.g. precipitated particles with a diameter greater than 0.22 micrometres). As an alternative to dead-end filtration, tangential microfiltration can be used.

Diafiltration: The process of the invention may involve a step of diafiltration after the precipitation of proteins and/or nucleic acids. Tangential flow diafiltration may be used. The filtration membrane should thus be one that allows passage of impurities while retaining the capsular polysaccharide. A cut-off in the range 10kDa-30kDa is typical. Smaller cut-off sizes can be used but higher cut-off sizes advantageously allow removal of other contaminants without leading to loss of the capsular saccharide. At least 1 cycle of diafiltration may be performed e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more.

In some embodiments, the method further comprises a step of removing contaminating nucleic acids and/or proteins. Particularly contaminating nucleic acids and/or proteins may be removed by the use of precipitation. Yet more

particularly, contaminating nucleic acids and/or proteins may be removed from the capsular polysaccharide in aqueous form by the use of alcoholic precipitation. When a step of alcoholic precipitation is included, an alcohol and an aqueous metal cation may be used to precipitate the nucleic acids and/or proteins leaving the polysaccharide in solution. When a step of alcoholic precipitation is included, the method may include a further step of separating the precipitated material from the polysaccharide. Particularly the precipitated material may be separated from the polysaccharide by filtration and yet more particularly the method may comprise a step of diafiltration after the precipitation of nucleic acids and/or proteins. In some embodiments the alcohol is ethanol or isopropanol. In some embodiments the aqueous metal cation is $CaCl_2$. Particularly, methods of the invention will comprise one or more steps of filtration, for example, ultrafiltration and/or gel filtration. In particular embodiments, gel filtration using Sephacryl ® is performed, for example using Sephacryl ® S-500 gel.

Further treatment of the capsular polysaccharide: 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). For example, further precipitation steps may be used. Where an aqueous re-solubilisation was performed then this precipitation will typically use an alcohol, as described in the preceding section; conversely, where an alcoholic re-solubilisation was performed then this precipitation will typically use an aqueous cation solution, as described in the preceding section. The precipitated saccharide can then be separated from any remaining aqueous contaminants e.g. by centrifugation. The precipitated material is stable and can be stored for future use. Further rounds of precipitation and filtration can also be performed. Depth filtration can also be used e.g. as an alternative to centrifugation. Depth filtration will typically be used after solubilisation in alcohol.

The precipitated material may be subjected to vacuum drying. This treatment will typically be used not to stabilise the saccharide for storage, but to dry the saccharide and remove any residual alcohol. The method produces a purified Streptococcus agalactiae capsular polysaccharide. Particularly the capsular polysaccharide is a sialylated capsular polysaccharide, in other words it is neither

partially nor fully de-N-acetylated. Yet more particularly, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to about 100% of the repeating units of the purified capsular polysaccharide comprise side chains terminated by N-acetylneuraminic acid (sialic acid; Neu5Ac) α2,3 linked to galactose (Gal).

The 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 saccharide to a carrier molecule, such as a protein. In general, covalent conjugation of saccharides to carriers enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens, thus allowing priming for immunological memory. Conjugation is a well known technique. Thus the methods of the invention may include the further step of conjugating the purified saccharide to a carrier molecule. The invention may also provide 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. 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 saccharides 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.

General

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 "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do no materially alter the basic and novel characteristics of the claimed composition, method or structure. The term "consisting of" is generally taken to mean that the invention as claimed is limited to those elements specifically recited in the claim (and may include their equivalents, insofar as the doctrine of equivalents is applicable).

The term "about" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. For example, "substantially free" from Y can be understood as a composition containing not more than 5% Y, not more than 4% Y, not more than 3% Y, not more than 2% Y, not more than 1% Y, or not more than 0.1% Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

As used herein, unless otherwise clear from context the term "or" is understood to be inclusive and can be used interchangeably with the term "and/or".

The term "mutant" refers to a gene or gene product that displays modifications in sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product.

All GenBank Accession numbers provided herein are incorporated by reference in the version available on the date of filing the instant application.

The term "recovering" means the isolation of the capsular polysaccharide in different purities, for example between 5% and 100% purity, preferred purities are in the range of 10% and 99%, 20% and 99%, 30% and 99%, 40% and 99%, 50% and 99%, 60% and 99%, 70% and 99%, 80% and 99%, 90% and 99%. Particular purities are greater than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99%. Recovering may also be referred to as extracting or purifying. The term "purity" takes the general meaning used in the art to refer to the percentage of the in-hand, isolated sample is actually capsular polysaccharide.

This invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLES

Bacterial strains and growth conditions.

GBS 515 (Wessels, et al. 1993) and its isogenic derivatives were grown in Todd-Hewitt broth (THB medium; Difco Laboratories) at 37°C, 5% CO_2. Tryptic soy broth (Difco Laboratories), 15 g/L agar (TSA) was used as solid medium. Strains were stored at -80°C in THB medium + 15% glycerol. MAX Efficiency® DH5α™ Competent Cells (Invitrogen) and competent HK100 *E.coli* cells prepared in house were used for transformation, propagation, and preparation of plasmids. *E. coli* was grown at 37°C with agitation (180 rpm) in Luria-Bertani (LB, Difco laboratories) broth, or on 15 g/L agar plates (LBA). Erythromycin (Erm) was used for selection of GBS (1 µg/ml) or *E. coli* (100 µg/ml) containing the pJRS233-(Perez-Casal et al. 1993) derived plasmids used for mutagenesis. Kanamycin (Kan) was used for selection *E. coli* (50 µg/ml) containing the pET24b-derived plasmids (Novagen) used for initial mutagenesis of inserts, prior to transfer into pJRS233.

Construction of CpsA plasmids for mutagenesis and chromosomal complementation

To prepare each mutant strain, the shuttle vector pJRS233 (Perez-Casal J, *et al.* 1993) containing the gene locus with an in-frame deletion or a codon substitution was constructed. Mutant strains developed are described in the Table 1:

Mutant name	Description	Mutated protein

Δ*cpsA*	*cpsA* deletion	Deletion of aa 11-452 (total=458)
CpsA(Δext_domain)	deletion of the CpsA extracellular domain	Deletion of aa 96-458 (total=458)
CpsA(ΔLyt-R)	Deletion of the CpsA LytR domain	Deletion of aa 236-458

Primers used are listed in Table 2 below:

Name	Sequence	Description
amplification of *cpsA* gene+flanking regions		
NotA5Fsoe	TAAA**GCGGCCGC**CTCTATCACTGACAACAATGG	Forward, 894 bp up-stream *cpsA* start, NotI RS
XhA3Rsoe	TATC**CTCGAG**GAAGAAGTATATTGTGGCGTA	Reverse, 916 bp down-stream *cpsA* end, XhoI RS
***ΔcpsA* mutagenesis**		
KOA5Rsoe	TTGTTGACGGCGCGAATGATTAGACATTGTAA	Reverse, overlapping KOA3Fsoe
KOA3Fsoe	TCGCGCCGTCAACAAAAGAACACAATGGAGGAATAAC	Forward, overlapping KOA5Rsoe
CpsA(Δext_domain) mutagenesis		
M1A5Rsoe	TCCATATAAAGTAGTAGCAACGAAAATAGAAGC	Reverse, overlapping M1A3Fsoe
M1A3Fsoe	ACTACTTTATATGGATAACAAGAATGATTGATATTCATTC	Forward, overlapping M1A5Rsoe
CpsA(ΔLyt-R) mutagenesis		
M2A5Rsoe	ACCGCTAATATAGATATTAAATACCCCTTCTTTATG	Reverse, overlapping M2A3Fsoe
M2A3Fsoe	TCTATATTAGCGGTTAACAAGAATGATTGATATTCATTCTC	Forward, overlapping M2A5Rsoe
qRT-PCR		
SAN_1047F	AGGTTTACTTGTGGCGCTTG	Forward, annealing to *gyrA*
SAN_1047R	TCTGCTTGAGCAATGGTGTC	Reverse, annealing to *gyrA*
SAK_1262F	TCAACTGGACAACGCTTCAC	Forward, annealing to *cpsA*
SAK_1262R	AAGTTGAGCTCCTGGCATTG	Reverse, annealing to *cpsA*
SAK_1258F	TGCTCATATGTGGCATTGTG	Forward, annealing to *cpsE*
SAK_1258R	AGAAAAGATAGCCGGTCCAC	Reverse, annealing to *cpsE*

Constructs for genes with codon substitutions were prepared using a splicing by overlap extension PCR (SOEing-PCR) strategy. Briefly, the two parts of the gene up- and downstream of the codon substitution were amplified from 515 gDNA using the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies). 900-1000 bp upstream and downstream the coding sequence of the gene were added to the inserts. Primers used to amplify the two parts of the genes have 15 bp overlapping tails and introduce the codon substitution in the two PCR products that are then joined together by SOEing-PCR. The resulting fragment was ligated into pJRS233 using BamHI and XhoI restriction sites.

Constructs for genes with in-frame deletions were prepared using the Polymerase Incomplete Primer Extension (PIPE) method. Briefly, the gene plus 900-1000 bp up- and downstream the coding sequence were amplified from 515 gDNA and cloned into pET24b using NotI and XhoI (*cpsA* inserts) or BamHI and

XhoI (*cpsD* inserts) restriction sites. In frame deletions of the genes were developed by amplifying the plasmid using primers with 15 bp overlapping tails annealing at the two sides of the region to delete. Linear plasmids were transformed into HK100 competent cells able to re-circularize the plasmid. The resulting inserts were then transferred into pJRS233 plasmid by restriction digestion and ligation.

Constructs for chromosomal complementation were prepared transferring into pJRS233 the wild type inserts cloned into pET24b.

Construction of CpsD plasmids for mutagenesis and chromosomal complementation

To prepare each mutant strain, the shuttle vector pJRS233 (Perez-Casal J, *et al.* 1993) containing the gene locus with an in-frame deletion or a codon substitution was constructed. Mutant strains developed are described in the Table 3:

Mutant name	Description	Mutated protein
Δ*cpsD*	*cpsD* deletion	Deletion of aa 11-225 (total=232)
CpsD(K49A)	Point mutation in the autokinase active site	Lysine to alanine in position 49 (total=232)
CpsD(ΔP-Tyr)	Phosphoacceptor site C-terminal deletion	Deletion of aa 213-224 (total=232)

Primers used are listed in Table 2 below:

Name	Sequence	Description
amplification of cpsD gene+flanking regions		
BaD5Fsoe	TTTA**GGATCC**CAAAAAGAACGGGTGAAGGAA	Forward, 1018 bp up-stream cpsA start, BamHI RS
XhD3Rsoe	TCTA**CTCGAG**CTACCATTACGACCTACTCTA	Reverse, 966 bp down-stream cpsA end, XhoI RS
Δ*cpsD* mutagenesis		
KOD5Rsoe	GCTATCAACTATTTCTAAACGAGTCATTATATTCTC	Reverse, overlapping KOD3Fsoe
KOD3Fsoe	GAAATAGTTGATAGCAAAAGGGATAGAAAAAGGAAGTAA	Forward, overlapping KOD5Rsoe
CpsD(K49A) mutagenesis		
M1DmutF	GGAAGGGGAAGGA**GC**ATCCACTACTTCA	Reverse, overlapping M1DmutR
M1DmutR	TGAAGTAGTGGAT**GC**TCCTTCCCCTTCC	Forward, overlapping M1DmutF
CpsD(ΔP-Tyr) mutagenesis		
M2D5Rsoe	AACAGATTCACTAACTTTATTAAGAATAATACCTAAGAAC	Reverse, overlapping M2D3Fsoe
M2D3Fsoe	GTTAGTGAATCTGTTGGAAAAAGGGATAGAAAAAGG	Forward, overlapping M2D5Rsoe
qRT-PCR		
SAN_1047F	AGGTTTACTTGTGGCGCTTG	Forward, annealing to *gyrA*
SAN_1047R	TCTGCTTGAGCAATGGTGTC	Reverse, annealing to *gyrA*
SAK_1262F	TCAACTGGACAACGCTTCAC	Forward, annealing to *cpsA*
SAK_1262R	AAGTTGAGCTCCTGGCATTG	Reverse, annealing to *cpsA*
SAK_1258F	TGCTCATATGTGGCATTGTG	Forward, annealing to *cpsE*
SAK_1258R	AGAAAAGATAGCCGGTCCAC	Reverse, annealing to *cpsE*

Constructs for genes with codon substitutions were prepared using a splicing by overlap extension PCR (SOEing-PCR) strategy. Briefly, the two parts of the gene up- and downstream of the codon substitution were amplified from 515 gDNA using the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies). 900-1000 bp upstream and downstream the coding sequence of the gene were added to the inserts. Primers used to amplify the two parts of the genes have 15 bp overlapping tails and introduce the codon substitution in the two PCR products that are then joined together by SOEing-PCR. The resulting fragment was ligated into pJRS233 using BamHI and XhoI restriction sites.

Constructs for genes with in-frame deletions were prepared using the Polymerase Incomplete Primer Extension (PIPE) method. Briefly, the gene plus 900-1000 bp up- and downstream the coding sequence were amplified from 515 gDNA and cloned into pET24b using NotI and XhoI (*cpsA* inserts) or BamHI and XhoI (*cpsD* inserts) restriction sites. In frame deletions of the genes were developed by amplifying the plasmid using primers with 15 bp overlapping tails annealing at the two sides of the region to delete. Linear plasmids were transformed into HK100 competent cells able to re-circularize the plasmid. The resulting inserts were then transferred into pJRS233 plasmid by restriction digestion and ligation.

Constructs for chromosomal complementation were prepared transferring into pJRS233 the wild type inserts cloned into pET24b.

Construction of isogenic mutants and chromosomally complemented strains

An insertion/duplication and excision mutagenesis strategy was used both to obtain the in-frame deletion/codon substitution in the genes and to replace the mutations to obtain the chromosomally complemented strains. Briefly, pJRS233-derived plasmids purified from *E. coli* were used to transform electrocompetent 515 cells by electroporation. Transformants were selected by growth on TSA + Erm at 30°C for 48 hours. Integration was performed by growth of transformants

at 37°C (non-permissive temperature for the suicide shuttle vector) with Erm selection. Excision of the integrated plasmid was performed by serial passages in THB at 30°C, and parallel screening for Erm-sensitive colonies on plate. Mutants were verified by PCR sequencing of the loci.

To obtain the chromosomally complemented strains, pJRS233-derived plasmids containing the wild type version of the genes and the flanking 900-1000 bp up- and downstream were purified from *E. coli* and complementation of the respective mutant strains was performed as described for mutagenesis.

qRT-PCR analysis

Bacteria were harvested at two time points: at OD_{600}=0.4 (log phase) and OD_{600}=1.7 (early stationary phase). To rapidly arrest transcription, 10 ml of bacteria were cooled on ice and added to 10 ml of frozen THB medium in a 50 ml conical tube. GBS cells were then collected by centrifugation for 15 min at 4000 rpm, 4°C, and resuspended in 800 µl of TRIzol (Invitrogen). Bacteria were disrupted mechanically by agitation with Lysing matrix B in 2 ml tubes (DBA Italy) using a homogenizer (Fastprep-24, Millipore) for 60 sec at 6.5 m/s for two cycles, and kept on ice for 2 min between the cycles. Samples were then centrifuged for 5 min at 8000 x *g*, 4°C and RNA was extracted with Direct-zol™ RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. RNA samples were treated with DNase (Roche) for 2 h at 37°C and further purified using the RNA MiniPrep kit (Qiagen), including a second DNase treatment on the column for 30 min at room temperature (RT), according to the manufacturer's instructions. cDNA was prepared using the Reverse Transcription System (Promega) by using 500 ng of RNA per reaction. Real time quantitative PCR (qRT-PCR) was performed on 50 ng of cDNA that was amplified using LightCycler® 480 DNA SYBR Green I Master, (Roche). Reactions were monitored using a LightCycler® 480 instrument and software (Roche). Three technical replicates were monitored for each strain/condition analyzed. To quantify *cps* operon transcription level primers annealing on *cpsA* and *cpsE* were used respectively for *cpsA* mutants. The transcript amounts in each condition were standardized to an internal control gene (*gyrA*) and compared with standardized expression in the wild-type strain ($\Delta\Delta C_T$ method).

Quantification of the capsular polysaccharide attached to the cell surface

An overnight culture was used to inoculate (1:1000) 50 ml of fresh THB and bacteria were grown at 37°C for 8 hours. GBS cells were collected by centrifugation for 15 min at 4000 rpm at 4°C, resuspended in 1.1 ml of PBS + 0.8 M NaOH and incubated at 37°C for 36 hours. Samples were neutralized and pelleted by centrifugation for 10 min at 4000 rpm, 4°C. 850 µl of the supernatant were diluted in 7.15 ml of water, and centrifuged for 10 min at 4000 rpm at 4°C. 7.2 µl of the supernatant were loaded on a Vivaspin 10 tube (Sartorius Stedim Biotech) which was centrifuged at 4000 rpm until most of the solution passed through the membrane. After two washes with 1 ml of water, the CPS extract was recovered from the membrane and resuspended in 1.6 ml of water. The amount of CPS present in the extract was estimated by measuring the sialic acid content using the colorimetric resorcinol-hydrochloric acid method (Svennerholm *et al*, 1957). Briefly, 120 µl of extract were mixed with 380 µl of water and 500 µl of freshly prepared solution R3 (resorcinol 0.2%, copper sulfate 0.3 mM, HCl 30% in H_2O). Samples were boiled for 20 min and then cooled to room temperature before being transferred into 1 ml cuvettes to measure their absorbance at 564 nm. The sialic acid content of the samples was then calculated using a concomitantly prepared standard curve using serial dilutions of purified sialic acid. CPS extracts were prepared three times from independent growths to minimize the biological variability.

Quantification of the capsular polysaccharide released in the growth medium

An overnight culture was used to inoculate (1:1000) 10 ml of fresh THB and bacteria were grown at 37°C for 8 hours. GBS cells were pelleted by centrifugation for 15 min at 4000 rpm at 4°C, and the growth medium was collected and filtered using a 0.22 µm Nalgene Syringe Filter (Thermo Scientific). The amount of capsular polysaccharide released in the growth medium was estimated by dot blot. Purified serotype Ia CPS 10 mg/ml was used as standard. Eight serial dilutions were prepared in a 96-well plate by diluting the standard and the growth media in PBS (dilution ratios 1:2 for media, 1:4 for the standard). 2 µl of each serial dilution were spotted onto a nitrocellulose membrane. The

membrane was dried for 20 min and blocked by soaking in 5% (w/v) skim milk in PBS-Tween 0.05%. The membrane was then probed with a primary mouse monoclonal anti- serotype Ia CPS-CRM conjugated antibody (30E9/B11) used at 1:2000, washed 3 times in PBS-Tween 0.05%, and incubated in 1:15000 of secondary goat anti-mouse antibody conjugated to horseradish peroxidase. Detection was performed using Thermo Scientific Pierce ECL Western Blotting Substrate according to the manufacturer's instructions.

Western blot on the capsular polysaccharide released in the growth medium

20 µl of medium were mixed with 10 µl of 3x NuPAGE® LDS Sample Buffer + Reducing Agent and boiled for 5 min. Then, 20 µl were loaded on a NuPage 4-12% Bis-Tris Gel 1.0 mm, 12 well (Life Technologies) (running buffer: MOPS 1x) and run at 150V until the band corresponding to 28 kDa of the SeeBlue® Plus2 Pre-stained Protein Standard (Life Technologies) reached the bottom of the gel. The samples separated on the gel were transferred to a nitrocellulose membrane using the iBlot® 7-Minute Blotting System (Life Technologies). The membrane was blocked blocked by soaking in 5% (w/v) skim milk in PBS-Tween 0.05%. The membrane was then probed with a primary mouse monoclonal anti- serotype Ia CPS-CRM conjugated antibody (30E9/B11) used at 1:2000, washed 3 times in PBS-Tween 0.05%, and incubated in 1:15000 of secondary goat anti-mouse antibody conjugated to horseradish peroxidase. Detection was performed using Thermo Scientific Pierce ECL Western Blotting Substrate according to the manufacturer's instructions.

Purification of capsular saccharide from culture media

Supernatant from culture medium was collected by centrifugation following culture of group B *streptococcus* CpsA mutants. A mixture of aqueous ethanol (30%) and CaCl2 (0.1M) was added to the culture medium. A precipitate rapidly formed, which was removed by centrifugation. Sialic acid assays showed that the capsular saccharide remained in the supernatant. The supernatant was subjected to dead-end microfiltration in regenerated cellulose filters (0.22µm cut-off). The supernatant was than subjected to a tangential flow filtration (TFF) using a 30kDa cut-off cellulose membrane against Tris 50mM/NaCl 500mM pH8.8 and followed

by diafiltration against NaPi 10mM, pH7.2. The precipitate was again removed by centrifugation. A further step of gel filtration using Sephacryl S-500 resin was used. The polysaccharide may be recovered as a pool of fractions, that in some cases, are dried, for example, by vacuum drying.

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

REFERENCES

Wessels, M. R., Paoletti, L. C., Rodewald, A. K., Michon, F., DiFabio, J., Jennings, H. J. & Kasper, D. L. (1993) Infect. Immun. 61, 4760–4766.

Perez-Casal J, Price JA, Maguin E, Scott JR. (1993) Mol Microbiol 8, 809-819.

Svennerholm L., (1957) Biochim Biophys Acta. 24(3), 604-611.

INDEX OF SEQUENCE IDs

SEQ ID NO:1 - CpsA DNA sequence

SEQ ID NO: 2 - CpsA amino acid sequence

SEQ ID NO: 3 - CpsA LytR amino acid sequence (aa236-458)

SEQ ID NO: 4 - CpsA ΔLytR polynucleotide sequence

SEQ ID NO: 5 - CpsA ΔLytR amino acid sequence

SEQ ID NO: 6- ΔcpsA Nucleotide sequence:

SEQ ID NO: 7 - ΔcpsA Amino acid sequence:

SEQ ID NO: 8 - CpsA(Δext_domain) Nucleotide sequence:

SEQ ID NO: 9 - CpsA(Δext_domain) Amino acid sequence:

SEQ ID NO: 10 - cpsD wild type Nucleotide sequence:

SEQ ID NO: 11 - cpsD wild type Amino acid sequence:

SEQ ID NO: 12 - ΔcpsD Nucleotide sequence:

SEQ ID NO: 13 - ΔcpsD Amino acid sequence:

SEQ ID NO: 14 - CpsD(K49A) Nucleotide sequence:

SEQ ID NO: 15 - CpsD(K49A) Amino acid sequence:

SEQ ID NO: 16 - CpsD(ΔP-Tyr) Nucleotide sequence:

SEQ ID NO: 17 - CpsD(ΔP-Tyr) Amino acid sequence:

SEQ ID NO: 18 - Primer NotA5Fsoe

SEQ ID NO: 19 – Primer XhA3Rsoe

SEQ ID NO: 20 – Primer KOA5Rsoe

SEQ ID NO: 21 – Primer KOA3Fsoe

SEQ ID NO: 22 – Primer M1A5Rsoe

SEQ ID NO: 23 – Primer M1A3Fsoe

SEQ ID NO: 24 – Primer M2A5Rsoe

SEQ ID NO: 25 – Primer M2A3Fsoe

SEQ ID NO: 26 – Primer BaD5Fsoe

SEQ ID NO: 27 – Primer XhD3Rsoe

SEQ ID NO: 28 – Primer KOD5Rsoe

SEQ ID NO: 29 – Primer KOD3Fsoe

SEQ ID NO: 30 – Primer M1DmutF

SEQ ID NO: 31 – Primer M1DmutR

SEQ ID NO: 32 – Primer M2D5Rsoe

SEQ ID NO: 33 – Primer M2D3Fsoe

SEQ ID NO: 34 – Primer SAN_1047F

SEQ ID NO: 35 – Primer SAN_1047R

SEQ ID NO: 36 – Primer SAK_1262F

SEQ ID NO: 37 – Primer SAK_1262R

SEQ ID NO: 38 – Primer SAK_1258F

SEQ ID NO:39 – Primer SAK_1258R

SEQ ID NO:40 – CpsA LytR Domain aa248-395

SEQ ID NO: 41 – CpsA PPF/PFAM Domain aa72-187

CLAIMS

1. A method for producing a capsular polysaccharide from *Streptococcus agalactiae* comprising: culturing in a suitable culture medium a CpsA or CpsD mutant that exhibits increased secretion of capsular polysaccharide compared to the wild-type strain and recovering the polysaccharide from the culture medium.

2. The method of claim 1 wherein the CpsA mutant comprises:

(a) an altered CpsA protein wherein the nucleotide sequence that encodes an amino acid sequence comprising the CpsA protein (SEQ ID NO:2) is deleted or partially deleted; or

(b) an altered CpsA protein wherein the nucleotide sequence that encodes (i) the LytR domain of SEQ ID NO:40 or SEQ ID NO:3 and/or (ii) the PFAM domain of SEQ ID NO:41 is deleted; or

(c) an altered CpsA protein, the altered CpsA protein having a substitution of at least one amino acid residue selected from the group consisting of: R271, R366, D375, R378 and Q382 numbered according to CpsA protein (SEQ ID NO: 2); and/or

(d) an altered CpsA protein, the altered CpsA protein having a substitution of at least one amino acid residue selected from the group consisting of: D238, R248, D250, R271 and R368 numbered according to CpsA protein (SEQ ID NO: 2);

and wherein the CpsA mutant exhibits increased secretion of capsular polysaccharide compared to the wild-type strain.

3. The method of claim 1 wherein the CpsD mutant comprises:

(a) an altered CpsD protein wherein the nucleotide sequence that encodes an amino acid sequence comprising SEQ ID NO:13 in wild-type *Streptococcus agalactiae* is deleted; or

(b) an altered CpsD protein, the altered CpsD protein comprising a substitution of at least one amino acid residue selected from the group consisting of K49, S50, D73 and P154 numbered according to SEQ ID NO:11; or

(c) an altered CpsD protein encoded by the nucleic acid sequence SEQ ID NO: 16;

wherein the CpsD mutant exhibits increased secretion of capsular polysaccharide compared to the wild-type strain.

4. The method of claim 1, 2 or 3, wherein the mutant exhibits an increase in the level of secreted polysaccharide that is at least 10 percent, at least 20 percent, at least 30 percent, at least 40 percent, at least 50 percent, at least 60 percent, at least 70 percent at least 80 percent, at least 90 percent, at least 100 percent or greater than 100 percent more than a wild-type cell of the same serotype.

5. The method of any preceding claim, wherein the polysaccharide is from a *Streptococcus agalactiae* serotype selected from the group consisting of Ia, Ib, II, III, IV, V, VI, VII or VIII, particularly from serotypes Ia, Ib, II, III or V.

6. The method of any preceding claim, wherein the polysaccharide has a molecular weight greater than 800kDa.

7. The method of any preceding claim, wherein the saccharide is neither partially nor fully de-N-acetylated.

8. The method of any preceding claim, further comprising the step of removing contaminating nucleic acids and/or proteins by the use of precipitation.

9. The process of claim 8, wherein the process comprises the steps of: (a) removing contaminating nucleic acids and/or proteins from the capsular polysaccharide in aqueous form by the use of alcoholic precipitation, wherein an alcohol and an aqueous metal cation are used to precipitate the nucleic acids and/or proteins leaving the polysaccharide in solution; (b) separating the precipitated material from the polysaccharide.

10. The method of claim 8 or 9, wherein the process further comprises a step of diafiltration after the precipitation of nucleic acids and/or proteins.

11. The process of claim 9 or 10, wherein the alcohol is ethanol or isopropanol.

12. The process of claim 11, wherein the aqueous metal cation is $CaCl_2$.

13. The process of any one of claims 8 to 12, further comprising one or more steps of filtration.

14. A purified *Streptococcus agalactiae* capsular polysaccharide obtained by the process of any preceding claim.

15. An immunogenic composition, particularly a vaccine composition, comprising the capsular polysaccharide of claim 14.

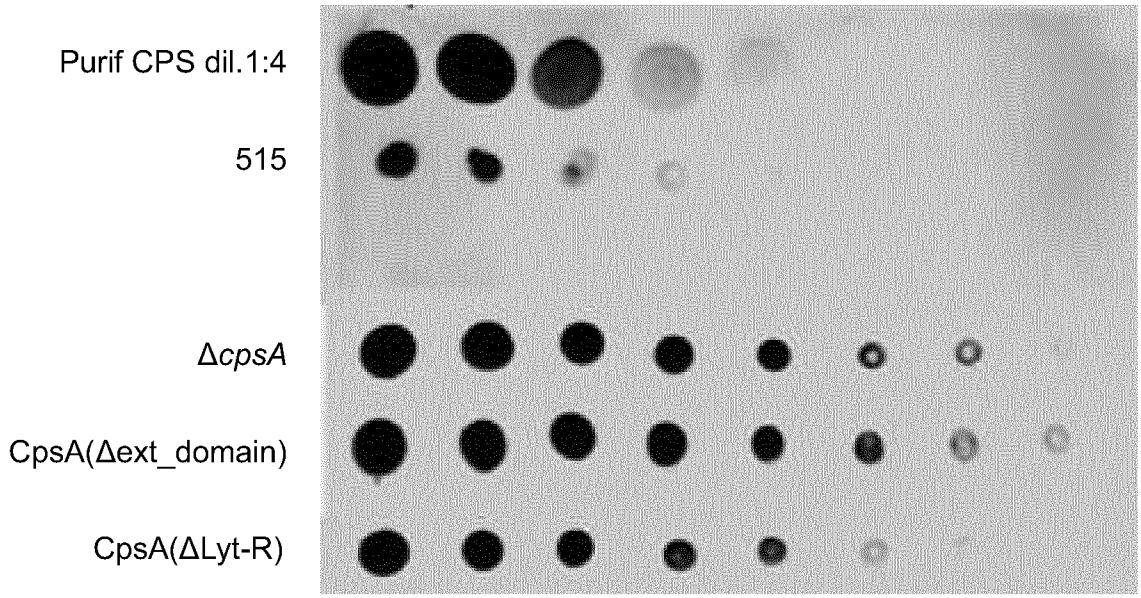


Fig.1

Capsule attached to the bacterial surface (resorcinol assay)

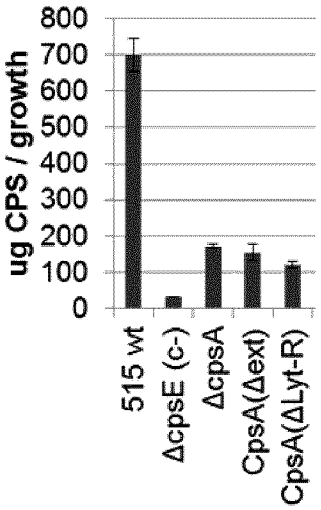


Fig. 2

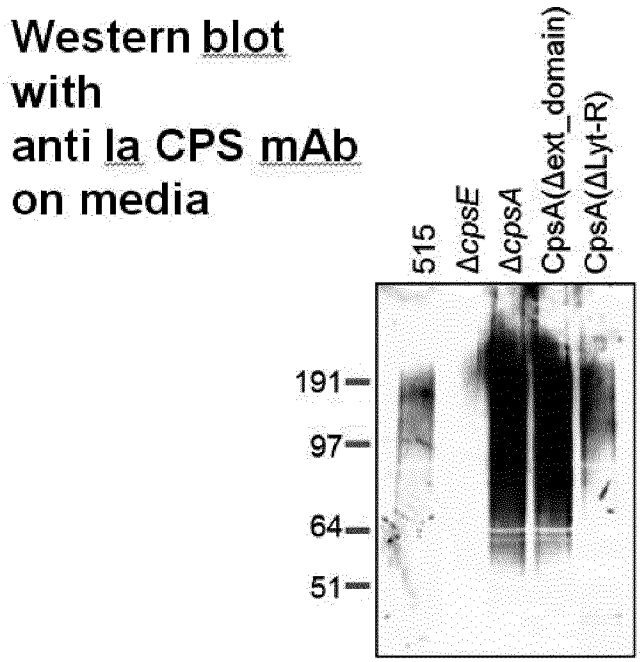

Western blot
with
anti la CPS mAb
on media
515
ΔcpsE
ΔcpsA
CpsA(Δext_domain)
CpsA(ΔLyt-R)
191
97
64
51

Fig. 3

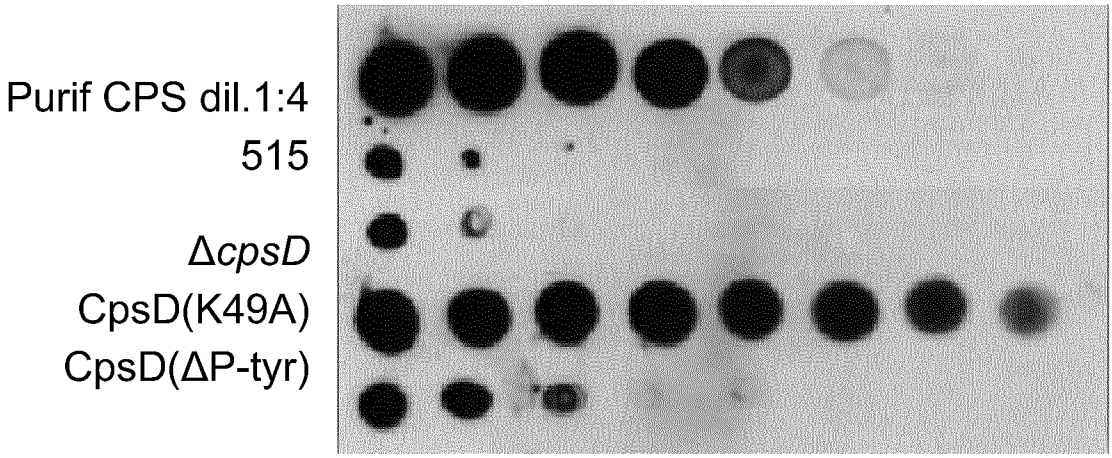


Fig.4

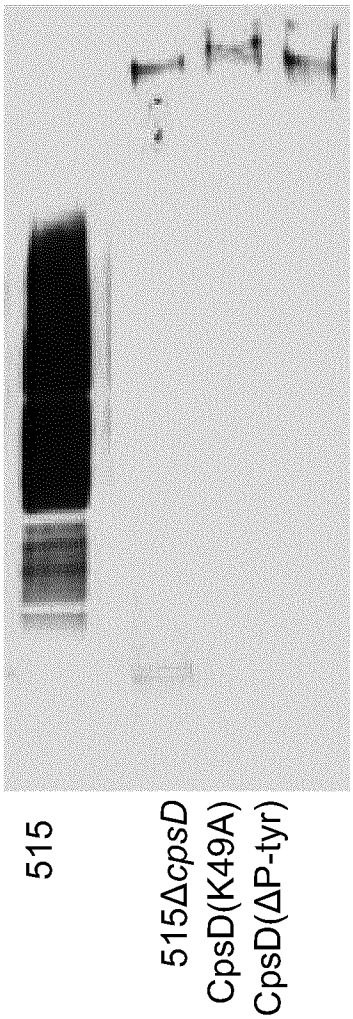


Fig.5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/059773

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/04 A61K39/09 C08B37/00 C07K14/315
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P A61K C08B C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	B. R. HANSON ET AL: "Functional Analysis of the CpsA Protein of Streptococcus agalactiae", JOURNAL OF BACTERIOLOGY, vol. 194, no. 7, 1 April 2012 (2012-04-01) , pages 1668-1678, XP055150264, ISSN: 0021-9193, DOI: 10.1128/JB.06373-11	14,15
A	page 1677	1-13

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A	WO 2006/065137 A2 (SCHAPPEN STICHTING TECHNOLOGIS [NL]; NIEROP GROOT MASJA NATHALIE [NL];) 22 June 2006 (2006-06-22) the whole document	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2015	30/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schneider, Patrick

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/059773

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