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Novel protein vaccine candidates against Group B streptococcal infection identified using alkaline phosphatase fusions

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Abstract

Using an alkaline phosphatase-based genetic screening method, we identified a number of proteins that are potentially located on the outer surface of Group B streptococcus (*Streptococcus agalactiae*). In an enzyme-linked immunosorbent assay, antisera raised against two of the proteins, the streptococcal *yutD* homologue and a subunit of an ABC transporter, recognised clinically important serotypes of Group B streptococcus. In a neonatal rat model, purified IgG from the sera conferred significant levels of protection against a lethal challenge infection. The proteins identified show potential as protein subunit candidates for vaccines against Group B streptococcal disease in neonates.

Keywords: Group B streptococcus; Vaccine; Functional genomics; *Streptococcus agalactiae*

1. Introduction

Group B streptococcus (GBS, *Streptococcus agalactiae*) is the causal agent of a broad range of human diseases. Of these, the most prominent are septicemia, pneumonia and meningitis of neonates. The major component of the cell surface is a polysaccharide capsule of which there are at least 10 antigenically distinct types. Although antibodies against capsule are protective, the polysaccharide capsule itself is associated with a number of difficulties for use as a vaccine candidate. As well as a need for the inclusion of at least five serotypes, it is poorly immunogenic. To elicit a good immune response, each polysaccharide would need to be independently conjugated to a carrier protein. However, whole GBS cells contain a complement of proteins embedded throughout this capsule. The identification of these proteins may reveal candidates for vaccines against GBS infection. Many proteins have been identified using direct methods. These include the tandem repeat proteins, alpha-C protein [1] and Rib protein [2], the beta-C protein [3], X proteins [4], glutamine synthetase [5], α-enolase [6], Hsp70 [7] and Sip [8]. A recent proteomic screen of the outer surface proteins also revealed the presence of several other proteins [9], including non-phosphorylating glyceral-

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dehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, ornithine carbamoyltransferase, glucose-6-phosphate isomerase and purine nucleoside phosphatase.

An alternative method to identify outer surface proteins is to interrogate the DNA sequence. Using a bioinformatic approach, the sequence of the genome is examined for signal sequences [10]. Generation of the genome sequence and analysis of hypothetical hits is time-consuming and labour-intensive. Further, it requires knowledge of the appropriate sequences responsible for protein export in that particular organism. Many of the proteins named above would not have been identified as being present on the outer surface of an organism based on such an *in silico* approach. Consequently as an alternative, we undertook a biological screen of the genomic DNA of GBS using the alkaline phosphatase reporter system [11].

Molecular fusion of DNA to an appropriate reporter gene has been used extensively in studies on cellular location of proteins [12], topology of proteins [13] and to screen for unknown exported proteins in several different bacteria. These include *Streptococcus pneumoniae* [14], *Staphylococcus aureus* [15], *Helicobacter pylori* [16], *Mycobacterium tuberculosis* [17] and *Actinobacillus actinomycetemcomitans* [18,19]. Using a homologous system, a method for screening for signal sequences in GBS has also been described [20]. This study only identified a limited number of proteins, and also admitted that there was little difference between the results obtained from the homologous system and a heterologous one where the proteins were expressed in *Escherichia coli*. Consequently to identify further outer surface proteins of GBS that may be vaccine candidates, we undertook a study to search for genes with signals capable of exporting proteins using an *E. coli* system. Of the proteins identified in this study, two were selected for further analysis. Their location as outer surface proteins was confirmed prior to the demonstration of in vivo efficacy as vaccine candidates.

2. Materials and methods

2.1. Bacterial strains

S. agalactiae serotypes and strains used were Type III (strain M732 (8)), Type I a/c (RF76, an isolated pathogenic strain which, due to patient consent restrictions, we are unable to release), Type I b/c (strain H36b, ATCC), Type II (strain 18RS21, NCTC), Type IV (strain R80405, ATCC), Type I a/c (strain A909, ATCC) and Type V (Prague strain supplied by PHLS, Collingdale). All strains used were maintained on Columbia horse blood agar plates (Oxoid, Basingstoke, UK). Liquid cultures for sample preparation were grown overnight in Todd–Hewitt broth (Oxoid) at 37°C. Prior to harvesting, cells were checked for serogroup specificity by latex agglutination test (Oxoid).

2.2. Construction of GBS DNA-phoA libraries

A 20 min partial restriction of 250 μg of GBS genomic DNA (strain M732) was performed using 18 units of *Sau*3AI (NEB, Hertfordshire, UK). The resulting fragments were separated over a 10–40% sucrose gradient which was spun for 24 h at $85\,000\times g$ in an SW40 swing-out rotor (Beckman Ultracentrifuge, Beckman Coulter, Buckinghamshire, UK). Following analysis by agarose gel electrophoresis, fractions containing fragments of less than 1 kb in size were pooled, dialysed and the DNA precipitated prior to ligation into the vectors. Three vectors were used based on *pFW*-phoA [21], which features a leaderless *E. coli* phoA gene downstream of a multi-cloning site. The basic vector, *pFW*-phoA1, differs from the others in the addition of one (*pFW*-phoA2) or two (*pFW*-phoA3) base pairs in the multi-cloning site, resulting in fusions in the three possible reading frames. The vectors were restricted with *Bam*HI and the GBS DNA fragments were ligated into them. The products of each ligation reaction were separately transformed into the *E. coli* phoA-host strain, DH5α.

2.3. Analysis of clones exhibiting alkaline phosphatase activity

The products of the transformation reactions were plated onto L-agar containing 100 μg ml^{-1} spectinomycin and 40 μg ml^{-1} of the chromogenic substrate, XP (5-bromo-4-chloro-3-indoyl-phosphate; Roche Diagnostics, Sussex, UK). Individual positive (blue) colonies were picked and re-streaked on XP-containing plates. Finally, to confirm the phenotype, plasmid DNA from the positive cultures was isolated and retransformed into DH5α cells. The transformants were once again assayed for activity. Plasmid DNA from the positive cultures was sequenced using an ABI 310 automated sequencer (ABI, Warrington, UK) running an ABI big-dye terminator mix chemistry (ABI). The GBS sequence present in the clones was compared to DNA sequences deposited in publicly accessible databases for putative identification. Unless specifically stated, all sequence accession numbers used in this text can direct the reader to the sequence through the trEMBL supplement to the SWISS-PROT database (http://www.expasy.org/sprot/).

2.4. Generation of full-length sequence

From the list of sequences generated, two genes were chosen for further analysis. The sequence obtained from the screen was used to design DNA primers. These were used to sequence genomic GBS DNA in an 'outwards' direction (i.e. towards the 5′ and 3′ ends of the gene). The termini of the genes were determined at the 5′ end by the presence of a ribosome binding site [22] positioned appropriately in relation to a 'start' codon, and at the 3′

end by repeated sequencing of putative termination codons.

2.5. Cloning, expression and purification of proteins

Primers were designed to amplify the full-length genes, and included restriction endonuclease recognition sites to facilitate subsequent cloning steps. Due to the high AT content of the GBS sequences (and GBS genomic DNA in general), the primers designed were unusually long to allow a raised annealing temperature to be used. The sequences of the primer pairs used to amplify Pho3-1 were 5′-CCATATGCGAAAAGAAGTGACACC-3′ and 5′-GCGGCCGCTATTATCCTTTCTTATTTTTCTTAC-G-3′, and for Pho2-2, 5′-CCTCTAGAAATAATTTTG-TTTAACTTTAAGAAGGAGATATATACATATGGG-CCAAGAACCTATCATCG-3′ and 5′-GCGGCCGCT-ATTACAAATCTTCCTTCTTCAAAG-3′.

The restriction enzyme recognition sites (underlined) were designed to allow excision and cloning of Pho3-1 by *Nde*I and *Not*I at the 5′ and 3′ ends, respectively, whilst the enzyme used to digest the 5′ end of the Pho2-2 gene was *Xba*I, with *Not*I being used at the 3′ end. The use of the *Xba*I site as a cloning site necessitated the inclusion of a ribosome binding site (which is removed from the expression vector following digestion with *Xba*I and *Not*I). Following polymerase chain reaction (PCR) of the GBS DNA with the appropriate primer pairs, amplified fragments were cloned into an appropriate commercially viable expression system. Gene identity was verified by sequencing.

Pho3-1 was expressed in a soluble form when induced with a low concentration (100 μM) of isopropyl thiogalactose (IPTG) and grown at reduced temperatures (20°C). Following sonication, protein was purified by two rounds of subtractive ion exchange chromatography. Protein was equilibrated to pH 7.2, then passed first through a Q Sepharose Hi-Trap column (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then pooled flow-through containing Pho3-1 was passed through an SP Sepharose Hi-Trap column. The flow-through was collected, protein-concentrated using a vivaspin centrifugal ultrafiltration device (Sartorius) and identity of the purified protein was confirmed by amino acid analysis (Alta Bioscience, University of Birmingham). Purity was estimated by analysis of a Coomassie blue-stained SDS–PAGE (polyacrylamide gel electrophoresis) gel.

Pho2-2 accumulated in insoluble inclusion bodies in culture. Inclusion bodies were purified by pelleting, and then dissolved in 10 mM Tris pH 7.2, 8 M urea. Following renaturation by dilution with 10 mM Tris pH 8.5, 5 mM EDTA, 1 mM oxidised glutathione, 5 mM reduced glutathione, the protein was loaded onto a Q Sepharose column. The protein was eluted with 10 mM Tris pH 8.5, 1 M NaCl. Following dilution to reduce the NaCl concentration, the protein was concentrated and identity and purity confirmed as for Pho3-1.

2.6. Sera and analysis

Fractions containing recombinant protein were used to immunise New Zealand White rabbits (AbCam, Cambridge, UK). Rabbits were immunised with 100–150 μg of protein plus Freund's complete adjuvant for the initial immunisation and for the three subsequent booster injections, each at 28-day intervals, Freund's incomplete adjuvant and protein. Antisera were tested for specificity by probing Western blots of recombinant protein and GBS outer surface proteins prepared from strain M732 (prepared and verified as being free from intracellular contamination as previously described, [9]). Proteins were analysed on a 12% criterion SDS polyacrylamide gel (Bio-Rad, Hertfordshire, UK) prior to blotting onto 0.2 mm nitrocellulose membrane (Bio-Rad). Blots were blocked with 3% bovine serum albumin (BSA) prior to probing with a 1:5000 dilution of the test or pre-immune sera. Blots were decorated with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma, Dorset, UK), and the blot was developed with Western blue stabilised substrate (Promega, Southampton, UK).

2.7. Whole cell enzyme-linked immunosorbent assay (ELISA)

The sera directed against the recombinant proteins were used to challenge GBS in whole-cell ELISAs. Six strains of GBS, representing the major clinical serotypes, were used. The cells were grown to mid-exponential phase, harvested, washed with phosphate-buffered saline (PBS) and used to coat ELISA plates (Nunc Immunosorb, Life Technologies, Paisley, UK). The supernatant from the wells was carefully aspirated following overnight coating at 4°C, and assayed for the presence of the intracellular marker, aldolase [23]. All subsequent incubations were performed at 37°C for 1 h unless otherwise stated. After blocking with 3% BSA, the plates were probed with 50 μl of 10-fold serial dilutions of pre- and post-immune sera. Antibody was decorated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) at a dilution of 1:5000 with PBS, which was in turn detected with *p*-nitrophenyl phosphate (Sigma). Plates were incubated at 37°C for 30 min, then read at 405 nm.

2.8. Animal protection

The antibodies were tested for ability to protect against GBS disease in a passive neonatal rat model. IgG directed against the recombinant proteins was purified by Protein A affinity chromatography (Protein A Sepharose, Amersham Pharmacia Biotech), concentrated by ammonium

Table 1
Results of bioinformatic analysis of Group B streptococcal DNA sequences responsible for export of alkaline phosphatase reporter molecule

Clone	Database homologue	Similarity (%)	Identity (%)	Range (amino acids)
Pho1-3	Diacylglycerol kinase *Streptococcus mutans* (O51806)	98	81	59
Pho1-5	SceA precursor *Staphylococcus carnosus* (O54494)	56	53	41
Pho1-9	Putative transmembrane protein *Mycobacterium tuberculosis*	66	65	30
Pho1-10	LMRP integral membrane protein *Lactococcus lactis* (Q48658)	62	61	49
Pho1-11	Rib protein *Streptococcus agalactiae* (P72362)	100	100	46
Pho1-12	Phosphopentomutase *L. lactis* (O32808)	80	64	68
Pho1-13	ClpP protease *Streptococcus salivarus* (P36398)	97	96	92
Pho1-14	No significant hit			
Pho1-15	No significant hit			
Pho2-2	Spermidine/putrescine transport ATP-binding protein *Haemophilus influenza* (P45171)	46	61	45
Pho2-7	Probable tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase *Chlamydia trachomatis* (O84289)	83	64	56
Pho2-10	KUP system potassium uptake protein *Escherichia coli* (P30016)	63	46	84
Pho2-11	Ribonuclease III *Bacillus subtilis* (P51833)	64	41	51
Pho2-15	Phosphotransferase system enzyme EIIC *E. coli* (P37189)	56	28	83
Pho2-16	ATP-dependent helicase *Bacillus stearothermophilus* (P56255)	91	80	35
Pho3-1	Hypothetical protein, YutD *B. subtilis* (O32127)	65	46	77
Pho3-6	Modification methylase *E. coli* (Q59380)	53	36	52
Pho3-10	PRPP synthetase *B. subtilis* (O33924)	95	91	23
Pho3-14	Transmembrane sugar transport protein *M. tuberculosis* (O53484)	54	26	92
Pho3-17	No significant hit			
Pho3-18	Hypothetical transmembrane protein *Mycoplasma pneumoniae* (P75528)	56	33	30
Pho3-19	E1–E2 ATPase *B. subtilis* (O34431)	66	44	190
Pho3-20	No significant hit			
Pho3-21	Basic surface protein *Lactobacillus fermentum* (O06530)	59	38	105
Pho3-22	Cytoplasmic membrane lipoprotein precursor *Staphylococcus equisimilis* (O05471)	50	33	51
Pho3-23	Putative permease *Streptococcus mutans* (O68573)	63	40	85
Pho3-24	Integral membrane protein *S. mutans* (O68577)	89	77	240
Pho3-25	Uracil permease *Bacillus caldolyticus* (P41006)	92	61	57
Pho3-29	Outer cell wall protein precursor *Bacillus brevis* (P09333)	59	45	22
Pho3-30	Muconate transport protein *Acinetobacter calcoaceticus* (P94131)	64	42	42
Pho3-35	DTDP-4-keto-6-deoxyglucose-3,5-epimerase *Streptococcus pneumoniae* (O54547)	95	85	69
Pho3-50	Penicillin binding protein 2B *Streptococcus mitis* (Q54502)	70	54	107

Sequences were subjected to BLAST searches, and the results are reported in terms of closest similarities, and the percentage similarity and identity the query protein has to the database hit, and the length of the sequence over which the similarity/identity was seen.

sulphate precipitation, resuspended and dialysed against PBS. The concentration of purified IgG in the samples was between 25 and 35 mg ml^{-1}. Previous experiments had demonstrated no protective activity of pre-immune sera, so negative controls were supplied by PBS-injected pups. Positive control serum was raised against whole GBS (A909 strain) (anti-whole cell) using a protocol designed to raise a response against the carbohydrate capsule [24].

50 μl IgG containing solution was injected via the intraperitoneal route into 24-h old pups, with 30 pups being used in each group. In all cases, pups from first-time mothers were used in order to eliminate experimental complications due to the presence of anti-GBS antisera in the mothers [25]. The pups were removed from their mothers and the litters were randomised prior to treatment with antisera to avoid bias in survival due to differing levels of maternal care. The pups were rested for between 5 and 7 h prior to challenge to allow for entry of the IgG into the circulation. We demonstrated (data not shown) that this was a sufficient amount of time in order to allow maximum concentration of antibody to be present in the circulation. Pups were challenged subcutaneously with 5×10^4 cfu Type I a/c (A909) GBS (determined empirically to represent an LD_{80-100} dose, data not shown) and observed for 64 h. The experiments were repeated independently nine times for the PBS control group, five times for treatment with anti-whole cell, seven times for treatment with anti-Pho2-2 IgG, and three times for treatment with anti-Pho3-1 IgG.

Pups were scored when an end point had been reached. This was empirically realised and defined as two of the following symptoms being observed: excessive pallor, generalised erythema, subcutaneous haemorrhaging, redness at site of bacterial injection, reduced body temperature, or increased muscle tone. Once an end point was reached, the pups were humanely killed.

The animals were housed and maintained in accordance with the Code of Practice for the Housing and Care of Animals used in Scientific Procedures issued by the UK Home Office. The experiments were carried out under the authority of a Project Licence granted under the Animals (Scientific Procedures) Act 1986.

3. Results and discussion

3.1. Alkaline phosphatase screen

Three GBS–phoA gene fusion libraries were created by the insertion of *Sau*3AI-digested GBS chromosomal DNA fragments into the plasmids *pFW*-phoA1, *pFW*-phoA2 and *pFW*-phoA3. Approximately 30 000 transformants were screened, and 83 which possessed alkaline phosphatase activity were shown to be stable and contain GBS DNA inserts. Of these, 42 were unique. 32 of these sequences were analysed using bioinformatic tools. Table 1 shows the results of this analysis.

Based on the analysis and other studies (data not shown), two of the proteins were selected for further analysis – Pho2-2 and Pho3-1.

3.2. Cloning of full-length genes for Pho2-2 and Pho3-1, and expression of recombinant proteins

The full-length sequences for the Pho2-2 and Pho3-1 genes have been assigned as GenBank accession numbers CAC09093 and CAC09097, respectively. Neither of these genes have been previously described in GBS. *In silico* translation of these sequences enabled a search of the trEMBL database to be performed, and in agreement with the results of the initial screen, the highest homologies to Pho2-2 (for proteins with confirmed identity) are against the ATPase subunit of ABC transport systems such as that from the L-carnitine transporter in *Listeria monocytogenes* (AAF91342), the glycine betaine/carnitine/choline transporter from *S. aureus* (BAB96237) or the proline/glycine betaine transport system of *Clostridium acetobutylicum* (Q97F91). Walker A and B motifs can be identified in this protein, as can an ATP active binding domain, confirming the identity of the protein.

Pho3-1 is one of a growing number of hypothetical proteins identified from genome sequencing projects. These include *Bacillus subtilis* (O32127), *Streptococcus pyogenes* (AAL98118) and *S. pneumoniae* (AAK99479). Pho3-1 shows at least 50% identity and 68% similarity to all of these proteins. There is no significant homology of these proteins to any with an assigned function.

Both proteins expressed to high levels when the genes were cloned into the pET29a expression vector. The yield of purified protein from 250 ml of starting culture for Pho3-1 was approximately 2.75 mg, and for Pho2-2, approximately 9.75 mg. In both cases it was estimated (by Coomassie blue staining of SDS–PAGE gels, data not shown) that the protein samples were over 90% pure. Amino acid analysis of the purified samples matched the predicted composition of the proteins, confirming identity and intactness.

3.3. Sera directed against Pho3-1 and Pho2-2 react against whole GBS

The individual purified proteins were used to immunise New Zealand White rabbits. Fig. 1 shows Western blots using the sera, reacted against blots of both the recombinant protein and a preparation of GBS outer surface proteins. As can be seen, the size of both recombinant protein and the reacting band in the GBS outer surface protein preparation are essentially the same, indicating that the recombinant proteins against which the antibodies were raised were full-length. Additional, lower molecular-mass bands are visible reacting to proteins in the OSP preps,

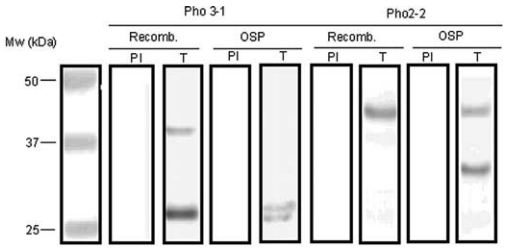

Fig. 1. Western blot analysis of purified recombinant Pho2-2 and Pho3-1 (Recomb.) and GBS outer surface proteins (OSP) probed with pre-immune (PI), anti-Pho3-1 and anti-Pho2-2 antisera (T). Proteins were analysed by SDS–PAGE, blotted to nitrocellulose and probed with a 1:5000 dilution of pre-immune-phase sera or antisera. Blots were decorated with anti-rabbit alkaline phosphatase-conjugate secondary antibody and were developed with Western blue substrate (Promega). Mw, molecular mass.

which could represent breakdown products. Additionally, a higher-weight molecular mass protein in the recombinant protein preparation is recognised by the anti-Pho3-1 antibody, possibly due to read-through of the transcript, past the stop codon.

Using the same sera, whole GBS cells were screened in an ELISA to give additional confirmation that these antigens were located on the surface of the cell and that they were present in representative strains from the clinically important GBS serotypes. Further confirmation of the presence of the two genes across different serotypes was obtained by BLAST searching the recently elucidated genomes for GBS Type III and Type V using the Pho3-1 and Pho2-2 amino acid sequences. This showed the identity levels were 84% and 99%, respectively, in both strains. To confirm that the cells had maintained their integrity on the ELISA plate, the supernatant (following coating) was carefully aspirated and assayed for the presence of the intracellular marker, aldolase [23]. Results (not shown) demonstrated that less than 3% of the cells lysed during the process.

Results of the ELISA with primary anti-sera at a dilution of 1:1000 are shown in Fig. 2. For both of the antisera investigated, the post-immune sera reacted to a much greater level than the pre-immune sera. Furthermore, the sera reacted with all of the serotypes tested, indicating the presence of the antigen on the surface of all the major serotypes. The comparatively low levels of reactivity against the Type III (M732) strain has been seen for other sera raised against outer surface proteins [9], and probably reflects masking of the protein by the thick carbohydrate capsule [26], which in turn is influenced by growth conditions [27].

3.4. *Anti-Pho2-2 and anti-Pho3-1 antibodies protect against GBS infection*

As it was demonstrated that the Pho2-2 and Pho3-1 antigens were located on the surface of GBS, we postulated that the proteins might be candidates for vaccines against the disease. To test this, a passive immunisation experiment was performed using purified anti-Pho2-2 and Pho3-1 IgG, to test for protection against infection. The results are shown in Fig. 3.

The anti-whole-cell immunoglobulin was able to provide >90% protection against infection, as would have been anticipated – whilst 80% of those animals which had been treated with PBS succumbed to the infection. Using Dunnett's test for analysis, the odds of survival in both the anti-Pho2-2 and anti-Pho3-1 groups were significantly greater than the odds of survival in the PBS control group with 95% confidence, though not as great as that observed for the anti-whole-cell-treated animals.

The identification of outer surface proteins is a process by which novel candidates for vaccines against bacterial diseases may be discovered. This is particularly the case where it is known that the main immunogenic determinant of the bacteria is not a protein, and is serotype-specific as is the case with GBS. A protective response against the GBS capsular carbohydrate does not provide cross-type protection [28]. Vaccination with a protein present in a range of serotypes would overcome this issue.

Outer surface proteins may be identified by one of several methods. We have previously described work undertaken to examine the major proteins on the cell surface by direct sequencing of enzymatically extracted proteins [9]. Whilst this proteomic method is powerful and resulted in the identification of protective outer surface proteins, only the most abundant 20 or so proteins were identified. An alternative method is to examine the genome for proteins with known signal sequences for protein export, or for membrane anchorage. This methodology has also been used to identify potential targets in *Neisseria meningitidis* [29]. However, only proteins which conform to the current cannon of signal sequences will be identified. Further, the genome sequence needs to be available and that of GBS has only recently been released [30,31]. Consequently, we have employed a functional method, relying on identification of genetic fusions of GBS export signals with a leaderless PhoA molecule.

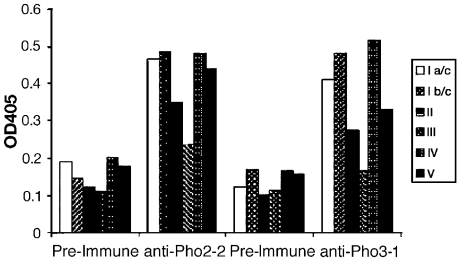

Fig. 2. Sera directed against Group B streptococcal Pho2-2 and Pho3-1 recognises GBS in a whole-cell ELISA. Cells of six GBS serotypes, Type I a/c (RF76a), Type I b/c (H36b), Type II (18RS21, Type III (M732), Type IV (R80405) and Type V (Prague strain) were used to coat ELISA plates, which were subsequently challenged with rabbit pre-immune sera, or antisera raised against Pho2-2 or Pho3-1. The reacting antibody was decorated with secondary (goat-anti-rabbit, alkaline phosphatase-conjugated) antibody, and the reaction developed with PNPP and read at 405 nm.

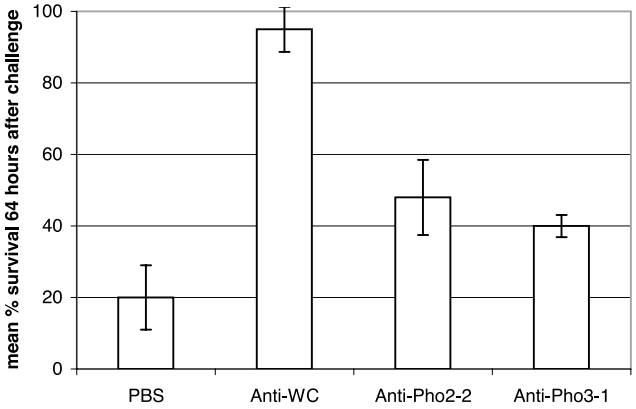

Fig. 3. Anti-Pho2-2 and anti-Pho3-1 IgG offers statistically significant protection against infection with a lethal challenge of GBS in a neonatal animal model. Purified IgG raised against whole GBS cells (WC), Pho2-2 or Pho3-1 proteins were used to passively immunise neonatal rat pups. After a rest period the pups were challenged with a lethal dose of GBS. The monitored animals were scored as non-survivors when a predefined end point had been reached. Means and standard deviation of nine (PBS), five (WC), seven (Pho2-2) and three (Pho3-1) independent experiments are shown.

All the evidence presented here indicates that both the Pho2-2 and Pho3-1 molecules are resident on the outer surface of the GBS bacteria. They confer an export phenotype to the leaderless PhoA, antisera raised against the recombinant proteins recognise an appropriately sized protein on a Western blot of outer surface proteins, the antisera recognise whole cells and, most convincingly, IgG directed against the proteins protects against GBS infection in an in vivo model. In neither case were these proteins identified by proteomic examination of the outer surface protein profile. It is unlikely that either would they have been identified by reverse vaccinology. Bioinformatic analysis (PIX analysis) demonstrates that Pho3-1 does not possess a classical signal motif, whilst Pho2-2 has a possible, but ambiguous signal. In addition, the lack of N-terminal signal-motif or the Gram-positive anchor motif LPXTG for either of the proteins raises the question of how they are attached to the cell surface, or the mechanism of export. As such, Pho2-2 and Pho3-1 join a growing list of such proteins [32].

Bioinformatic analysis of Pho3-1 offers little indication as to its function. Homology searches using this sequence reveals that it is a member of a protein family which has been identified in several Gram-positive bacterial species from genomic sequencing projects. The homologous proteins have been given various nomenclatures, including yutD (*B. subtilis*), SPy1534 (*S. pyogenes*), SP0767 (*S. pneumoniae*), and ybiC (*Lactobacillus lactis*). There are no proteins with significant homology to Pho3-1 to which a biological function has been assigned. Identification of this protein as being present on the outer surface of the bacteria should aid in studies designed to elucidate the function.

The Pho2-2 protein is of particular interest. The bioinformatic search identifies the protein as the Walker-motif-containing ATP binding protein of an ABC cassette. The topology of these multi-unit proteins identifies the ATP-binding domains as being intracellular [33]. It is therefore surprising to have identified this subunit as being present on the outer surface of the bacteria.

Whilst this was a surprising result it is not without precedent. The presence of a Walker-motif-containing portion of an ABC protein on the surface of *S. aureus* has been reported previously [34], and polyclonal antisera raised against the protein proved efficacious as an immunotherapeutic agent. Together with the data presented here, this suggests that the ATPase is, indeed on the outer surface of the cell. It has been hypothesised for other ABC proteins that the ATPase spans the membrane, with portions being available to the external environment [35–39]. However, other comprehensive studies have demonstrated that the ATPase is not accessible to the external environment [40]. It is possible therefore that when on the outside of the cell, the protein is not part of the ABC multi-subunit protein, and is acting in a different, independent role. The presence of proteins on the bacterial cell surface which have alternate intracellular roles has been widely reported [5–7,9] and suggests that these proteins may have more than one role. This has been demonstrated, for example, for intracellular enzymes which also display extracellular binding functions [6,41]. It is tempting to speculate therefore that Pho2-2 is performing a role other than that of an ABC protein-associated ATPase on the surface of GBS.

Using the PhoA fusion technology, we have successfully identified two novel vaccine candidates, Pho2-2 and Pho3-1 for GBS. Antisera directed against both these candidates provide significant protection in an in vivo model. It is thus possible that the proteins themselves may be efficacious in preventing the disease, if administered as an active protein subunit vaccine either singly or in combination. As the antisera recognise examples from the clinically important serotypes, this protection should be more broad-ranging than that offered by capsular carbohydrate.

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