OUTER SURFACE PROTEINS, THEIR GENES, AND THEIR USE

Field of the Invention

This invention relates to the identification of outer surface proteins, their genes, and their use. More particularly, it relates to their use in therapy, for immunisation and in screening for drugs.

Background to the Invention

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is the causative agent of various conditions. In particular, GBS causes:

Early onset neonatal infection.

This infection usually begins *in utero* and causes severe septicaemia and pneumonia in infants, which is lethal if untreated and even with treatment is associated with a 10-20% mortality rate.

Late onset neonatal infection.

This infection occurs in the period shortly after birth until about 3 months of age. It causes a septicaemia, which is complicated by meningitis in 90% of cases. Other focal infections also occur including osteomyelitis, septic arthritis, abscesses and endopthalmitis.

Adult infections.

These appear to be increasingly common and occur most frequently in women who have just delivered a baby, the elderly and the immunocompromised. They are characterised by septicaemia and focal infections including osteomyelitis, septic arthritis, abscesses and endopthalmitis.

Urinary tract infections.

GBS is a cause of urinary tract infections and in pregnancy accounts for about 10% of all infections.

Veterinary infections.

GBS causes chronic mastitis in cows. This, in turn, leads to reduced milk production and is therefore of considerable economic importance.

GBS infections can be treated with antibiotics. However, immunisation is preferable. It is therefore desirable to develop an immunogen that could be used in a therapeutically-effective vaccine.

Summary of the Invention

The present invention is based on the identification of a series of genes in GBS, and also related organisms, the products of which may be associated with the outer surface of the organism and may therefore be useful as targets for immunotherapy.

According to one aspect of the invention, a peptide is encoded by an operon including any of the genes identified herein as MS4, MS10, MS11, MS14 and MS16, obtainable from Group B *Streptococcus*, or a homologue or functional fragment thereof. Such a peptide is suitable for therapeutic use, e.g. when isolated.

The term "functional fragments" is used herein to define a part of the gene or peptide which retains the activity of the whole gene or peptide. For example, a functional fragment of the peptide may be used as an antigenic determinant, useful in a vaccine or in the production of antibodies.

A gene fragment may be used to encode the active peptide. Alternatively, the gene fragment may have utility in gene therapy, targetting the wild-type gene *in vivo* to exert a therapeutic effect.

A peptide according to the present invention may comprise any of the amino acid sequences identified herein as SEQ ID NOS. 2, 4, 6, 8, 10 and 12, or a functional fragment thereof.

Because of the extracellular or cell surface location, the peptides of the present invention may be suitable candidates for the production of therapeutically-effective vaccines against GBS. The term "therapeutically-effective" is intended to include the prophylactic effect of vaccines. For example, a vaccine may comprise a peptide according to

the invention, or the means for its expression, for the treatment of infection.

This vaccine may be administered to females either prior to or during pregnancy to protect mother and neonate against infection by GBS.

According to another aspect of the invention, the peptides or genes may be used for screening potential antimicrobial drugs or for the detection of virulence.

A further aspect of this invention is the use of any of the products identified herein, for the treatment or prevention of a condition associated with infection by a Group B *Streptococcal* strain.

Although the protein has been described for use in the treatment of patients, veterinary uses of the products of the invention are also considered to be within the scope of the present invention. In particular, the peptides or the vaccines may be used in the treatment of chronic mastitis, especially in cows.

Description of the Invention

The present invention is described with reference to Group B *Streptococcal* strain M732. However, all the GBS strains and many other bacterial strains are likely to include related peptides or proteins having amino acid sequence homology with the peptide of M732. Organisms likely to contain the peptides include, but are not limited to, *S. pneumoniae*, *S. pyogenes*, *S. suis*, *S. milleri*, Group C and Group G *Streptococci* and *Enterococci*. Vaccines to each of these may be developed in the same way as described for GBS.

Preferably, the peptides that may be useful for the production of vaccines have greater than 40% sequence similarity with the peptides identified herein. More preferably, the peptides have greater than 60% sequence similarity. Most preferably, the peptides have greater than 80% sequence similarity, e.g. 95% similarity.

Having characterised a gene according to the invention, it is possible to use the gene sequence to

establish homologies in other microorganisms. In this way it is possible to determine whether other microorganisms have similar outer surface products. Sequence homologies may be established by searching in existing databases, e.g. EMBL or Genbank.

Peptides or proteins according to the invention may be purified and isolated by methods known in the art. In particular, having identified the gene sequence, it will be possible to use recombinant techniques to express the genes in a suitable host. Active fragments and homologues can be identified and may be useful in therapy. For example, the peptides or their active fragments may be used as antigenic determinants in a vaccine, to elicit an immune response. They may also be used in the preparation of antibodies, for passive immunisation, or diagnostic applications. Suitable antibodies include monoclonal antibodies, or fragments thereof, including single chain fv fragments. Methods for the preparation of antibodies will be apparent to those skilled in the art.

The preparation of vaccines based on attenuated microorganisms is known to those skilled in the art. Vaccine compositions can be formulated with suitable carriers or adjuvants, e.g. alum, as necessary or desired, and used in therapy, to provide effective immunisation against Group B *Streptococci* or other related microorganisms. The preparation of vaccine formulations will be apparent to the skilled person.

More generally, and as is well known to those skilled in the art, a suitable amount of an active component of the invention can be selected, for therapeutic use, as can suitable carriers or excipients, and routes of administration. These factors will be chosen or determined according to known criteria such as the nature/severity of the condition to be treated, the type or health of the subject etc.

The products of the present invention were identified as follows:

Todd-Hewitt broth was inoculated with GBS and allowed to grow overnight at 37°C. The cells were harvested by centrifugation and washed with Phosphate Buffered Saline (PBS). The cells were resuspended in an osmotic buffer (20%(w/v) Sucrose, 20mM Tris-HCl pH 7.0, 10mM $MgCl_2$) containing protease inhibitors (1 mM PMSF, 10 μM Iodoeacetic Acid, 10 mM 1,10-Phenanthroline, 1 μM Pepstatin A) and Mutanolysin at a final concentration of 4 Units per microlitre. This was incubated (shaking) at 37°C for 2 hours.

Cells and debris were removed first by high speed centrifugation, then ultra-centrifugation for 1 hour. The resultant supernatant containing cell wall proteins was concentrated under pressure using an ultrafiltration device (10,000 molecular weight cut-off).

The sample was dialysed against ultra high quality water and lyophilised. After resuspension in loading buffer, the proteins were separated by preparative 2-Dimensional-Gel Electrophoresis. Following electrophoresis an individual spot was chosen for study. The spot was subjected to in-gel tryptic digestion. The resulting peptides were extracted from the gel and purified using microbore RP-HPLC. Fractions were collected every 45 seconds and a portion of these consistent with the regions of UV absorbance were analysed by Delayed Extraction-Matrix Assisted Laser Desorption-Time of Flight Mass Spectrometry (DE-MALDI-TOF-MS). Peptides not observed in a blank preparation were then subjected to sequencing using Nanospray-MS/MS

Using this peptide sequence information, degenerate oligonucleotides were designed to be used in a polymerase chain reaction (PCR) to amplify the DNA segment lying between the peptide sequences identified.

PCR amplification resulted in the production of several polynucleotide fragments, each of which was cloned into the pCR 2.1-TOPO vector (Invitrogen BV, Netherlands) according to manufacturers protocol.

The DNA fragment in each plasmid was identified by sequencing and then used to obtain the full-length gene sequence, as follows.

Using the identified DNA fragment, oligonucleotide primers were designed for genomic DNA sequencing. These primers were designed so as to sequence in an `outward' direction from the obtained sequence. Once read, the sequence obtained was checked to see if the 5' and 3' termini of the gene had been reached. The presence of these features was identified by checking against homologous sequences, and for the 5' end the presence of an AUG start codon (or accepted alternative) preceded by a Shine-Dalgarno consensus sequence, and for the 3' end, the presence of a translation termination (Stop) codon.

Upon identification of the full-length gene, primers were designed for amplification of full-length product from GBS genomic DNA. Primers used included restriction enzyme recognition sites (NcoI at the 5'end and Eco0109I at the 3' end) to allow subsequent cloning of the product into the Lactococcal expression system used.

PCR was carried out using the primers, and the products cloned into a pCR 2.1 cloning vector (In Vitrogen). Following confirmation of the presence of the cloned fragment, the DNA was excised using the restriction enzymes NcoI and Eco0109I.

The vector into which this fragment was inserted was a modified version of pNZ8048 (Kuipers, O. P. *et al.* (1998) J. Biotech 64: 15-21). This vector, harbouring a lactococcal origin of replication, a chloramphenicol resistance marker, an inducible nisin promoter and a multicloning site was altered by the replacement of the multicloning site with two 10X His tags, flanked on the 5-most end with an NcoI site, split in the middle with a multicloning site (including an Eco0109I site), and a Stop (termination) codon at the 3'end of the His tags.

The gene of interest was inserted so that a 10X His tag was in the 3' position relative to the coding region.

Following transformation of the recombinant plasmid into *L.lactis* (strain NZ9000 - Kuipers, O. P. *et al.* (1998) *supra)*, a 400 ml liquid culture was set up and translation of the protein was induced by the addition of nisin to the culture. After a 2 hour incubation, the cells were harvested and lysed by bead beating. The resultant lysate was cleared by centrifugation, then passed over a metal affinity (Talon, Clonetech) column. The column was washed repeatedly before bound proteins were eluted with Imidazole.

To identify fractions containing the His-tagged recombinant protein, an aliquot from each fraction was analysed by SDS-PAGE, Western blotted and probed with anti-His antibodies.

The recombinant protein obtained was then used to immunise New Zealand white rabbits, with pre-immune sera being harvested prior to immunisation. Following a boost, the rabbits were sacrificed and sera collected. This sera was used in Western blots, ELISA and animal protection models.

Using the sera obtained from the animal studies, immunosorption studies were carried out.

Group B *Streptococcus* was grown in 20ml Todd Hewitt broth (THB) for 8 hours, harvested and resuspended in 5ml PBS. 50µl aliquots of this were used to coat wells in a 96 well plate (Nunc Immuno-Sorb). This was left at 4°C overnight to allow for absorbance of the bacteria onto the plate. Plates were washed twice with PBS, then blocked with 3%BSA in PBS for 1hr at 37°C. Plates were again washed. Serial 10 fold dilutions of the sera were made in PBS and 50µl of these dilutions were added to the wells of the plate, in duplicate. The plate was covered and incubated for 1 hr at 37°C. The plate was washed, then 50µl anti-rabbit alkaline phosphatase conjugated secondary antibody at a concentration of 1:5000 was added to each well. Following incubation at 37°C for an hour, the plate was washed again. 50µl substrate (PNPP) was added to each

well, and the reaction allowed to proceed for 30min before the absorbance was read at 405 nm.

Animal protection studies were also carried out to test the effectiveness of protection on the immunised rabbits.

GBS M732 was grown up in THB until mid-log phase was reached - approximately 5 hours. Cells were counted in a counting chamber, and bacteria were diluted to give a concentration of $2x10^7$ bacteria per ml in pre-immune or test sera. 50µl of this was injected via the intraperitoneal route into 0-1 day old mice. The mice were observed for survival over 48 hours.

The following Examples illustrate the invention.

Example 1

A first plasmid was termed MS4. The cloned DNA fragment was sequenced and the nucleotide and deduced amino acid sequence (SEQ ID NO. 1 and 2) was used to search protein databases.

Homologues to the GBS MS4 gene product can be identified in *Clostridium perfingens*, *Haemophilus influenzae*, *Neisseria flavescens* and *Thermatoga maritima*. In all cases the homologues are the genes for Ornithine Carbamoyltransferase (OCT). In eukaryotic systems this enzyme catalyses the second step in the Urea cycle, the conversion of ornithine to citrulline, a reaction requiring carbomyl phosphate. In prokaryotes, ODC is one of the three enzymes involved in Arginine Deaminase activity - a system which protects bacteria from acid damage. In particular, ODC is responsible for the conversion of citrulline to ornithine and carbamoyl phosphate (the opposite role to that in eukaryotes) (Casiano-Colon, A and Marquis, R. E. 1988. Appl. Environ. Microbiol. 54: 1318-1324, Cunin, R. *et al*. 1986. Microbiol. Rev. 50: 314-352).

Animal protection studies were carried out as described above. The results are as follows:

Treatment	# pups	# pups surviving at time (hrs)	
		24	48
PBS	15	6	0
Pre-Immune	41	18	1
Test	41	33	14

Example 2

A second plasmid was termed MS11. The nucleotide and deduced amino acid sequence (SEQ ID NOS. 3 and 4) were used to search protein databases.

Homologues to the GBS MS11 gene product can be identified in *Lactobacillus delbrueckii, Thermotoga maritima, Clostridium acetobulylicum, Bacillus megaterium, Triticum aestivium and Synechocystis PCC6803*.

In all cases the homologues are the genes for the protein Phosphoglycerate Kinase (PGK). PGK is a major enzyme in the glycolytic pathway, being involved in the conversion of Glyceraldehyde-3-phosphate to Phosphoenolpyruvate. In particular, it is involved in the catalysis of the reaction between Glycerate-1,3-diphosphate and 3-Phospho-Glycerate, releasing a phosphate in the forward reaction.

Example 3

A third plasmid was termed pMS16. The 5' and 3' cloned DNA fragments were sequenced and the nucleotide and deduced amino acid sequences for each are shown as SEQ ID NOS. 5 and 6 for the 5' fragment and SEQ ID NOS. 7 and 8 for the 3' fragment.

Homologues to the GBS MS16 gene product can be identified in *Bacillus stearothermophilus, Bacillus subtilis* and *Mycoplasma genitalium*.

In all cases the homologues are the genes for the protein Glucose-6-Phosphate Isomerase (GPI).

The enzyme Glucose-6-Phospate Isomerase catalyses the reaction between Glucose-6-phosphate and Fructose-6-Phosphate in both glycolysis (G6P to F6P) and

gluconeogenesis (F6P to G6P). Mutations in the *gpi* gene have been shown to confer purine analogue sensitivity to organisms.

Example 4

A fourth plasmid was termed pMS14. The cloned DNA fragment was sequenced and the nucleotide and deduced amino acid sequence (SEQ ID NOS. 9 and 10) was used to search protein databases.

Homologues to the GBS MS14 gene product can be identified in *Bacillus subtilis*, *Bacillus stearothermophilus*, *Mus musculus*, *Bos taurus* and *Zea mays*. In all cases the homologues are the genes for the protein Purine Nucleoside Phosphatase (PNP). The function of this enzyme is to cleave the nucleosides guanosine or inosine to their respective basis and sugar-1-phosphate molecules in the presence of orthophosphate.

Example 5

A fifth plasmid was termed pMS10. The cloned DNA fragment was sequenced. The nucleotide and deduced amino acid sequence (SEQ ID NOS. 11 and 12) was used to search protein databases.

Homologues to the GBS MS10 gene product can be identified in *Streptococcus mutans*, *Nicotiana plumb*, *Pisum sativum* and *Zea mays*. In all cases the homologues are the genes for the protein Nonphosphorylating, NADP-Dependent Glyceraldehyde-3-Phosphate Dehydrogenase (NPGAP-3-DH). NPGAP-3-DH has been reported as being an important means of generating NADPH for biosynthetic reactions in *S. mutans* (as opposed to NAD-specific GAP-3-DH which satisfies the requirements of the glycolytic pathway) (Boyd, D.A., Cvitkovitch, D. G. and Hamilton, I. R 1995 J. Bacteriol. 177: 2622-2727).

CLAIMS

1. A peptide encoded by an operon including any of the genes identified herein as MS4, MS10, MS11, MS14 and MS16, obtainable from Group B *streptococcus*, or a homologue thereof or a functional fragment thereof.

2. A peptide according to claim 1, comprising any of the amino acid sequences identified herein as SEQ ID NOS. 2, 4, 6, 8, 10 and 12.

3. A peptide according to claim 1 or claim 2, for therapeutic use.

4. A polynucleotide encoding a peptide according to claim 1 or claim 2, for therapeutic use.

5. A host transformed to express a peptide according to claim 1 or claim 2.

6. A vaccine comprising a peptide according to claim 1 or claim 2, or the means for its expression.

7. Use of a product according to any of claims 1 to 5, for screening potential drugs or for the detection of virulence.

8. Use of a product according to any of claims 1 to 5, for the manufacture of a medicament for use in the treatment or prevention of a condition associated with bacterial infection.

9. Use according to claim 8, wherein the infection is a Group B *streptococcal* infection.

10. Use according to claim 8 or claim 9, wherein the infection is a focal infection.

11. Use according to claim 8 or claim 9, wherein the infection is a urinary tract infection.

12. An antibody raised against a peptide according to claim 1 or claim 2.