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Molecular genetic analysis of virulence factors from *Streptococcus pneumoniae*

Diplomová práce

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Annotation:

The work focuses on the significance of pneumolysin in contribution to virulence of *Streptococcus pneumoniae* serotype 1 isolates. Methods include bioinformatics as well as *in vitro* assays. A SNP within nucleotide sequence of the second virulence factor, hyaluronidase, is a subject for screen of meningitis isolates.

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Introduction

1. The advantage of a simple organism

It all started when G. M. Sternberg and Louis Pasteur independently isolated the bacterium later called *Streptococcus pneumoniae* in 1881. Study of this micro-organism revealed that the bacteria can be surrounded by a polysaccharide capsule (Dochez *et* Avery, 1917). It was the transformation of unencapsulated (rough) to encapsulated (smooth) bacteria which proved that DNA is responsible for carrying and expressing genetic information (Avery *et al.*, 1944), (Avery *et al*., 1979). Ever since that discovery, it is now more than ever obvious that the simpler the organism, the more adaptable and flexible. All living organisms are threatened by many enemies all the time. There is a need to be able to update their genomes so they can develop new defence strategies. It is never ending race, sexual reproduction affords more diversity to create new „locks"(antibodies) against „keys"(antigens) (Ridley, 2007). Simple organisms such as *Streptococcus* that can naturally take up foreign DNA from environment and incorporate it into their genome (transformation) (Griffith, 1928), can obtain exogenous genetic material from bacteriophages (transduction) or plasmids (conjugation), even exchange it with other species and undergo homologous recombination (HR), these are often pathogens trying to invade a host (Spratt *et al.*, 2001). The source of variation lies within recombination and/or point mutations. In bacteria, besides binary fission which does not allow much recombination between alleles, HR contributes to genome plasticity and diversity to a much wider extent than point mutations do. An estimated rate of recombination versus mutation per allele for *Streptococcus pneumoniae* is 10:1 in favour of recombination (Feil *et al.*, 2000). Genomes can diversify more rapidly leading to increased microbial evolution.

What seems to be quite frequent is horizontal gene transfer (HGT). This could explain how strains from basically commensal species become pathogenic (Spratt *et al.*, 2001). For example, HGT of penicillin-binding proteins (PBPs) genes has been demonstrated among natural populations of pneumococci contributing to spread of resistance to penicillin (Watson *et al*., 1993). Although the introduction of mass vaccination and antimicrobial treatment such as antibiotics reduced the burden of pneumococcal disease, it has also accelerated the evolution of the pneumococcal genome, from which more virulent and antimicrobial-resistant strains have arisen (Ambur *et al.*, 2009).

Streptococcus pneumoniae as a member of the phylum Firmicutes is a fastidious pathogen of humans and occasionally other hosts, facing up to its environment with a small but hyperdynamic genome. There is evidence that HGT can occur even from eukaryotes to certain bacterial species (Aravind *et al.*, 1999). Many genes can be obtained from a large supragenome pool. *S. pneumoniae* has pan-genome much larger than the genome of any individual strain (Hiller *et al.*, 2007). It is known that some genes of significance in virulence might be activated only by HGT from the accessory genome and also that mobile genetic elements contribute to the accessory gene make-up (Embry *et al*., 2007), (Burne *et al.*, 2007).

Ding *et al* found that the genome evolution of *Streptococcus pneumoniae* is largely driven by host adaptation (Ding *et al.*, 2009). *Streptococcus pneumoniae* is responsible for more than 2.6 million annual deaths in children younger than 5 years (Muench *et al*., 2010). Pneumococcal mechanisms of invasion are of

great interest, especially the action of its virulence factors. One of the most studied and historically one of the most important virulence factors, pneumolysin, has undergone some major changes without affecting pneumococcal virulence. Other factors such as hyaluronidase, which might play a role in pneumococcal meningitis, for example, are subjects of this work. The combination of bioinformatics and conventional laboratory techniques is a promising method to unveil links between even minor changes within genotype and their effects on phenotype.

2. Streptococcus pneumoniae

2.1. Classification & determination

Streptococcus pneumoniae is facultative anaerobic chemo-organotrophic Gram positive bacteria that form characteristic chains (Hardie *et* Whiley, 1997). It belongs to the lactic acid bacteria with essentially a fermentative metabolism. On blood agar, the pneumococcus can be distinguished from other members of the *Streptococcus mitis–Streptococcus oralis* group of viridans streptococci by zones of α-haemolysis responsible for the greenish red blood cells (RBCs) when incubated anaerobically (Canvin *et al*., 1997). Susceptibility to optochin (ethylhydrocupreine) is used to confirm purity of a studied pneumococcal strain on blood agar plates (Williams, 1958). Diplococcal colony morphology, bile solubility which is the most accurate phenotypic test, and agglutination with anti-pneumococcal polysaccharide capsule antibodies are techniques used to differentiate pneumococci from other streptococci that can be found, for example, in the oral cavity (*S. mitis*) (Kawamura *et al*., 1999). The pneumococcus grows in rich medium and requires simple sugars as a carbon source (Whatmore *et al*., 2000).

From DNA-probe-based methods, the Accuprobe test is a rapid and highly accurate culture confirmation method for identification of *Streptococcus pneumoniae* with sensitivity and specificity of 100%. It utilizes a chemiluminescent DNA probe which hybridizes with target ribosomal RNA (rRNA) in solution. It can even detect colonies of the pneumococcus in mixed cultures and from positive blood cultures (Denys *et* Carey, 1992).

2.2. Identification & genome composition

The Pneumococcus is usually completely surrounded by a polysaccharide capsule on which antigenic basis we now recognize more than 90 different serotypes. The capsular serotype is determined by the Quellung reaction first used by Neufeld at the turn of the century (Bednar, 2009). The capsule itself is made of repeating units of polysaccharides in a complex composition covalently bound to the outer side of the cell wall. Genes responsible for expressing specific capsule types are located at the *cps* locus and they are arranged in so called cassettes that can be transformed via HR because both ends of this locus are flanked by IS elements (Dillard *et al*., 1995). Since a precise identification of a strain is crucial for epidemiological studies, more sophisticated and elaborated techniques are being included. Sequential multiplex PCR approach proved to be helpful especially in situations where culture is insensitive (Pai *et al*., 2006).

Nevertheless, taxonomists may have to accept that a perfect classification of such organism that is constantly exposed to possibility of HGT can never be achieved.

Besides the serotype, recent techniques allow us to go further and assign each pneumococcal isolate its own sequence type (ST). A new technique has been developed on the basis of multilocus enzyme electrophoresis (MLEE) in the laboratory of Brian Spratt (Maiden *et al*., 1998). Multilocus sequence typing (MLST) is a simple technique for strain characterization at the molecular level and an excellent method for identifying clonal background. The mechanism is based on comparing sequences of seven pneumococcal housekeeping genes; *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* (Enright *et* Spratt, 1998). Each encodes a fundamental metabolic function, they are very well conserved and each and every one bacterial cell must have them in order to live (Maiden, 2006). Once the seven polymorphic genes are sequenced, they can be uploaded. Sequences of each fragment are compared with all the previously identified sequences. They are allocated allele numbers at each of the seven loci. The combination of the seven allele numbers defines the allelic profile of the strain and each different allelic profile is assigned as an ST used to describe the strain (Aanensen *et* Spratt, 2005). However, there is no certainty that two isolates with the same ST do not differ in other regions besides housekeeping genes. A database for *S. pneumoniae* is growing (Multi Locus Sequence Typing, 2009). With MLST as a golden standard, almost all pathogenic or non-pathogenic bacterial species and many other haploid organisms can be typed. The advantage of such an approach lies within its portability which makes it much easier to readily compare the results between laboratories via the Internet. MLST is a powerful resource for global epidemiology; it has been validated for identification of hyper-virulent clones of *Streptococcus pneumoniae* as well as for defining genuine pneumococci and distinguishing it from closely related species (Hanage *et al*., 2005a). MLST can be used for determining population structures, searching for recombination and/or investigating phylogenetic relations between isolates by means of dendrograms. In order to discern the relatedness and patterns of evolutionary descent among isolates, the eBURST programme, an algorithm on MLST website, can be used. It is especially useful to explore how bacterial clones diversify and to predict the founding genotype of each clonal complex (Feil *et al*., 2004).

The *Streptococcus pneumoniae* (pneumococcal) genome comprises of approximately 2.1 – 2.2 megabase pairs (bp) arranged in a single circular chromosome (Tettelin *et al*., 2001). The complete genome sequence of a pneumococcal representative isolate TIGR4 (TIGR4 stands for The Institute for Genomic Research), has been assigned a GenBank accession number AE005672 or NCBI reference sequence NC_003028 (J. Craig Venter Institute, 2009). TIGR4 was isolated in Norway in the 1990s as a highly virulent strain. The genome is rich in repeated sequences such as insertion sequences (IS) whereas plasmids are rare. Repeated-sequence elements are strategically located with respect to virulence genes suggesting a possibility of coordinated regulation of important genes by these elements (Watson *et al*., 1993). DNA elements alongside BOX and RUP elements, described as small, dispersed DNA repeats, may act as an efficient conduit for intra- as well as inter-genomic recombination. A possible reason for their abundance might be their contribution to genomic plasticity typical for the pneumococcus making it an organism highly adaptable to various environmental changes (Tettelin *et* Hollingshead, 2004). Simple sequence repeats also contribute to phase variation in bacteria further more expanding its variability (Varvio *et al*., 2009).

Furthermore, the pneumococcal genome is often enriched by temperate bacteriophages (pneumophages) (Romero *et al*., 2009).

2.3. Reasons to study pneumolysin and hyaluronidase

Both pneumolysin and hyaluronidase are pneumococcal virulence factors. They differ from each other in terms of function, localization and variation. There can be minor changes within their nucleotide sequences with possible global impact. Given that pneumococcal disease is still a big issue not only in developing countries, it is important to focus on parameters that drive this microorganism further and to study its virulence factors from the very core, the genes.

For that purpose, polymerase chain reaction is perfect when one needs to check the presence of a gene and quality of generated DNA. Polymerase chain reaction (PCR) is a technique widely used by many scientific branches. One of its purposes is an *in vitro* amplification of original molecule of DNA by enzymatic replication so it can be detected. DNA molecule serves as a template. During melting step, the reaction solution is heated to 95°C in order to separate strands of dsDNA from each other. When the temperature is lowered to 55°C, primers of specific nucleotide sequence bind to complementary sequence within the DNA, this step is called annealing. Nucleotides are incorporated by DNA polymerase produced by hot spring bacterium *Thermus aquaticus* (*Taq*) which is a thermo-stabile enzyme that can withstand high temperatures required for PCR method. Once the incorporated nucleotides stabilize the bond between primers and DNA, temperature is set up to 72°C which is ideal for *Taq* DNA polymerase, and it extends the new strand, hence extension step. These steps are being repeated and copies of DNA molecules double in every cycle. Successfully amplified DNA can be analysed by agarose gel electrophoresis described below.

Genome variation is important in the ability of pneumococci to interact with the host. So-called single-nucleotide polymorphisms (SNPs) contribute to diversity of more than 2000 genes in the pneumococcus (Mitchell *et* Mitchell, 2010). SNP is an abundant form of genetic variation; more genetic diversity means a larger pool of SNPs. It is a single base pair position in genomic DNA (gDNA) at which different sequence alternatives (alleles) exist in a population. SNP is caused by substitution of a single nucleotide in coding or non-coding region. SNPs are usually point mutations that remain due to certain evolutional advantage. A SNP does not necessarily lead to a change of an amino acid coded by the triplet affected, those are silent mutations. Other SNPs may change it and those are missense mutations. It differs from a rare variation in requirement for 1% or greater frequency of a given allele in a population (Brookes, 1999). SNPs thus do not include insertion or deletion polymorphisms (Kim *et* Misra, 2007). In bacteriology, SNPs analysis with special applications can be used for genotyping of species for which there are MLST databases with sufficient comparative sequence data (Robertson *et al*., 2004). Given that SNPs are stable and widespread, they can be utilized to link sequence variation to phenotypic changes.

In order to identify SNP, a number of nucleotide sequences must be aligned so that the polymorphism is apparent. This can be done in bioinformatics programmes such as CLC Genomic Workbench version 3.6.5. CLC Main Workbench which was used for this project. These computing

programmes allow researchers to import, view, align, edit and analyze sequencing results from automated sequencing machines.

In this work, real-time PCR (RT-PCR) was used for SNP genotyping. It relies on the ability to measure product accumulation during the exponential phase of amplification. Real-time means that progress and increasing numbers of DNA copies can be observed any time during the reaction. This is possible thanks to variable fluorescent reporters that bind to newly synthesized dsDNA. Hence as the number of gene copies increases during reaction so the fluorescence increases (Kwok, 2001). The output from RT-PCR reaction is in a form of a graph showing the number of cycles, where one cycle envisages denaturation, annealing and extension, against the increasing fluorescence. The cycle number at which the amplification plot crosses a threshold is called the Ct value. The lower the Ct value the greater the starting amount of DNA (Yu *et al*., 2006). It is a high-throughput screening tool that has a potential to detect outbreaks, unveil new dangerous variants of strains, control infection in healthcare facilities, and monitor food-borne disease. RT-PCR is capable of detecting biologically relevant SNPs in clinical isolates. It distinguishes between two different bases within a known sequence by means of allele-specific primers (Kwok, 2000).

2.3.1. Pneumolysin

Pneumolysin (Ply) is a multifunctional toxin produced by *Streptococcus pneumoniae* (Alexander *et al*., 1998). The gene is annotated SP_1923 in TIGR4 genome, Ply appears to be chromosomally coded. There is an increasing body of evidence that Ply is directly involved in pathogenesis. The 53-kDa protein is slightly curved and belongs to the family of thiol-activated toxins (Boulnois, 1992). Genes for these toxins have undergone extensive divergence from a common ancestor (Boulnois *et al*., 1991). Ply shows a sequence variation of 3.3%. Antibodies detected by enzyme-linked immunosorbent assay (ELISA) provided evidence that the toxin is produced *in vivo* and that it acts within extracellular environment (Kanclerski *et al*., 1988). It is a heat labile cytoplasmic enzyme irreversibly inhibited by cholesterol (Jedrzejas, 2001). As Ply is a member of the cholesterol-dependent cytolysin (CDC) family, it can only bind to membranes that contain cholesterol. In fluid solution, free cholesterol blocks pneumolysin's linkages so it will no longer bind to cholesterol-containing membranes of host cells. Ply is missing an N-terminal signal sequence for transport out of the cytoplasm, which confirms its intracellular localization (Walker *et al*., 1987). Ply from a representative strain is made of 1416 nucleotides encoding 471 amino acids. A sequence of 11 amino acids including the unique cysteine (Cys_{428}) and histidine (His_{367}) is the largest contiguous stretch of identity among CDC proteins. This highly conserved Cys motif (ECTGLAWEWWR) at the C-terminal end of Ply is thought to be important for the cytotoxic activity (Berry *et al*., 1999). Ply also possesses limited sequence homology with human C-reactive protein (CRP) and it has Fc-binding capacity. The Ply gene can be successfully expressed to high levels in *Escherichia coli* (Mitchell *et al*., 1989).

Secretion of Ply is very controversial. New information keeps occurring. One possibility is that Ply is located in cytoplasm. It was previously believed that autolysin (LytA) is responsible for Ply release during autolysis in the stationary phase (Lock *et al*., 1992). Balachandran *et al* shown that few strains of serotypes 3, 4 and 6A do not require LytA for the release and that Ply was present in supernatant of these strains'

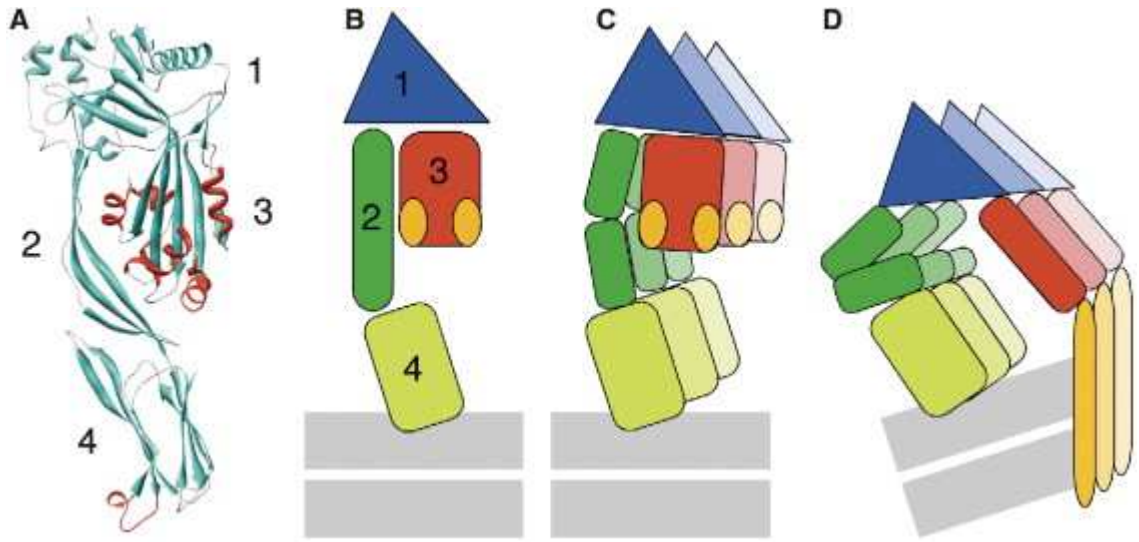
cultures prior to mid-log phase. Experiments proved that Ply was secreted into the external medium in the absence of autolysis, and the release was not caused by a lysogenic bacteriophage. Some unidentified nonlytic mechanism could explain this phenomenon. It is also possible that various pneumococci have developed distinct expression kinetics of Ply (Balachandran *et al*., 2001). Another hypothesis is that competent cells (CC) trigger release of virulence factors including Ply from non-competent cells (NC) within the population of the same bacterial species. This phenomenon was named allolysis. Allolysis is a complex process involving at least 6 genes. Upon the intimate contact with NC, CC can activate autolytic enzymes from targeted cells. CC themselves are equipped with an element that confers immunity to the competence-mediated lysis thereby protects them. CC can benefit from NC lysis, because they use nutrients as well as released virulence factors. Allolysis is probably a tool of competition among strains, a predation strategy conserved among transformable streptococci (Guiral *et al*., 2005). Apparent strain to strain variation in the contribution of LytA to Ply release suggests that a further lytic enzyme, glucosaminidase, may contribute to the release (García *et al*., 1989). According to the last discovery, Ply may be located at the cell surface. The placement might be under control of two conserved genes (SP_1924 and SP_1925) upstream of Ply (SP_1923) (Yadav *et al*., in preparation).

Despite many detrimental properties, it is not unreasonable to speculate that Ply preferably promotes carriage, maybe its virulence potential is only manifested within normally sterile body sites or that cytotoxicity is just a result of highly sensitive recognition system and subsequent excessive defence.

So far known impacts of Ply presence include haemolysis, activation of classical pathway of complement in the absence of specific antibodies, inhibition of antimicrobial functions of human polymorphonuclear leukocytes (PMNLs), bactericidal activity of neutrophils, lymphocyte proliferation and synthesis of all immunoglobulins classes, slowing of cilial beating of respiratory epithelium, stimulation of proinflammatory cytokines production, disruption of pulmonary tissue barriers, and other regulatory and cytopathic effects within anatomical sites such as central nervous system, inner ear, eye, lymphoid tissue, upper and lower respiratory tract (Berry *et al*., 1995). Ply alone can induce salient histological features of pneumonia when inhaled into murine lungs. What Ply does not seem to be involved in is the nasopharyngeal colonisation. It disposes of a high affinity to the lung tissue (Rubins *et al*., 1998a).

This major pneumococcal virulence factor induces lysis of eukaryotic cells including RBCs. Higher concentrations of Ply are cytolytic for eukaryotic cells with cholesterol in their membranes and erythrocytes are particularly susceptible to the toxin. Native Ply has a haemolytic activity of between 5×10^5 and 1×10^6 haemolytic units per milligram of protein (HU/mg). A structurally similar perfringolysin from *Clostridium perfringens* prompts that Ply consists of four domains. The process of pore-formation is depicted in Picture 1. Domain 1 provides a solid base for the rest of the protein, allows oligomerization once the protein is incorporating into the membrane, and its negative charge determines orientation of formed oligomers in the membrane. Domain 4 harbors conserved loop rich in tryptophans (Trp), it is responsible for membrane binding and pore formation. Side directed mutagenesis studies have revealed that a motif with the only Cys and Trp are somehow important for haemolytic activity, even though the overall structure is essential rather than single residues (Saunders *et al*., 1989). The bottom of domain 4 only participates in penetrating the

hydrophobic bilayer. Membrane binding promotes oligomerization in which domain 2 is involved creating so-called pre-pores. Once the oligomers are created, they no longer need cholesterol to penetrate the membrane. To complete the pore formation, domain 3 dissociates from domain 2 and approaches membrane. Domain 2 contains sites involved in self-association of Ply monomers (Andrew *et al*., 1997). The last step involves unfolding of domain 3 helical regions and inserting them into membrane. Insertion of the protein into the lipid bilayer is in contrast to binding to it a temperature-dependent process. Membrane is believed to serve as an anchor for Ply monomers so they can assemble, create transmembrane channels of 230 – 260 Å and break the membrane (Tilley *et al*., 2005).



Picture 1. Crystal structure of the soluble Ply monomer (A) and major stages in pore formation: membrane binding (B), oligomerization (C), pore formation (D) (Tilley *et al*., 2005).

Probably only a small number of pores is sufficient to disrupt membrane integrity which leads to osmotic disbalance, leakage of high-molecular weight cytoplasmic molecules and eventually cell death. A pore unit has size of 30 – 35 nm and it can consist of up to 50 monomers (Boulnois, 1992). Cell lysis is important during sepsis, for acute lung and neuronal injury, bacterial growth in the lungs 3 to 6h after i.t. inoculation, and neutrophil recruitment (Benton *et al*., 1997a), (Marriott *el al*., 2008). Point mutations in His_{367} and Trp_{433} eliminate haemolytic activity and improve survival rate in mice during first 6h post infection (p.i.) (Jounblat *et al*., 2003). Surprisingly, substitution of His_{156} by Tyr results in loss of 98% of haemolytic activity. Another study describes mutations at residues from 433 to 436 within conserved Cys motif as affecting neither cell binding nor oligomerization formation; however, these mutations did affect haemolysis: mutated Ply was discovered to form more large channels than did wt Ply. Predominantly small channels caused by wt Ply tend to close in response to bivalent cations and this is no longer true for large channels. Trp_{433} has the most profound effect on the haemolytic activity of Ply (Korchev *et al*., 1998).

Ply activates classical pathway of complement independently on antibodies, a property common to Ply and CRP (Boulnois, 1992). It is the region of a partial amino acid homology with the CRP that is responsible for binding of Fc region of IgG as well as for complement-activating property (Mitchell *et al*.,

1991). Ply increases complement activity by binding C1q. A question why pneumococcal toxin would activate a host defence mechanism might be explained by a presumption that Ply released from bacteria will sidetrack the attention of complement from the living cells. It would also deplete complement compounds so there would not be enough molecules to opsonise the living bacterial cells. Also, enhanced complement activity contributes to inflammation, host tissue damage, and promotes virulence and influx of nutrient-containing serum to the site of infection due to increased vascular permeability (Hammerschmidt *et al*, 2007). The complement-activation property was shown to be important for bacterial growth in the lungs and blood 24h after intratracheal (i.t.) inoculation, bacterial persistence within the lungs, T-cell recruitment, inhibition of PMN-mediated killing of pneumococci, and for accumulation of immune cells in the lesions. Complement activation and antibody binding mechanisms are thought to be related. Tyrosine (Tyr_{384}) and aspartic acid (Asp_{385}) located in one of the regions alike CRP may be important for both (Benton *et al*., 1997a). The complement-activating property seems to be irrelevant when bacteria are introduced intraperitoneally (i.p.) while intranasal route (i.n.) requires both complement-activating and lytic properties for full virulence. Ply works in concert with pneumococcal surface protein (PspA) in order to overcome complement-dependent innate immunity. PspA decreases the intensity of C3 deposition on *S.p.* and Ply ensures a lower proportion of opsonised bacteria by C3 (Yuste *et al*., 2005). Activation also occurs with membrane-fixed toxin and this may result in direct complement-mediated attack to host cells (Paton, 1996).

Haemolytic and complement-activating functions work independently on each other. Unlike complement-activating property, cytotoxic activity can be inhibited by cholesterol-treatment, which in turn seems to promote complement-activating property (Paton *et al*., 1984). A question how much is Ply really important for overall virulence of the pneumococcus has been asked. Both of these functions described above support early growth of bacteria in lung tissue, cause damage to the host by inducing inflammation, and confer resistance to antimicrobial mechanisms of the host but that is it. Even though substitutions of His_{367} → arginine (Arg), Asp_{385} → asparagine (Asn), Cys_{428} → glycine (Gly) and Trp_{433} → phenylalanine (Phe) that abrogate cytotoxic and complement activating properties increase survival of mice after i.n. inoculation, it is noteworthy that those mutations no longer make any difference in bacterial multiplication rate after 48h p.i. in comparison to wild type (wt) strain bacterial loads (Jounblat *et al*., 2003). In conclusion, Ply aids pneumococci by preventing host responses during colonisation or the early stages of infection, but in contrast, when disease is firmly established, Ply contributes to virulence and survival of the pneumococcus at two distinct stages of infection by helping drive excessive inflammatory responses (Kadioglu *et al*., 2000).

One of the strongest antimicrobial capacities of human immunological system is the oxygen-dependent antimicrobial activity of PMNLs. Reactive oxygen metabolites such as H_2O_2 released from PMNLs result in metabolic changes and subsequent respiratory burst that promotes inflammation during pneumococcal infection. The burst helps opsonisation-mediated killing of bacteria. However, should the PMNLs work; first they must approach bacteria at closer distance. This is when another Ply function comes on the scene. Low concentrations of Ply with no effect on cell viability significantly block PMNLs migration and chemotaxis thereby inhibit killing of pneumococci. A region of a toxin important in the lytic mechanism is believed to be involved in these effects on PMNLs (Mitchell *et* Andrew, 1997b). Establishment of bonds

between Ply and PMNLs can be abolished by means of cholesterol pre-treatment (Paton *et* Ferrante, 1983). Higher concentrations of Ply have been implicated in early polymorphoneutrophils (PMNs) recruitment. PMNs are believed to contribute to bacterial dissemination resulting in bacteraemia and early death. Apoptotic PMNs attracted by Ply tend to persist in the lungs and aggravate the inflammation; they do not seem to control the bacterial burden (Marks *et al*., 2007).

Cough reflexes, filtration, secretions and mucociliary transport are examples of non-specific mechanisms. At concentrations higher than 5 ng/ml, Ply reduces the beating of cilia on human respiratory tract epithelium hereby facilitates bacterial entry to the lower respiratory tract (Rubins *et* Janoff, 1998b). This activity is associated with haemolytic property and appears at the late stationary phase of culture and during autolysis. Epithelial disruption, separation of tight junctions, impaired cilia and stimulated mucus production are favourable conditions for bacterial colonisation. Ply alters alveolar permeability which could lead to nutrients and antioxidants influx (Steinfort *et al*., 1989). Other ciliated cells like those of brain ependyma are negatively affected by Ply cytotoxic property too (Marriott *el al*., 2008), (Hirst *et al*., 2004). Cytopathology of cerebral endothelial cells depends on *de novo* synthesis of Ply, tyrosine phosphorylation, and partially on caspase activation (Zysk *et al*., 2001).

The early response to the pneumococcus is guaranteed by T-cells. These CD4+ cells are attracted by pneumococcal toxin Ply. Neither prior CD4+ cells activation nor the haemolytic property of Ply is required for the migration process, not even a close proximity of the cells and bacteria. Another yet not known molecule probably cooperates only with Ply from *in vivo*-grown wt pneumococci (Kadioglu *et al*., 2004).

Antibodies elicited against Ply confer partial protection once the infection is established but do not prevent colonisation (Marriott *el al*., 2008). Antibodies produced by mice exposed to the toxin are largely of IgG class. The fact that the host immune mechanism recognizes Ply is evidence that it truly is a factor of virulence significance. There are at least three epitopes within Ply that react with neutralizing antibodies. Such interactions result in neutralization of Ply cytotoxicity. Epitopes are situated within the cell binding and oligomerization sites in domain 2 (Toyos *et al*., 1996).

It has been discovered that human monocytes increased their production of tumor necrosis factor alpha (TNF-α) and interleukine-1ß (IL-1ß) when exposed to sublytic doses of Ply. These cytokines are the major mediators of inflammation during meningitis bacterial infections; they also activate endothelial and epithelial cells which present more platelet-activating factor (PAF) receptors at their surfaces. The PAF receptor belongs to a G-protein-coupled superfamily proteins and it normally binds PAF via its natural ligand choline. It is hypothesized that the PAF receptor provides a mechanism of cell entry, a way how to get through the endothelial barrier, i.e. the blood-brain barrier (BBB) (Cundell *et al.,* 1995b). Only virulent pneumococci can engage the PAF receptor; a fact that enhances the physiological significance of the pneumococcus-PAF receptor interaction (Cundell *et al*., 1995c). Ply is even a better stimulator of TNF- α production than the cell wall components, but not as strong stimulator as cell wall fragments containing teichoic acid in case of IL-1 ß (Houldsworth *et al*., 1994). Monocytes-produced TNF-α provides protection against lung infections with wt strains and IL-1ß is important in survival beyond 5 days p.i. (Benton *et al*., 1998).

Ply was found to initiate nitric oxide (NO) production in macrophages. Besides its function as a neurotransmitter and a vasodilator, NO is also a strong antimicrobial immunity element contributing to septic shock and cochlear damage. Ply from a wt strain produces dose- and time-dependent NO production by murine macrophages and this production is strictly dependent on gamma interferon (IFN-γ), the best known activator of macrophages (Braun *et al*., 1999). Ply modulates IFN- γ production by spleen cells by a mechanism different from pore formation. More over, it seems that cytolytic property somehow interferes with the induction of IFN- γ. As we already know, should the cytotoxicity manifest, the overall molecular structure of the toxin must be complete. Therefore it is likely that changes seen in nonhaemolytic proteins render the protein different skills, i.e. IFN- γ induction (Baba *et al*., 2002).

Ply interacts with so-called Toll-like receptors (TLRs), specifically TLR4 on the surface of macrophages and this physical interaction does not involve either lipopolysaccharides (LPS) or pore-forming domains. TLR4 subsequently trigger nuclear translocation and activation of transcriptional factor NF-ƙB. Signalling pathway leads towards release of inflammatory molecules such as NO and TNF-α, and eventual cell death. Recognition of Ply by TLR4 confers protection against infection with *Streptococcus pneumoniae* after nasopharyngeal challenge (Malley *et al*., 2003). Ply-stimulated TLR4 activate apoptotic processes in pulmonary cells. Apoptosis of alveolar macrophages in the upper airways during nasopharyngeal pneumococcal exposure is a wanted event because it promotes clearance of the bacteria by killing the infected phagocytes at an early stage of pneumococcal murine pneumonia but only when the original inoculum is low in. It partially depends on caspases as well as on the cytotoxic properties of Ply. On the other hand, apoptosis during meningitis is deleterious to the host' nervous system (Srivastava *et al*., 2005). Exogenously given Ply does not induce apoptosis but Ply produced by living bacteria does. Apoptosis is beneficial during both colonisation and infection when it enhances the bacterial clearance and regulates the inflammatory response, respectively. However, macrophage apoptosis is no longer beneficial in lethal infections when damage to tissue is too extensive (Marriot *el al*., 2008).

Studies with Ply negative mutant (PLN) strains point towards interesting findings; in all possible routes of infection ((i.n.), (i.p.), (i.t.), intravenous (i.v.)), PLN strains show reduced virulence in comparison to wt strains, in case of i.n. route, PLN strains are avirulent (Berry *et al*., 1992). Reduction of virulence is accompanied with lower inflammation, slower multiplication within the lung and in the blood, decreased injury of alveolar-capillary barrier, less intense penetration of interstitium of the lung, and delayed onset of bacteremia which becomes chronic after reaching 10^7 colony forming units per millilitre of the blood (cfu/ml) (Berry *et al*., 1989). When compared to wt strain in the course of *in vivo* infection which was capable of exponential net growth up to lethal $10^{9\text{-}10}$ (cfu/ml), PLN stopped growing at $10^{6\text{-}7}$ cfu/ml regardless of the initial dose. This implies that Ply interferes with either function or generation of inflammation-induced immunity and permits continued exponential net growth of pneumococci (Benton *et al*., 1995). The major effect of Ply deletion is a delay in time to death, which results of an overall debilitation (Wellmer *et al*., 2002). However, it eventually happens anyway despite that infected hosts might be able to control the level of sepsis. Neither cytoplasmic nor extracellular fluctuating production of Ply contributes to differences seen in virulence among strains of various serotypes, namely types 2, 3, 4, 5, and 6. More likely,

a basic minimal level of multiplication, serotype and other virulence factors are responsible for the discrepancies. It has been documented that fully functional Ply produced by wt strains can compensate for Ply missing in mutant strains during *in vivo* infections suggesting Ply's effects exerted at a distance (Benton *et al*., 1997b).

Ply changes gene transcription in eukaryotic cells. A vast spectrum of consequences from this effect can be drawn. Ply directly as well as indirectly affects host transcriptional factors that may change host responses, cell signalling, adherence, defence and survival. For example, sublytic doses of pore-forming toxin open calcium-dependent pores, alter membrane charge and activate p38 mitogen-activated protein kinase (MAPK) in epithelial cells that leads to amended signalling and transcription pattern (Marriott *el al*., 2008). A recent study noted that different opacity phenotype plays a role in Ply gene expression. It was shown that Ply gene is consistently induced in transparent variants of bacteria attached to middle ear mucosa unlike opaque variants (Li-Korotky *et al*., 2009). On the contrary, deletion of iron-dependent transcriptional regulator (PsaR) gene seemed to have an impact on Ply gene expression too, which was downregulated during *in vitro* growth of TIGR4 strain. The virulence in murine model of pneumonia was the same for wt and the PsaR mutant. The only implication whatsoever was that mice infected with the mutant expressing less Ply had substantially lower bacterial loads at 24h p.i. (Hendriksen *et al*., 2009).

It was found that absence of Ply prevents development of sepsis, retards onset of bacteremia and subsequently the time to death. It was shown that PLN strains asymptomatically persisted within the lungs for at least 11 days (264h) pointing towards possibility that maybe PLN strains posses a kind of advantage from not expressing Ply that allows them to survive in the bloodstream and lungs without being eliminated. Although mutations in Asp_{385} → Asn, Cys_{428} → Gly and Trp_{433} → Phe abolish Ply cytotoxic as well as complement-activating properties, such a mutated pneumococcal strain still retains nearly full virulence in bronchopneumonia model of infection that is higher than that of a PLN strain. These findings strongly suggest an existence of yet unknown function of Ply and its significance in causing IPD (Berry *et al*., 1999). The triple mutant maintains the Fc binding capacity, induction of IFN-γ secretion, and proinflammatory responses by macrophages resulting from interaction between Ply and TLR4. It is inevitable to speculate whether these remaining abilities play a greater role in bacteremia than either haemolysis or complement activation (Benton *et al*., 1997a).

Ply exists in number of allelic forms and it is probably more variable than previously thought. Fourteen Ply variants have been recognized in a set of 121 clinical isolates of *Streptococcus pneumoniae* (Jefferies *et al*., 2007). It is worth explaining that there might be many more variants of Ply alleles than just these 14 protein types since variation on DNA level does not have to be necessarily reflected in amino acid sequence variation. Ply type denoted by number 1 is assigned to D39 laboratory strain of serotype 2. Ply 2 belongs to TIGR4 strain of serotype 4. Ply 5 is present in all serotype 1 ST 306 strains tested plus in serotype 8 strains. The only difference between Ply 5 and 3 is an extra mutation at position 150 in Ply 5 that changes Tyr to His. It is a mutation unique to Ply 5. A thousand times decreased haemolytic activity implies that although Tyr_{150} is an important residue, other mutations (threonine (Thr_{172}) → isoleucine (Ile)) are needed to abrogate haemolytic activity completely (Lock *et al*., 1996). Ply 5 produces a nonhaemolytic Ply. ELISA

ruled out possibility that observed nonhaemolysis is because those strains do not express Ply. In a mice model of infection, haemolytic activity did not seem to play a role in virulence of serotype 1 strains of either ST 227 or ST 306 (Kirkham *et al*., 2006).

2.3.2. Hyaluronidase

Streptococcus pneumoniae hyaluronate lyase was first described in Meyer's laboratory in 1937 (Meyer *et al*., 1941) as 'spreading factor.' It is commonly known as hyaluronidase (Hyl), a bacterial spreading enzyme that catalyzes degradation of hyaluronan (HA), a glycosaminoglycan in the extracellular matrix of connective tissue, chondroitin and chondroitin sulphates. Its multi-functionality in an organism and high turnover makes this association interesting. Clearing of these substances promotes bacterial invasion further into the host. Degradation products such as glucose and glucuronic acid can also be utilized by bacteria as a source of carbon. The Hyl gene has been partially cloned and it is annotated as SP_0314 in TIGR4 strain genome. There is only one copy of this gene in the *Streptococcus pneumoniae* chromosome (Mitchell *et al*., 1997a). The full-length Hyl has a molecular weight of 107 kDa. The partially cloned but active Hyl protein expressed in *E. coli* is approximately 89kDa and it best works at acid pH 5.6 – 5.8 (Jedrzejas, 2001).

Crystallographic studies revealed that Hyl molecule has two domains connected by a linker; the N-terminal α-domain which forms a major part of a cleft in the middle of Hyl where binding and degrading of HA takes place, and the C-terminal ß-domain which modulates Hyl's activity rather than degrades HA directly (Ponnuraj *et* Jedrzejas, 2000). Amino acids Asn_{349}, His_{399} and Tyr_{408} make up the active centre of the enzyme. His_{399} is a key catalyzing residue and Tyr_{408} acts as a proton pump providing proton from surrounding water molecules to break down a glycosidic bond (Lin *et al*., 1997). Positive and negative charges in the cleft and HA, respectively; hold the whole structure together during ß-elimination reaction (Ludowieg *et al*., 1961). Weak binding interactions such as salt bridges, ionic interactions and hydrogen bonds help keep the reaction going. The degradation process is endolytic; once the enzyme binds the substrate, it progressively cuts it from the reducing end until it leaves only mostly unsaturated disaccharides being the smallest degradation products of HA (Li *et al*., 2000). Residues concerning substrate binding and degradation are highly conserved among streptococcal lyases (Jedrzejas *et al*., 2002).

Hyl belongs to the family of cell wall-anchored proteins showing the C-terminal LPXTG motif without the N-terminal signal peptide. It is found in both pneumococcal culture supernatant and the cell-associated fraction. Hyl is actively secreted during log-phase growth *in vitro.* It is produced by a vast majority of clinical isolates but with a considerable variation in activity (Meyer *et al*., 1941).

2.4. The success of serotype 1 ST 306 strains

It has been suggested that pneumococci are prone to a freely recombining population structure with epidemic spread of successful clones (Hall *el al*., 1996). Primacy in causing invasive disease changes in time and varies with geographical locations. More recently, it seems that the severe pneumococcal disease is most often caused by serotype 1 strains initially classified in 1913 (Sandgren *et al.,* 2004). In contrast, serotype 1

is very rarely or never found among healthy carriers because it is short-lived. Serotype 1 strains were isolated from different parts of the world. Brueggemann *el al* observed an inverse relationship between disposition to cause invasive disease and duration of carriage: the more invasive isolate, the less commonly carried in the nasopharynx. They didn't find any evidence of temporal or geographical differences in potential for invasiveness among isolates (Brueggemann *el al*., 2004). In every way, serotype 1 has a high attack rate and therefore is at the forefront of scientists' attention.

Pneumococcal serotypes are made up of genetically distinct clones (Jefferies *et al*., 2004). A study focusing on geographical distribution of serotype 1 strains revealed that a few highly virulent pneumococcal clones appear to be circulating around the world. These clones are capable of natural serotype switching. MLST clustered them into 3 major lineages; lineage A represents exclusively European and North American isolates, lineage B contains strains from Africa and Israel, and lineage C isolates come mainly from Chile. Apart from England, ST 306 and ST 217 are prevalent clones of serotype 1 in Europe, Africa, and Israel, whereas ST 227 is the most common clone in England. However, these clones do not represent the main cause of invasive disease in North America thus it is not possible to conclude which clone is responsible for the prevalence of serotype 1 disease worldwide (Brueggemann *et* Spratt, 2003b). Serotype 1 ST 227 isolates were also found among indigenous Australian children with unexpectedly high carriage rates. This raises concerns about potential serotype replacement with nonvaccine serotypes that is more likely to happen during carriage (Smith-Vaughan *et al*., 2009**).** It seems that clonal selection occurs among strains that cause bacteremia but not among those that cause meningitis. A Swedish study from the period 1987 – 1997 points out that serotype 1 with predominating ST306 is on the rise. Increasing serotype 1 clones are penicillin-susceptible which means that the rapid emergence cannot be attributed to selection by antibiotic usage (Normark *et al*., 2001).

Another important pneumococcal serotype with propensity to cause disease in older children and adults is serotype 8 (Marks *et al*., 2007). Sequenced nucleic acid from wild type strain ATCC6308 revealed some similarities with the allele serotype 1 strains virulence factor pneumolysin. That is why one of the serotype 8 strains was added to the analysis.

Nonhaemolytic Ply has been found in serotype 1 ST 306 isolates in lineage A, and serotype 8 ST 53, a clone already dominant in causing invasive disease in Scotland. A recently studied serotype 1 strain ST 3018 from lineage C with Ply designated as Ply 4496 is hypervirulent too but its Ply possesses low haemolytic activity. What is surprising is that these strains with nonhaemolytic Ply were isolated from patients suffering invasive pneumococcal disease caused by serotypes 1 and 8 known to be associated with outbreaks. Unexpectedly, Ply 4496 strain grew faster in the blood than the wt strain with fully haemolytic Ply. Alignment of Ply 4496 with Ply 5 from ST306 serotype 1 and 8 strains revealed that Ply 4496 contains the same SNPs (Thr_{172} → Ile, lysine (Lys_{224}) → Arg, alanine (Ala_{256}) → serine (Ser)) and deletions (valine (Val_{270}), Lys_{271}) as Ply 5. However, it lacks Tyr_{150} → His which could explain the residual haemolytic activity of Ply4496 compared to nonhaemolytic Ply 5 strains. Additionally, Ply 4496 has a mutation Thr_{378} → Asn that has not been reported previously. The full haemolytic activity may actually slow down early proliferation in the blood. These findings suggest a role for haemolytic activity in severity of bacteremia

whereas nonhaemolytic properties of Ply may be important for proliferation at the beginning of infection in the blood (Harvey *et al*., 2011). Jefferies *et al* propose a hypothesis that this newly observed feature of Ply is maintained by the pneumococcus itself since it seems to correlate with certain clones that have undergone expansion. Ply 5 may confer some yet unknown advantage in combination with expression of other proteins working in accordance and this may actually drive such clonal expansion. Studies carried out by Alexander *et al* and Rubins *et al* indicate that even a residual haemolytic activity makes a great difference in virulence in comparison to completely nonhaemolytic or PLN strains. No relationship was seen between the total haemolytic activity and virulence. Perhaps a solid and intact structure of domains involved in pore formation and production of a minimum threshold level of Ply is sufficient for virulence (Alexander *et al*., 1994), (Rubins *et al*., 1995).

2.5. Host colonization & colony morphology

Naturally, *Streptococcus pneumoniae* is a commensal in the human throat and the nasopharynx (Jenkinson, 1997). It is possible though that pet animals living in close contact to humans can be infected by human isolates. It is not known whether pneumococci residing in animals can re-infect humans (Linden *et al*., 2009). The prevalence of usually 6 weeks lasting asymptomatic carriage is much higher than the incidence of invasive disease that is rather a rare event. Persons who have only recently become colonized have higher chances to progress into state of invasive disease (Paton *et al*., 1993). A patient may carry up to four different serotypes. The invasive disease is always preceded by at least short colonisation. Sometimes, the only one but a significant difference between a colonizer and an invader is the invader's ability to gain access to a niche within a host and to invade barriers that separate it (Dagerhamn, 2009).

The colony phenotype can be visually differentiated on either opaque or transparent variants by their appearance when viewed on transparent solid agar because of differences in transmission of light. The opaque appearance is characterized by bigger and bulgy colonies with more production of polysaccharide capsule and a surface protein A; it has an advantage over the transparent phenotype once in the bloodstream during systemic infection. The transparency is usually accompanied with lower amount of capsular polysaccharide, smaller, transparent and colourless colonies with bigger amount of teichoic acid in cell wall as well as a choline-binding protein A (CbpA). Transparent colonies better adhere to surfaces; they possess a selective advantage in the colonisation of the nasopharynx (Cundell *et al*., 1995a). Transparent phenotype resembles pneumococci in the process of autolysis, which would support the hypothesis, that during colonization of the nasopharynx, bacteria have better conditions to share the gene pool than during invasive disease in the blood stream (Saluja *et* Weiser, 1995). Those two phenotypes can be switched spontaneously back and forth at frequencies of 10^{-3} to 10^{-6}. This process is called the phase variation and it is believed to exist in order to help bacteria to deal with the challenges encountered in the nasopharynx and in the blood. Since those two are physiologically completely different niches, even though they are within one individual host, each phenotype may provide a selective advantage depending on where the organism is located at the time. The capsular type plays a little role in the phase variation if any at all; the presence of capsule seems to attenuate invasion of endothelial cells; that would explain the higher success rate of transparent bacteria with

less capsular material in colonisation as well as in invasion of the epithelium (Ring *et al*., 1998). However, some serotypes seem to be more prone to changes in the colonial morphology than other serotypes (Weiser *et al*., 1994). An assumption that it is rather the amount of teichoic acid than capsule that influences the phenotype variation has been made. It is the total amount of teichoic acid; thereby the choline itself that seems to matter. No difference in structure of lipoteichoic acids of either phenotype was observed (Kim *et* Weiser, 1998). Opacity locus has been discovered including BOX A-C element, which alters the rate of switching (Saluja *et* Weiser, 1995).

Especially young children are very likely to be colonized, sometimes by more than one serotype (Müller-Graf *et al*., 1999). It is assumed that children constitute a main reservoir for pneumococci and a source of spread to the adults and elderly individuals. With many other bacteria living there, the nasopharyngeal environment can therefore serve as a pool of genes that can be shared and exchanged within the microbiological community, which again promotes variation. Natural competence for genetic transformation is the best-characterized feature of the pneumococcus. The uptake of DNA provides a genetic pool to repair abundant mutations incurred by oxidative lifestyle during aerobic growth (Kadioglu *et al*., 2008). It is known that DNA release and competence are casually related processes.

It has been found that the overall diversity is higher among carriage strains than among isolates recovered from patients with invasive disease. In other words, isolates of serotypes with a high invasive disease potential (i.e., types 1 and 7F) are genetically highly related, whereas isolates belonging to serotypes with less invasive potentials (i.e., 23F and 19F) are much more diverse (Sjöström *et al*., 2006), (Sandgren *et al.*, 2004). Robison *et al* found that most invasive isolates were present among carriage isolates too. Apparently, the balance between the microbe and its host is very fragile and important to keep (Robinson *et al*., 2001). One of the other features of the capsule type is that it can predict duration of carriage as well as the attack rate of the pneumococcus in children. The longer the carriage lasts, the lower the attack rates are (Sleeman *et al*., 2006). In terms of energy costs of capsule production, a link between polysaccharide structure and carriage has been discovered. Those serotypes that produce metabolically inexpensive capsules have more amount of it and tend to stay in the nasopharynx for longer period which results in higher prevalence. This phenomenon may be explained by the fact that more amount of capsule means a better protection against neutrophil-mediated killing, which is one of the basic mechanisms of host immune system of clearing the pneumococci from the upper respiratory tract (Bruyn *et al.*, 1992). It is a kind of trade-off; pneumococci with more amount of capsule can evade neutrophils because it is well known that the capsule serves as a barrier rather than a target to neutrophils. That is why elimination by phagocytosis can not happen notwithstanding that respective cell wall antibodies are present on bacterial surfaces (Musher, 1992). These heavily encapsulated pneumococci interact less with the epithelium which they need to interact with in order to get deeper into the host. On the other hand, less encapsulated serotypes are more prone to be killed by neutrophils but they interact better with the mucosal surface and have better chances to invade the host in the end (Weinberger *et al*., 2009).

2.6 Pathogenesis

2.6.1. Invasiveness

The pneumococcus remains one of the top 10 most serious human pathogens with high morbidity and mortality (Coffey *et al*., 1998). It is particularly dangerous to the young, elderly and immunocompromised people in developed countries despite the existence of vaccine and antibiotics (Hoskins *et al.,* 2001). Apart from inducing local infections such as otitis media and sinusitis, the pneumococcus can cause life-threatening sepsis and meningitis and it is a leading agent in causing community-acquired pneumonia (Hammerschmidt *et al*, 2007). Both bacteraemia and meningitis can be associated with pneumonia. An emergence of penicillin- and multi drug-resistant pneumococcal strains doesn't help the global situation (Sutcliffe *et al*., 1996), (Brenwald *et al.,* 1998), (McGee *et al*., 2001). An example of such resistant worldwide spreading clone is the Spanish strain of serotype 23F (Croucher *et al*., 2009). Interestingly, the resistance doesn't necessarily lead to a higher invasiveness (Ewig *et al*., 1999). The outcome of the pneumonia doesn't depend only on the number of viable microorganism but also on factors such as male sex, low or high age, co-morbidities (immunity deficiencies, diabetes, bacteraemia, HIV infection), absence of capsular polysaccharide antibodies (Zysk *et al*., 2003), mechanical ventilation, smoking, alcoholism etc. that significantly increase the risk of severe disease and death (Fine *et al*., 1996), (Berg *et al*., 2006). According to Alanee *et al*, host factors are better predictors of the severity of invasive pneumococcal disease (IPD) than microbial factors (Alanee *et al*., 2007). Surviving pneumococcal meningitis often means dealing with long-term clinical neurologic sequelae and neurophysiological problems such as seizures, motor deficits, hearing loss, cognitive impairments and even mental retardation (Hammerschmidt *et al*., 2007).

2.6.2. Pneumococcal pneumonia – role of pneumolysin

Understanding the process of invasive disease is fundamental. Wt *Streptococcus pneumoniae* life cycle has three major events; transformation, adherence and autolysis (Tuomanen, 1997). Each phase is triggered at a specific cell density consistent with a quorum sensing paradigm. The first step towards bacterial pneumonia is the spread from the nasopharyngeal reservoir downwards to the lungs in aerosol state by inhalation. There the bacteria preferentially encounter the resting epithelial and type-II pulmonary cells (Cundell *et* Tuomanen, 1994). The adherence to eukaryotic cells is dose-dependent, quick, independent of capsular type, and it is believed to be mediated by special microbial proteins that link themselves to glycoconjugate-containing receptors on the surface of vascular endothelial cells (Wizemann *et al*., 1999). Pneumococci were proven to have toxic effects on epithelial cells; cytoplasmic blebs, mitochondrial swelling, cellular extrusion, and cell death were observed in studies with electron microscope (Steinfort *et al*., 1989). So far, transparent and opaque variants adhere continuously to inactivated host cells with more or less the same low efficiency. Once these eukaryotic cells are activated by cytokines during the onset of the disease, they upregulate expression of PAF receptors which seems to be binding only transparent

pneumococci in peaks-like fashion, a fact that links virulence to the ability to recognize new receptors. It results in enhanced internalization of the bacteria by receptor-mediated endocytosis and progression into the bloodstream (Cundell *et al*., 1995a).

Ply was found to facilitate intraalveolar replication of pneumococci, penetration into interstitium of the lungs, and the spread of the bacterium from the lungs to the blood during experimental pneumonia (Feldman *et al*., 1991). It has a critical role in sepsis during the first few hours p.i., however, once the chronic bacteremia is established, Ply is no longer able to act as a virulence factor. The role of the cytokines is complicated. Inflammatory cells release cytokines into surroundings in response to Ply *in vitro*. Ply attracts an excessive numbers of neutrophils into the lungs where they secrete IL-8 in dependence on extracellular Ca^{2+}. This chemokine recruits more neutrophils, activates them and may contribute to the exaggerated pulmonary inflammation (Cockeran *et al*., 2002). Neutrophils are the first and most abundant inflammatory cells accumulated in the lungs. On the other hand, Ply can also prematurely deactivate neutrophils, defer its avalanche as mentioned earlier, and disorientate them (Paton *et* Ferrante, 1983). This probably depends on different circumstances and concentrations. Ply is a double-edge sword because it both stimulates and inhibits host responses vital to the pneumococcus and it causes excessive pulmonary inflammation. Ply activates phospholipase A in pulmonary artery endothelial cells via forming transmembrane pores, phospholipase A releases free fatty acids and causes direct cytotoxicity (Marriott *el al*., 2008).

2.6.3. Pneumococcal meningitis - roles of pneumolysin and hyaluronidase

A similar pattern is seen in the development of meningitis. Pneumococcal meningitis is the most severe infection a child can catch in its early childhood. It occurs during high level bacteraemia and is accompanied by high fevers. The most serious complication during bacterial meningitis is the brain edema caused by microvascular changes and damage to the BBB (Small *et al*., 1986). The transparent phenotype shows significant increase in invasiveness to human brain microvascular endothelial cells (Ring *et al*., 1998).

Ply occurs at concentrations from 0.85 to 180 ng/ml in CSF of meningitis patients; such moieties are able to exert pathophysiological effects (Spreer *et al*., 2003). Since Ply disrupts the integrity of the cerebral endothelial cells, it is the main tool utilized by pneumococci when it comes to getting across the BBB (Zysk *et al*., 2001). Damage seen in meningitis infection is ascribed to the host inflammatory response and resultant neuronal apoptosis mainly in the dentate gyrus of the hippocampus. Apoptosis is a biological process that is most responsible for the injury to the nervous system and it is not influenced by polysaccharide capsule (Braun *et al*., 2002). Ply is supposed to play a role during the first phase with its direct cytotoxicity on neurons in concentrations above 0.1 μg/ml. Even though pneumococci tend to stay and multiply in the CSF, released Ply can diffuse into the parenchyma. The toxicity of Ply is more severe than of host-derived H_2O_2 *in vivo*. After the cell wall components are released, inflammation is triggered and caspase-dependent mechanism sustains ongoing apoptosis (Mitchell *et al*., 2004). Overall impact of Ply on central nervous system is often terminated by development of sensineural deafness, because both supporting cells and hair cells in the organ of Corti in the inner ear are especially sensitive to Ply's cytotoxicity (Comis *et al*., 1993). Ply defective in cytolytic but not complement-activating activity is not proapoptotic. Cells of eye retina seem

to be damaged by Ply's complement-activating function which is also important for CSF pleocytosis (Marriott *el al*., 2008). Friedland *et al* agree that Ply can stimulate inflammatory cascade in the CNS, and that it promotes invasion of the BBB, however, they claim that Ply is not necessary for the pathogenesis of meningeal inflammation which is more likely caused by released cell wall components (Friedland *et al*., 1995). Moreover, Orihuela *et al* suggest that Ply is decreased once the bacteria reach CSF because they adapt so as to decrease damage to neurons (Orihuela *et al*., 2004a).

Hyl is considered to be a potential virulence factor (Berry *et al*., 1994). Even though interruption of Hyl gene was found to have no impact on virulence when injected i.p. into mice, double mutation in Ply as well as in Hyl genes resulted in even a lesser virulence than observed with only Ply mutant (Berry *et* Paton, 2000). On the contrary, disruption of Hyl gene in strains introduced i.n. caused a reduction in the virulence. These results indicate that in case of Hyl the outcome very much depends on the route of infection. Hyl is produced by all meningitis isolates, majority of otitis media strains and rarely by carriage strains indicating once again Hyl's role in breaching of the BBB. There is no correlation between capsular type and Hyl's activity, however, a loss of capsule leads to a loss of Hyl's activity (Kostyukova *et al*., 1995). Pathological conditions such as obstruction of Eustachian tube or aeration of the middle ear provoke Hyl expression upregulated in transparent-showing phenotype bacteria attached to the epithelium and opaque variants found in supernatant. It is thought that these conditions and also aerated environment trigger Hyl that promotes adherence and invasion (Li-Korotky *et al*., 2010). *Streptococcus pneumoniae* strains demonstrate a strong correlation between Hyl activity and the capacity to induce meningitis. The mechanism of Hyl action probably lies within its capability to loosen the barrier of the nasopharyngeal mucosa (Zwijnenburg *et al*., 2001).

2.6.4. The roles of serotype and sequence type

Although pneumococci belong to one of the most diverse species in terms of clonal and antigenic variability, only a few serotypes are associated with the invasive disease, for example serotypes 1, 4, 7F, 9V, 14 and 18C, and some are associated only with carriage, i.e. serotypes 3, 6B, 19F, 20, 23F and 27 (Brueggemann *et al*., 2003a), (Sandgren *et al*., 2005). Serotype 3 is heavily encapsulated and it acts like an opportunistic pathogen. However, when it does invade, it is more severe than a disease caused by less encapsulated strains; those are usually more invasive but cause no deaths, such strains behave as primary pathogens (Sjöström *et al*., 2006), (Weinberger *et al*., 2010).

It is inevitable to speculate to what extent the capability of pneumococci to cause invasive disease is determined by the capsular serotype or the sequence type. Undoubtedly, the capsule is one of the most significant virulence factor pneumococci possess. However, since we can determine the sequence type, a question whether it's genotype rather than serotype that plays the main role has been raised. So far the studies made clear that genetic aspects are in tight relation to serotype properties when it comes to invasiveness (Brueggemann *et al*., 2003a). Just like only a fragment of all existing serotypes causes invasive disease, few particular clones are overrepresented in disease compared to carriage. The fact that strains of same serotypes and STs yet behave differently in animal infection models can be explained by additional

quantitative and qualitative variations in virulence factors between isolates (Silva *et al*., 2006). For example, serotype 14 has been shown to include carriage clones as well as invasive disease clones (ST230 and ST307, respectively). A clone is a group of isolates that share identical sequence of all seven genes. It also works the other way round, when within 1 ST at least 2 different serotypes exist and this could be explained by a recombination event resulting in a phenomenon known as capsular switching. That is a case of isolate ST162 with serotypes 19F and 9V (Sandgren *et al.*, 2004). Capsular switching might happen more frequently then previously though. It is assumed that the longer strains stay in the nasopharynx (or the more often they are carried), the greater opportunity to switch their capsules by means of transformation of their capsular genes. Serotype replacement that occurs under the selective pressure of a host's immune system and current pneumococcal vaccine seems to be quite a frequent phenomenon among natural isolates and this could lead to a rise of the vaccine escapes (Kelly *et al*., 1994). It is of concern that serotype replacement may favour the emergence of successful strains, originally of vaccine serotype that have acquired a nonvaccine capsule through serotype switching. In truth, if vaccine serotypes are hardly carried, there is a little chance they would have enough time or opportunity to switch their capsules with those being carried in the nasopharynx and thereby less harmful. Logically speaking, higher levels of carriage of non-vaccine serotypes should not be a problem as long as they are poorly invasive (Hanage *et al*., 2005b). Experiments carried out with isolates of identical genetic background but different serotypes and with same serotype but distinct pulse-field-gel-electrophoresis (PFGE) patterns indicate that the extent of bacterial virulence cannot be predicted from its capsular type alone (Nebenzahl *et al*., 2004). For instance, 6A and 6B strains vary greatly in their disease potential. It is not known whether it is because their capsules have a little bit distinct structure (different linkage between the rhamnose and ribitol sugars), or whether variant STs are to blame. To cut a long story short, rather a combination of serotype, genetic background, and site of infection and host factors determines the outcome (Kadioglu *et al*., 2002).

2.7. Virulence factors

2.7.1. The capsule

Streptococcus pneumoniae is equipped with wide range of virulence factors. One of the most explored and studied one is the polysaccharide capsule. It clearly plays a role in disease; acapsular mutants are avirulent (Magee *et* Yother., 2001). Even though the capsule itself might be a barrier for adherence in the nasopharynx because it can hide some of the important adhesive molecules, at least a basal level of it is certainly needed for colonisation (Ring *et al*., 1998). It prevents mechanical removal by mucus on the basis of repulsion of negatively charged capsule and mucosal components (Zysk *et al*., 2003). Electron microscopy studies show that thickness of the capsule is reduced upon the intimate contact with respiratory epithelial cells, where it is no longer needed. Lesser amount of capsule enables adhesive molecules to be exposed, hence it promotes adherence (Talbot *et al*., 1996). But it is a double-edge sword. As the volume of capsule decreases, the pneumococcus becomes less pathogenic in terms of its capability to escape the immune

system. Changes in levels of encapsulation are probably regulated by genetic mechanisms in order to adapt to given conditions (Hammerschmidt *et al*., 2005). Capsule is a *sine qua non* of virulence on which the survival of bacteria in the bloodstream depends. Perhaps the most important role of this key virulence determinant is preventing phagocytosis thereby protecting the bacteria from ingestion by macrophages. It fixes complement and degrades its components; it can restrict autolysis and reduce exposure to antibiotics. Capsule is a kind of shield that protects putative bacterial surface proteins from being inactivated by circulating host antibodies. The capsule alone causes no inflammation (Tuomanen *et al*., 1995).

2.7.2. The cell wall

Underneath the capsule lies the cell wall. Besides the main component peptidoglycan, C polysaccharide and lipoteichoic acid strongly activate the alternative pathway of the complement cascade, bind C-reactive protein, promote coagulation, induce cytokines and PAF, draw leukocytes. It is well known that tissue injury during invasive disease is caused by host immune system, but it is the pneumococcal cell wall bioactive pieces that trigger that damaging inflammation when they are released during bacterial last phase of life, the autolysis (Mitchell *et al*., 2004). This situation resembles a final call; if a bacterial cell must die, at least its parts will help the rest of bacteria by increasing inflammation which seems to be a key prerequisite for invading human epithelial cells and facilitates further spread into other tissues (McCullers *et* Tuomanen, 2001). Above that, autolysis could be a mechanism how to exchange genetic material via the release of DNA. Once in the bloodstream, pneumococci interact with vascular endothelium. Both intact pneumococci and purified cell walls were found to adhere specifically to endothelial cells, cause cell separation, slowed ciliary beating and serious morphological damage resulting in eventual destruction of these cells in dose and time dependent manner. Single cell wall parts are stronger inflammation inducers than is the intact cell wall. This observation should be taken into consideration when administrating antibiotics because they may even augment the harmful effects of pneumococcal pneumonia and induce irreversible shock (Tuomanen *et al*., 1995).

2.7.3. Adhesins

Since hardly any strains possess any fibrils, another mechanism of attachment to host cells must be employed. Surface of the pneumococcus is bordered with a vast repertoire of proteins that may be somehow involved in pathogenesis. Since every single case of pneumococcal invasive disease or at least non-pathogenic colonisation starts with attachment to host cells, great attention is given to adhesins for a vaccine made of these adhesins would stop the invasion in its very beginning. Even better, adhesins are generally conserved antigens eliciting protecting IgG antibodies (Wizemann *et al*., 1999).

There are three classes of proteins in the genus pneumococcus.

(i) Surface proteins are characterized by LPXTGE motif that serves as a cleavage site and fixation of a protein to the cell wall by sortases. LPXTGE proteins are therefore important for the interaction with host cells and appear to be highly variable (Brückner *et al*., 2004). These include three neuraminidases (NanA,

NanB, NanC) (Cámara *et al*., 1994), Hyl, IgA1-protease, zinc metalloproteases (ZmpB, ZmpC and ZmpD), and surface proteins lacking the LPXTGE motif; enolase, glyceraldehyde-3-phosphate dehydrogenase, pneumococcal adherence and virulence factor A (PavA), and Ply (Hammerschmidt et al, 2007). NanA cleaves terminal sialic acid residues not only from glycoproteins, glycolipids and oligosaccharides on eukaryotic cell surfaces but also from LPS on microbial competitors in the nasopharynx such as *Neisseria meningitidis* and *Haemophilus influenzae* (Shakhnovich *et al*., 2002). Desialylation is an enzymatic reaction that unmasks ganglioside receptors possibly important for adherence; it also decreases the viscosity of lung mucus (King *et al*., 2005). This may explain why attachment of *S. pneumoniae* to respiratory epithelial cells is enhanced following infection with neuraminidase-producing influenza virus. Cleaving sugars from the mucosa provides a source of carbohydrates for bacterial metabolism. Adhesin-like function of this protein promotes invasion of the brain endothelium. IgA1-protease cuts human IgA1 into two fragments, Fab and Fc. Fab fragments chunked like that retain its antigen specificity and bound to bacterial surface preventing binding of an intact antibody. PavA and enolase bound with fibronectin and plasminogen, respectively, enolase potentiates degradation of the extracellular matrix thereby these promote colonisation.

(ii) The second class represents surface lipoproteins that bind to palmitic acid in the membrane. Pneumococcal surface antigen A (PsaA) is a divalent 37 kDa metal-ion binding lipoprotein component of an ATP-binding cassette (ABC) transporter system, which is required for processing and export of biologically active peptides.

(iii) Choline binding proteins (CBPs) as the third and most unique class are noncovalently and reversibly bound via the conserved choline-binding domain to phosphorylcholine in the cell wall (Gosink *et al*., 2000). The number of CBPs depends on a strain and it can vary from 13 to 16 CBPs. Basically, CBPs family includes members of pneumococcal surface proteins (PspA, CbpA) and cell wall hydrolyses (LytA). PspA is one of the most polymorphic pneumococcal proteins which sequence substantially varies among isolates (Sadowy *et al*., 2006). It is expressed by virtually all serotypes and is believed to have a protective potential. Interestingly, it functions oppositely to other pneumococcal virulence factors; as a matter of fact, PspA inhibits complement activation. Such paradox has a quite reasonable explanation; complement inhibition is desired at the beginning of the infection when pneumococci are still alive and multiplying. Later on, when bacteria lyse, complement activation leads to lysis and enhancement of the inflammation, pneumococci reach the stage of no return (AlonsoDeVelasco *et al*., 1995). CbpA is the largest and the most abundant of the CBPs. Also, it is the first known protein adhesin on pneumococcal surface. It creates a bridge between choline in teichoic or lipoteichoic acids and human glycoconjugates. This link is restricted to cytokine-activated human cells. CbpA clearly promotes the adherence. LytA is the major endogenous enzyme of pneumococcal cell wall hydrolyses. The enzyme is anchored to the cell membrane in an inactive state until the cell wall biosynthesis stops due to nutrient starvation or treatment with antibiotics. Its main ligand choline is necessary for conversion to active state. Nevertheless, high levels of choline in medium inhibit LytA actions. It specifically cleaves covalent bonds of the cell wall main constituent peptidoglycan inducing cell wall breakdown and eventually death, which is why it is called a suicidal enzyme. It is this enzyme's activity which the bile solubility test is based on. LytA participates in cells separation and it is overexpressed

during competence suggesting its promotional function during DNA release and competence. During invasive disease, human lysozyme, the first-line host bacteriocin that accumulates in secretions and inflamed tissues (Cottagnoud *et* Tomasz, 1993) triggers LytA activity whereby inadvertently worsens course of the disease, because the most important consequence of LytA action is the let out of intracellular proinflammatory components in high local concentrations at the site of an infection (López *et* García, 2004). Last but not least, a pilus has been identified in some clinical isolates (Barocchi *et al*., 2005). Pilus is a long, flexible, extracellular organelle covalently attached to the peptidoglycan in bacterial cell wall and it facilitates docking with receptors on the host cell surface mediated by adhesin protein located at the tip of the fimbriae (Abbot *et al*., 2007). Pili render the pneumococcus an advantage at adhesion to epithelial cells *in vitro*, at colonisation and invasion *in vivo*, and finally they augment the host inflammatory response. A second type of pilus was recently identified in strains of newly emerging highly invasive isolates of serotype 1 ST 306 and serotype 4 ST 205 that lack the first type pilus suggesting its role in invasive disease (Bagnoli *et al*., 2008), (Normark *et al*., 2008).

2.7.4. Regulation of virulence

As mentioned above, *Streptococcus pneumoniae* can damage various types of tissue. Such tissue tropism proposes an existence of tissue-specific virulence factors and their regulators. Using signature-tagged mutagenesis (STM) technique, Hava *et* Camilli identified several virulence factors: One-component regulatory system (RlrA) controlling genes responsible for mucosal attachment and two-component systems in charge of the adaptation to environment. Hava and Camilli used STM to track a pool of bacterial strains from one starting point to other sites in the animal. They found that the systemic infection was rather clonal due to a few strains recovered from the bloodstream than caused by a larger founder population. Other researchers also agree that the key point in pathogenicity is the ability to sense and respond to changes within host environment (Hava *et* Camilli, 2002).

Apart from virulence factors specific for pneumonia (LytA, NanA and Hyl), genes accountable for metabolic pathways are indispensably needed for adaptation and not for specific steps during infection. These include purine biosynthesis, anaerobic metabolism, nutrient requirements (glutamine synthesis), ABC transporters, proteases (IgA1 protease), DNA modification (mismatch repair), and adaptive responses (transcription regulator RegR). Mutations in these genes evinced low virulence in pneumonia model of infection (Polissi *et al*., 1998).

There is a fine line between a relatively harmless commensal and a quite formidable foe which the pneumococcus becomes when the watershed comes up and the bacteria undergo major switch in gene expression in order to adapt to different niches (the nasopharyngeal mucosa, lung mucosa, blood, CSF). The shift from a colonizer to an invader is not fully understood, however, it is considered that there are at least two distinct physiological states from the pneumococcal point of view. As it can be observed from *in vitro* studies, pneumococci take one of these two forms of a lifestyle; a planktonic or a biofilm-like state. Likewise, bacteria *in vivo* float in the bloodstream or survive as biofilm population in pneumonia or meningitis models of infection. Experiments confirmed the hypothesis that the overall pattern of gene

expression *in vivo* during sepsis resembles the one of *in vitro* during exponential growth in liquid culture, and that the genes upregulated in tissue infection are overexpressed on agar plates too. Specifically, Ply and PspA genes are upregulated in blood and cultures whereas NanA, ZmpB, ZmpC, IgA1-protease and competence stimulating peptide (CSP) expression is increased in pneumoniae and meningitis infections and on agar plates. Importantly, the switch from one lifestyle to another decides the fate of the course and disease severity (Oggioni *et al*., 2006).

2.8. Anti-pneumococcal immunity

The interplay between host defences and pneumococcal factors is complex. The innate immune system represents the first line of defence against the pneumococcus. Complement, neutrophils and macrophages are non-specific host weapons developed in order to react rapidly. The following adaptive immunity largely depends on how the innate system works and these two mechanisms are linked up (Paterson *et* Mitchell, 2006). The classical pathway is the most often and dominant pathway of complement activation during pneumococcal infection (Brown *et al*., 2002). C3 is the key complement component that binds to a bacterial cell membrane via a specific IgG. In its absence, C3 binding depends on both the classical and the alternative pathways and partially on natural IgM. This binding can be abrogated by the presence of pneumococcal capsule (Tuomanen *et al*., 1995). The main effect of complement is to prevent spread of the bacteria from the lungs to the blood, however, once the sepsis is established, the complement provides opsonisation (from the Greek word for “preparing food”) of the bacteria via C3b deposition on capsule that facilitates opsonophagocytosis by neutrophils and subsequent clearance of a pathogen from the systemic circulation in the spleen and liver. This mechanism is thought to be the most important tool for innate immunity against systemic and mucosal *Streptococcus pneumoniae* infections in mice (Bruyn *et al*., 1992). The pneumococcus has invented several mechanisms in order to avoid such elimination. Apart from the capsule, the already mentioned pneumococcal surface protein PspA prevents the alternative pathway of complement activation. A huge contribution of Ply to resistance to complement-mediated killing is discussed above.

When it comes to the adaptive immunity, immunoglobulins, T and B cells play the major role, however, complement is necessary to achieve effective clearance (AlonsoDeVelasco *et al*., 1995). CD4+ T cells otherwise known as T helper cells that present antigens on their surfaces also contribute to early response to the pneumococcus. They were shown to migrate to lungs during pneumococcal pneumonia. Ply strongly contributes to T-cells migration towards bacteria but it is not the only factor here (Brown *et al*., 2002). The best protection against the pneumococcus is warranted by type-specific long-lasting antibodies to polysaccharide capsule. Once on the bacterial surface, these antibodies bind directly to macrophages or activate C3; it deposits on bacterial surface and bacteria opsonised like this are then destroyed by macrophages which possess CrIg receptor for C3. Even thought other antibodies; i.e. to the cell wall constituents provide some degrees of protection, pneumococci visible by such antibodies are not phagocytised by neutrophils.

Prophylaxis of pneumococcal disease is represented by vaccination or antibiotics in more extreme cases such as outbreaks at day-care centres. In so much as the emergence of penicillin-resistant strains became a world wide problem ever since its first discovery in 1965 (Sibold *et al*., 1992), development of more effective and better vaccines has been initiated (McGee *et al*., 2001). Since 1983, when a 23-valent vaccine consisting of the most common 23 serotypes was introduced, attempts to upgrade the protection have been made (Watson *et al*., 1995). Not only this polyvalent vaccine does not confer protection against all other remaining serotypes which means that people from different geographical regions may be exposed to different prevalent serotypes and such vaccine would not protect them, but also purified capsular polysaccharides are thymus-independent antigens that elicit neither immunological memory nor class switch which means that mainly IgM antibodies are formed with no booster effect. Another limitation is that human response to polysaccharide antigens is related to age and that children less than 2 years old are not able to produce relevant IgG antibodies due to delayed maturation of B cells. These obstacles can be overcome by conjugation of polysaccharides with protein carriers. Proteins are thymus-dependent antigens so they are capable of CD4+ T cells stimulation which then helps B cells to yield antibodies (Bruyn *et al*., 1992). Admittedly, pneumococcal saccharide-protein conjugated vaccines can hold for only 11 serotypes and are likely to be more expensive which is not desirable in developing countries (Paton, 1998). Pneumococcal proteins as carriers signify light of hope in novel vaccine research. Many of them are being currently tested for potential vaccine candidates including PspA, PsaA, CbpA, NanA, Ply and more. Both membrane and cytoplasmic proteins were shown to be immunogenic during infection or previous colonisation (Zysk *et al*., 2000). For example, defined Ply toxoids with mutations abrogating its cytotoxicity are capable of eliciting opsonic antibodies just as effectively as wt Ply conferring protection against chosen serotypes (1 to 6, 7F, 8, 18C) even though differences in the degree of protection exist among serotypes and also varies with the route of infection. A further consideration relating to vaccine development is the route of administration of the antigen. Most experiments done to date have considered only systemic immunity. Since colonization of the nasopharyngeal mucosa by pneumococci is a requirement for development of pneumococcal disease, it may be that mucosal immunity is important in protection from disease (Mitchell *et al*., 1997b). An attenuated *Salmonella* strain capable of stably expressing a toxoided form of pneumolysin has been constructed and oral immunization of mice with this organism elicits both IgG and IgA antibodies to pneumolysin (Paton *et al*., 1993).

Objectives of the work

- Literature retrieval focused on the importance of genetic variation in virulence
- Growth of pathogenic microorganisms
- Isolation and preparation of pneumococcal gDNA, PCR, haemolytic assays, Western blot
- Analysis of mutations by quantitative PCR
- Bioinformatics computing for bacterial genome analysis

The diploma work is focused on the significance of pneumolysin in contribution to virulence of *Streptococcus pneumoniae* serotype 1 isolates. Even strains from the same serotype may vary greatly in invasiveness, a property bequeathed by the high level of pneumococcal genetic variability. The difference in virulence will be explained by analysis of the genomes in combination with gene expression studies and *in vitro* assays.

The second virulence factor of interest, hyaluronidase, is known to contain a SNP within its nucleotide sequence. The SNP genotyping method will be used to screen strains from a collection of meningitis isolates on the way to discovery of a true contribution of hyaluronidase to pneumococcal meningitis.

All *in vivo* work was performed by appropriately qualified personnel who are holders of Home Office Personal Licences under Prof. Mitchell's Project Licence. All animal procedures were performed in accordance with the United Kingdom Home Office Inspectorate under the Animals (Scientific Procedures) Act 1986. Ethical approval for these procedures was granted by the College of Medical, Veterinary and Life Sciences Ethical Review Committee at the University of Glasgow. All necessary procedures were performed under anaesthesia, and all efforts were made to minimise suffering.

Materials & Methods

1. Materials

The collection of strains used in experiments with Ply is depicted in Table 1. These strains were selected on the basis of observation of another group. Strains used in the study of Hyl SNP are listed in Table 3 in section Results.

Streptococcus pneumoniae was grown on blood agar plates (BAB). Preparation of approximately 20 plates included 16g of blood agar base 2 in a powder form and 400ml of distilled water. The mixture was shaken and autoclaved. Once the bottle had cooled sufficiently to approximately 40°C, 20ml of sterile defibrinated horse blood was added, mixed and poured straight away. The area of pouring the plates should be kept as sterile as possible by using Bunsen burner. When the plates were filled to three-quarters, incidental bubbles were removed by flaming the surface of the agar. BAB plates were kept on the bench for at least 30 minutes to solidify properly. Plates were further dried by placing them into an incubator upside down without a lid for 1h.

Pneumococcal liquid cultures were grown in brain heart infusion broth (BHI). Solid BHI powder weighting 7.4g was added to 200ml of distilled water. The mixture was shaken and autoclaved. When ready to use an appropriate volume was distributed into sterile Falcon or bijou tubes and used immediately.

For gDNA extractions, stock solutions were prepared: 1M Tris ((3-aminopropyl)-amine) pH 8.0, 0.5M EDTA (ethylenediaminetetraacetic acid) pH 8.0, 10% SDS (sodium dodecyl sulphate), 10M ammonium acetate, TE buffer (10mM Tris-Cl + 1mM EDTA + ddH_2O), DTT (dithiothreitol). Double distilled water (ddH_2O) was used thoroughly the preparations. SDS detergent was the only solution that could not be autoclaved. Lysis buffer (Tris + EDTA + SDS + ddH_2O) was made up fresh before use.

For SDS-PAGE gel, pre-cast gels were used **(**NuPAGE® Bis-Tris Gels) and solutions: loading buffer also called the Laemmli 2X buffer pH 6.8 (4% SDS + 10% 2-mercaptoehtanol + 20% glycerol + 0.004% bromophenol blue + 0.125 M Tris HCl) and running buffer (Tris + glycine + SDS), de-stain buffer (methanol + acetic acid + distilled water).

For Western blot analysis: transfer buffer (25mM Tris base + 192mM glycine + 20% methanol + distilled water) keep at 4°C, Tris NaCl (Tris base + NaCl + distilled water + HCl to adjust pH 7.4), 3% milk (milk powder + Tris NaCl pH 7.4), developer (4-chloro-1-napthol dissolved in methanol + Tris NaCl + H_2O_2) must be made just before use.

GENOME SEQUENCE STRAINS	SEROTYPE	SEQUENCE TYPE	PLY TYPE
TIGR4	4	205	2
INV104B	1	227	2
P1041	1	217	1
OXC141	3	180	2

INV200	14	9	2
03_2672	1	306	5
03_3038	1	306	5
06_1370	1	306	5
NCTC7465	1	615	2
ATCC700669	23F	81	1
TEST STRAINS	SEROTYPE	SEQUENCE TYPE	PLY TYPE
ATCC6308	8	unknown	5
09_2408	4	unknown	unknown
09_2458	4	unknown	unknown
1AL	1	615	2

Table 1. Strains used for Ply experiments and additional test strains

2. Microbiological techniques

First, a glycerol stock of working strains for this project was prepared. A sterile bacteriological loop was used to streak out *Streptococcus pneumoniae* strain from a previously stored glycerol stock (Mitchell Group strain collection) onto a BAB plate. An optochin disk was added in order to differentiate *Streptococcus pneumoniae* from other α-haemolytic Streptococcus species. Plates were incubated overnight at 37°C in candle jar to check the purity and identity of the content. Candle jars allowed anaerobic conditions. Simultaneously, all the volume (1ml) was transferred into a glass bijou bottle with 5ml of BHI and let grow statically overnight in 37°C water bath. After overnight incubation, 1ml of that grown pneumococcal culture was added into 19ml of BHI in Falcon tubes prepared already. Pneumococci were grown until the culture reached optical density of OD_{600nm} equalled 0.4 – 0.6 measured by spectrophotometry. The tubes were centrifuged for 20 minutes at maximum speed at 4°C and afterwards the supernatant was discarded. Meanwhile, sterile glycerol was prepared to a final concentration of 15% in BHI for a total volume 10ml, and added to the pellet. Aliquots of 1ml were taken and labelled cryovials were stored at -80°C for at least 16h. After that, the purity control check was done as described above. Viable counting of bacteria was performed as follows: a 96-well round-bottomed plate was filled with 180µl of sterile phosphate buffered saline (PBS) in adequate number of wells. Three cryovials were rapidly defrosted at 37°C water bath, gently vortexed for 5 seconds to separate the bacteria and centrifuged for 3 minutes at 13000rpm. Supernatant was removed and pellet was resuspended in 1ml PBS. The last step was repeated in order to wash the bacteria from residual glycerol and BHI. 20µl from each cryovial was added to the top well in the 96-well plate, mixed and transferred to the next well and so on down the plate taking a new tip each time. That resulted in serial dilution of sample from dilution 10^{-1} (first well) to 10^{-6} (last well). Three 20µl-drops of every dilution were spotted onto previously marked BAB plates. After overnight incubation at 37°C in candle jar, colonies of such dilution that gave between 10-50 colonies per spot were counted. The mean for

that dilution was counted, multiplied by 50 to give the count per ml, the result was multiplied by a reciprocal of a given dilution factor, and expressed as $\log_{10}$ value which gave us the colony-forming units per ml (cfu/ml).

Intraperitoneal passage was a required step for creation of standard inoculums which were used for *in vivo* studies. After long time of storage, a pneumococcal strain's original viability and virulence may need to be restored. Knowing the exact counts of viable bacteria from glycerol stocks, adequate volume to give a final concentration of $1x10^{\wedge 7}$cfu/ml necessary for challenge was calculated. This was for 6h i.p. passage. Steps included rapid thawing; 5 minutes spin at 13000rpm, and removal of supernatant, PBS wash step, gentle vortex, and addition of the volume to final concentration with extra sterile PBS. Dose must have been checked by 10 fold serial dilution before and after infection to ensure that bacteria did not perish during the procedure.

Blood from the mouse was placed into universal tube containing 20ml BHI and incubated at 37°C overnight. The blood cells were allowed to settle and 1 - 2ml of the upper layer with multiplying bacteria was transferred into BHI containing 20% fetal calf serum (FCS) 50ml-Falcone tube. Triplicates were done for each strain. Different strains grew at different rates, so the OD_{600nm} was checked at short time intervals so the culture was sampled in the logarithmic phase of growth. Standard inoculums were stored in labelled cryovials at -80°C for at least 16h after which the purity, identity and counts could be checked.

When a new strain was isolated, for example, from swabs from the throat, a bead stock for addition to the collection was prepared. The beads came in sterile Eppendorf tubes from a manufacturer. As much as possible of well-grown pneumococci were transferred from BAB plates with a sterile plastic loop to the bead suspension. The contents of the loop were transferred to the liquid in the vial and beads were mixed by shaking allowing bacterial transfer to the beads. The tube was deprived of the liquid, labelled and stored at -80°C.

3. Isolation and preparation of pneumococcal gDNA

Strains from the glycerol stock were grown in 50ml BHI at 37°C water bath. Once they reached the exponential growth phase (OD_{600nm} = 0.4 – 0.6), the culture was centrifuged at 4000rpm for 15 minutes at 4°C, supernatant was carefully removed without disturbing the pellet, and resuspended in 1ml of freshly made up lysis buffer. Then the content was splitted into two cryotubes and incubated at 37°C for 1h. 2.5µl of proteinase K was added into each tube and put into 50°C water bath for additional 3h. Next, 1µl of RNAse A was added per each tube and put back into 37°C water bath to incubate for 30 minutes. After this step, cryotubes could be put in a freezer and the procedure was stopped till following day. According to phenol/chloroform classical extraction protocol, the work was done in fume hood with double gloves, Eppendorf tubes were never used while manipulating chloroform because its lid could let some liquid out. 500µl of phenol:chloroform:isoamyl alcohol solution from the bottom of a bottle was taken and pipetted into each cryotube and inverted gently. A white suspension of proteins became visible. The tubes were spinned down at 13000rpm for 3 to 6 minutes. As much of the upper phase as possible was taken up without disturbing the lower phase, transferred to a new Eppendorf tube and previously splitted samples were poured

back together. 20µl of 10M ammonium acetate was added per 100µl of aqueous layer to precipitate DNA. 600µl of absolute ethanol was added to the mixture and gently inverted. Visible DNA precipitates were transferred by a pipette to a fresh tube in order to obtain as big DNA fragments of better quality as possible. The rest of the mixture was centrifuged at 13000rpm for 30 minutes. Pellets were left to air dry on bench. They were resuspended in 100 to 300µl of TE buffer so they would dissolve. Higher temperature and occasional flicking the bottom of tubes would help dissolving, so the tubes were placed into 37°C incubator for 2.5h. Thus prepared gDNA could be stored in a fridge (4°C) for few days or in a freezer (-20°C) for a long term.

Approximate amount and quality of isolated gDNA was checked by agarose gel electrophoresis. 0.4g of powdered agarose was dissolved by microwaving in 50ml of 1x Tris-acetate-EDTA buffer (TAE). 3µl of SYBR safe double-stranded DNA (dsDNA) dye was added, gel was poured into a gel tank with combs, covered by aluminum wrapping and left to solidify. In the meantime, 5µl of running samples was prepared: 2µl of loading buffer, 2µl of PCR water (extra clean) and 1µl of gDNA. Gel tank was filled with TAE buffer as well. Samples were applied into holes; one was left for DNA size ladder. The gel was run at 100V for 20 minutes. Electrophoresis is based on separating negatively charged nucleic acids in an electric field according to their sizes; larger fragments migrate slower. UviPro gel documentation system facilitates visualization of the DNA bands by detecting fluorescence excited by UV radiation from bound SYBR dye.

When approaching gDNA isolation from many different strains, an easier and faster method is required. The Nexttec^{TM} – Kit and method is column based and it is based on retaining unwanted substances such as proteins, detergents and low molecular weight compounds by the column resin. Briefly, membranes inside the columns were rehydrated by Prep buffer. Each bacterial strain was grown overnight on 2 BAB plates from the beads stock, a loopful of bacteria was put into a fresh tube with Lysis buffer 1 containing lysozyme and RNAse A, gently vortexed and sealed with Nesofilm. The tube was then incubated at 60°C for 20 minutes. Lysis buffer 2 containing EDTA, buffer 3, purified water, and DTT was added, sealed again, and incubated at the same temperature for 1h. Exactly 120µl of the lysate was transferred onto column, sealed, and incubated at room temperature for 3 minutes. A sterile plate was placed underneath it. Plate was centrifuged at 700rpm for 2 minutes. DNA passed through the column during that step, eluated DNA could be stored at -20°C.

Concentration of isolated gDNA is best measured on tabletop spectrophotometer Nanodrop. Nucleic acids absorb light at a wavelength of 260nm. Distilled water or TE was used as a blanking solution. Nanodrop measured the amount of light that passed through a sample and inferred the amount of light that was absorbed by it. OD_{260nm} = 1 correlated to a DNA concentration of 50ng/µl, so DNA concentration was easily calculated from OD measurements using only 1.5µl of a sample.

However, concentration of Nexttec^{TM} isolated gDNA could not be measured on Nanodrop because some substances from the buffers remained in DNA eluate, they would cause a higher UV absorption at 260 and 280nm and it would lead to an overestimation of DNA concentration. Qubit® fluorometer, on the other hand, quantifies DNA by means of fluorescent dyes that specifically bind only to target molecules and thereby emit corresponding signals. This method is highly sensitive and more accurate than UV absorbance.

Quant-iT™ Assay Kit provides buffers with fluorophores, DNA BR buffer was used as a blank, samples were prepared and vortexed, and a corresponding programme was set up according to used dilution.

4. PCR

The PCR method was used to confirm the presence of intact Ply and Hyl genes in the strains. It is also a good control of quality of isolated gDNA. By means of PCR, the lowest concentration of DNA suitable to use for the amplification was possible to determine, which 1ng/µl is. PCR products were obtained by using the following primer set: *Ply27R* (5´CTTGGCTACGATATTGGC3´) and *Ply27S* (5´TACTTAGTCCAACCACGG3´). PCR conditions for Ply gene were: 3 minutes incubation at 94°C to completely denature the template, followed by 30 cycles of 45 seconds of melting at 94°C, 30 seconds of annealing at 55°C, and 1.5 minute of extending at 72°C. Reaction mixture was further incubated additional 10 minutes at 72°C and then maintained at 4°C, samples were stored at -20°C. Hyl primers were used to amplify internal fragment of the gene; Hyl861For (GGATCCTATATCGACCACACCA) and Hyl861Rev (CCTAAAAAGGCGATCTTATC). Programme settings for Hyl gene were: 35 cycles, 3 minutes incubation at 94°C, 45 seconds of melting at 94°, 30 seconds of annealing at 52°, 1 minute of extension at 72°. Reaction mixture was also incubated additional 10 minutes at 72°C and maintained at 4°C, samples were handled the same way.

5. Quantitative haemolytic assay of pneumococcal lysates

Ply lyses any cells with cholesterol in the membrane, RBCs are easy to measure as haemoglobin release is visual and quantifiable by haemolytic assay. For this purpose, standard inoculums were used. One tube was quickly defrosted at 37°C water bath and centrifuged for 3 minutes at 13000rpm. Removal of supernatant was followed by addition of 1ml of PBS. Volume was transferred into 19ml of BHI containing Flacon tube. Culture was grown till it reached proper OD, and then it was spinned down 20 minutes at maximum speed at 4°C, deprived of supernatant again and frozen. The purity check of the culture was done. The culture was kept on ice during procedure to prevent protein degradation. For the same reason, protease inhibitor was added to 1ml of PBS which was than added to the frozen bacterial pellet. Since Ply is believed to be located in the cytoplasm, the cell walls must be disrupted. This was achieved by sonication. Sonication was carried out in the sonicator machine set according to desired outcome; 30 seconds of sonicating at amplitude of 10microns, 1 minute off, and this is repeated five times. Tube was kept on ice. Sonication was complete when the suspension went clear. Ten minutes of centrifugation at 13000rpm followed. Supernatant was collected. Pellet was frozen for further utilization. Measurement was done with the neat supernatant plus 1:50 dilution. Negative control with PBS and positive control with purified Ply were indispensable. Every well of round-bottomed 96-well plate was filled with 50µl of sterile PBS. 50µl of each solution (purified Ply, pneumococcal cell lysate neat, diluted, sterile PBS,) was added into the first column in duplicates. Wells were mixed by pipetting and 50µl was removed from column 1 and placed to column 2 which double-dilute the content. Tips were changed after each transfer. 50µl from the last column was discarded. Next, 50µl of

DTT was added into each well in order to make Ply monomer. Plate was covered with lid and put into 37°C dry incubator for 15 – 30 minutes. Meanwhile, the erythrocytes were prepared. 1ml of fresh sheep blood was centrifuged for 1 minute at 13000rpm. Supernatant was removed and cells were washed in the same volume of PBS. Wash step needed to be repeated until the supernatant was completely clear. Erythrocytes were used to prepare 2% solution in PBS just prior to reaction; the blood solution was kept on ice until then. 50µl of 2% erythrocyte solution was added to each well, covered by lid and incubated for 30 minutes at 37°C. Additional 50µl of PBS was added everywhere and the plate was centrifuged at 1000rpm for 1 minute. 100µl of supernatant from each well was carefully transferred into a fresh flat-bottomed 96-well plate. Spectrophotometer at 540nm was used to read levels of haemoglobin released from the erythrocytes by action of Ply. Programme Optima created an excel file from which Ply specific activity was calculated, which is defined as the dilution factor at which 50% haemolysis is detected, and expressed in HU/mg. Qubit® fluorometer was used to measure protein concentration in our samples in order to exclude the possibility that if a strain was not haemolytic, it was not because of absence of the protein.

6. Quantitative RT-PCR

Since screening up to 156 strains was planned, instead of generating gDNA by time-consuming phenol/chloroform extraction method, a simpler and more rapid extraction method with high throughput of clinical isolates, the Nexttec™, was optimized. RT-PCR was used to screen Polish meningitis isolates looking for a SNP within Hyl nucleotide sequence that causes its inactivation. Two reactions were run for each strain because two different primers were used; HI41FR1 for amplification of the wt sequence, and HI41FR2_for the mutated gene. 19µl of master mix containing only one or the other primer, distilled autoclaved water, and supplied mixture with fluorescence dyes was pipetted to the bottom of each tube in duplicates. 1µl of a sample was added into two labelled wells. The programme Opticon Monitor 3.0™ was initiated which controls the PCR programme and collects data for subsequent analysis. A standardised protocol using 40 cycles at was used. The sensitivity of this approach allows discrimination between strains that possess the SNP within Hyl gene or not by comparing Ct values: a lower Ct value occurs for the reaction with primer that matches the sequence better than the other primer.

7. SDS-PAGE gel & Western blot

Western blot is an analytical method used to detect a target protein in a sample made of mixture of proteins by means of a polyclonal or monoclonal antibody specific to that protein. It also allows investigators to determine the molecular weight of a protein.

SDS-PAGE gel separates proteins according to their sizes; SDS makes them all negatively charged while polyacrylamide provides heterogeneous environment through which smaller proteins travel faster. Bands of proteins were visualized by staining the gel with Coomassie Blue dye at room temperature for 20 minutes on shaker. The gel was decoloured using de-stain buffer for 20 to 50 minutes and placed into scanner. Coupled with Western blotting, it was used to determine the presence or absence of a given protein.

A reason to do Western blot analysis was to see whether Ply gene was translated or not. Samples were obtained by previously described procedure of sonication. Samples were prepared as follows: 200µl of PBS was added into each tube with pellet from which 72.5µl was taken out and mixed with 25µl of loading buffer and 2.5µl of DTT. 72.5µl of supernatant was mixed with 25µl of loading buffer and 2.5µl of DTT as well. 12.5µl of 1kb ladder was mixed with 40µl of loading buffer and 187.5µl of distilled water. Ladder can be stored at -20°C for a while. It provides a comparison of molecular weights for the bands of proteins. The samples were boiled at 90°C for 5 minutes, the pre-cast gel was put into tank and filled with running buffer and checked for leaks. Then the combs were removed. 2µl of a marker was added (SeeBlue®) to 10µl of each sample, 7µl of ladder, loaded it all into the gel and electrophoresis was run at 150V for 1h. Meanwhile, the pads, filter paper and nitrocellulose membrane were soaked in transfer buffer and arranged into given order of layers from the bottom: 3 pads, filter paper, future gel, filter paper, and 2 pads on the top. Any bubbles should be removed at that point. Once the SDS-PAGE gel was finished, gel cast was opened and the gel was carefully transferred, 100µl of antioxidant was added and it was run in transfer buffer in a new tank at 30V for 1h. Water was poured outside the tank to keep it cool. Then the membrane was put into a suitable container with 3% milk and blocked overnight in a fridge. The following day, the membrane was placed into a special foil, 15µl of primary anti-Ply rabbit antibody was added to 3ml of 3% milk, milk was poured in the foil and put on shaker at 37°C for 2h. The membrane was washed three times with Tris NaCl pH 7.4 on shaker at room temperature for 5 minutes each time. Again, the membrane was transferred to 3ml of 3% milk containing 3µl of second anti-rabbit antibody, sealed and shook at 37°C for 1h. Washing step was repeated three times, and then the membrane was placed into developing buffer in the dark. Once bands started becoming visible, the reaction was stopped with water. The second antibody, if bound to existing primary antibody that attached to Ply and was not washed out, caused that the product could be seen and photographed. In order to increase sensitivity and detect even low level of translated protein, ELC kit was used in following Western blots. Antibodies against PsaA confirmed the integrity and correctness of this procedure as well as the quality of the samples.

8. *In vivo* experiment

All the *in vivo* work animal work was performed by professional personnel. Six MF1 mice were infected by three selected strains in order to obtain the whole blood samples. Each mouse was infected intraperitoneally with a dose of 200µl containing $2x10^{\wedge 4}$ cfu. After 18h, the cardiac blood was pooled into sterile heparinised tube and kept on ice. 20µl of it was directly used to count the bacteria. Bacteria were harvested by 2-step centrifugation: first spin was performed at 820g at 4°C for 5 minutes, supernatant was transferred into a fresh tube and pellet was kept for further application. This supernatant was then again spinned at the same conditions, transferred into a fresh tube and pellet was kept. The last spin was at 15 000g speed at the same conditions and supernatant was detached from pellet. The second pellet was subjected to sonication, SDS page and Western blot analysis.

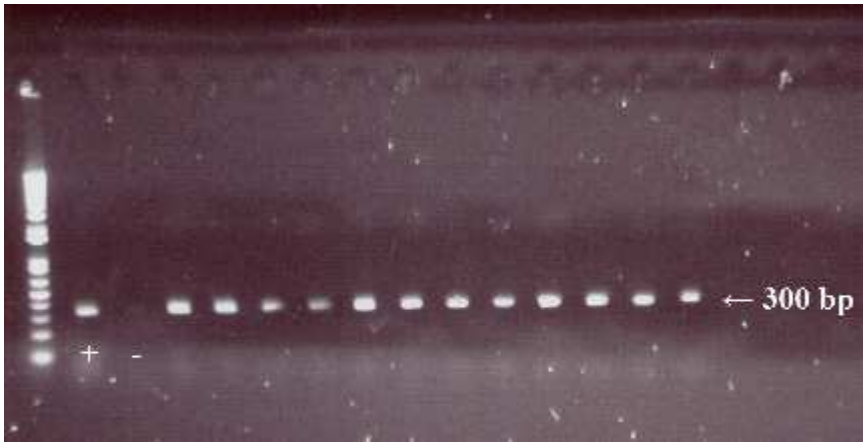
9. Bioinformatics

Comparative sequence analysis was performed in order to obtain an insight into pneumococcal genetic variation. All the pneumococcal genome sequences submitted to the Sanger had been selected, prepared for sequencing, and submitted by Prof. Mitchell's laboratory. Therefore the genomic information could be directly correlated with the biological activity of each strain. The latest versions of the genome assemblies were downloaded from Sanger website (Wellcome Trust Sanger Institute, 2009). Once the sequences were downloaded, it was possible to compare unknown sequences with those already published in the databases on the internet and identify them by calculating the sequence similarity. For this purpose, programme Blast can be used (Blast Assembled RefSeq Genomes, 2009). Blast is an acronym for basic local alignment search tool. It is a software package used for searching chosen databases with an input query. A website developed exclusively for *Streptococcus pneumoniae* bioinformatics is called Sybil (*Streptococcus pneumoniae* Comparative System, 2009). This has been developed by Prof. Tettelin and his group and is hosted by the University of Maryland, (USA). Sybil was used to do genome search and comparative genomics. Artemis was used to view if the genes of interest were present, and Act (Artemis comparison tool) to compare sequences with the reference sequence of TIGR4 strain using crunch files generated from Double Act, a programme to produce the input comparison file for comparing genomes within the Act provided by the Sanger Centre (Wellcome Trust Sanger Institute, 2009). These sequences and information could then be viewed within the programme CLC Genomics Workbench version 3.6.5 (CLC Bio, Aarhus, Denmark). Once the particular sequences were downloaded, ClustalW2, a general purpose multiple sequence alignment programme for DNA or proteins was used to compare/identify pneumolysin alleles in our strains (European Bioinformatics Institute, 2009).

Results

1. Pneumolysin gene is present in all the strains

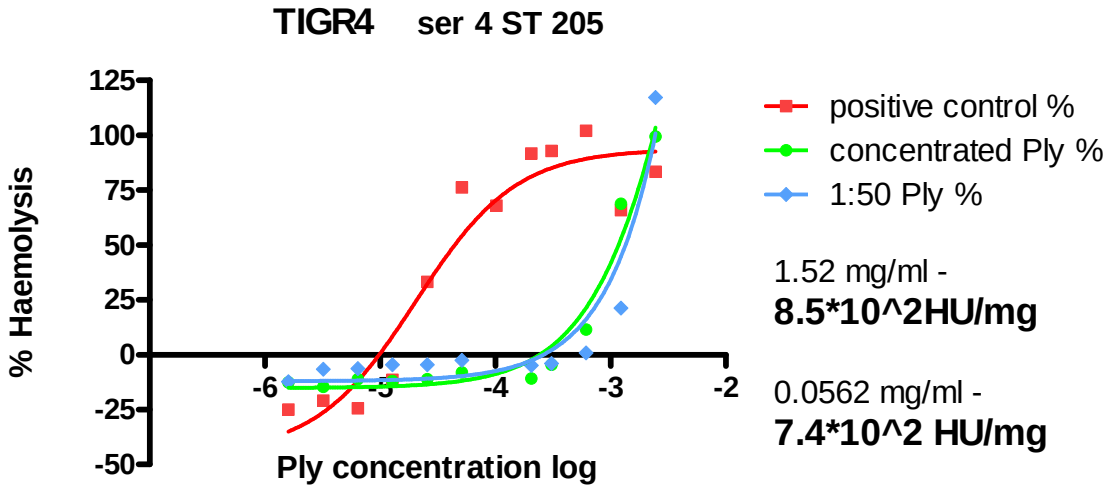
PCR confirmed presence and intactness of Ply in all the strains. Picture 2 shows amplified fragment of Ply gene of a representative sample of the strains. The genome sequenced strains are of different serotypes and STs, but with mainly serotype 1 as shown in Table 1. From previous work it is known that strains of serotype 1 ST 306 possess SNPs within the Ply gene affecting its haemolytic activity.



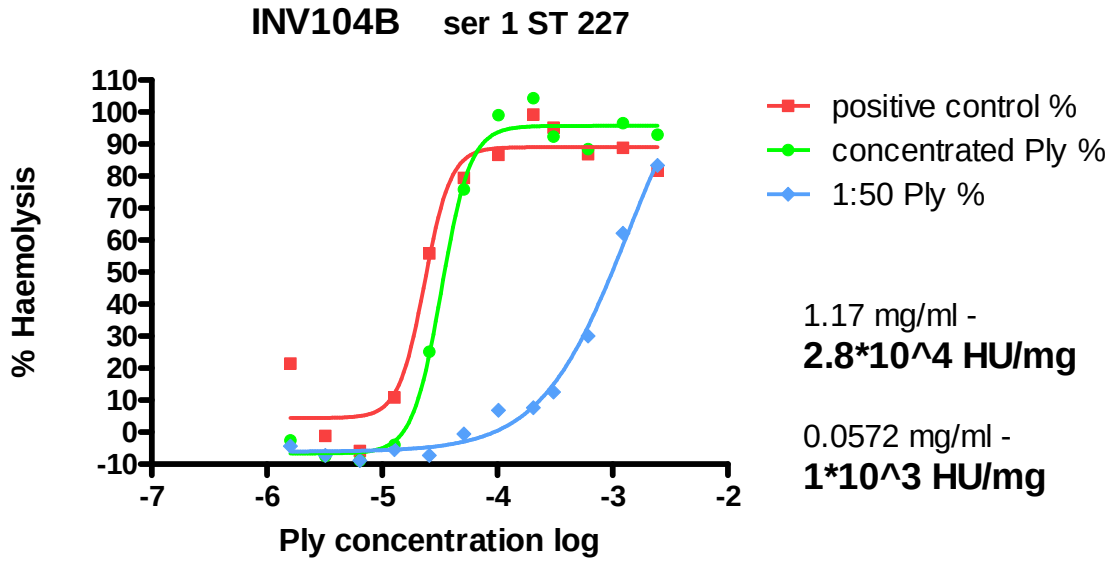
Picture 2. PCR amplification of Ply gene fragment

2. Pneumolysin genetic variability & haemolytic activity

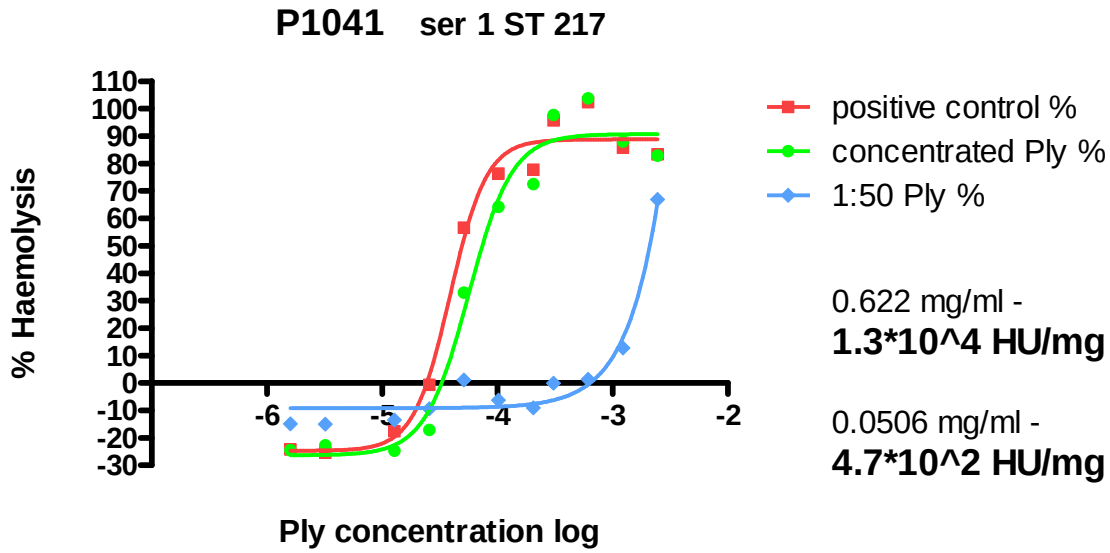
Since one of the main functions of Ply is cell lysis, quantitative haemolytic assays were performed in order to measure the lytic ability. Furthermore, protein concentration was measured in all the samples tested for haemolysis. It is shown in Graphs 1 – 12 bellow that serotype 1 ST 306 strains, two serotype 1 ST 615 strains and one strain of serotype 8 of unknown ST are all nonhaemolytic.



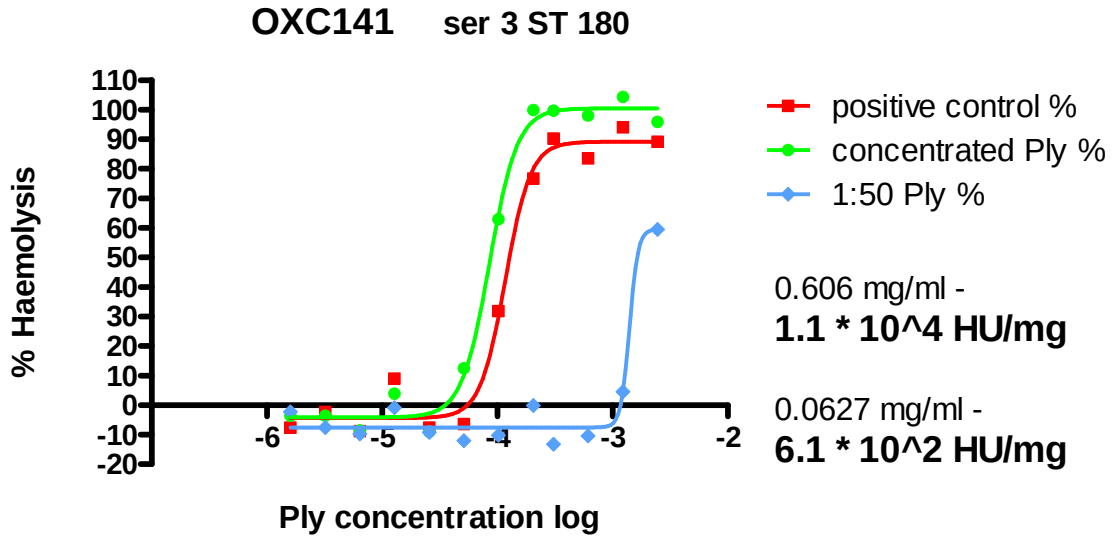
Graph 1. Haemolytic assay, protein concentrations and specific activities



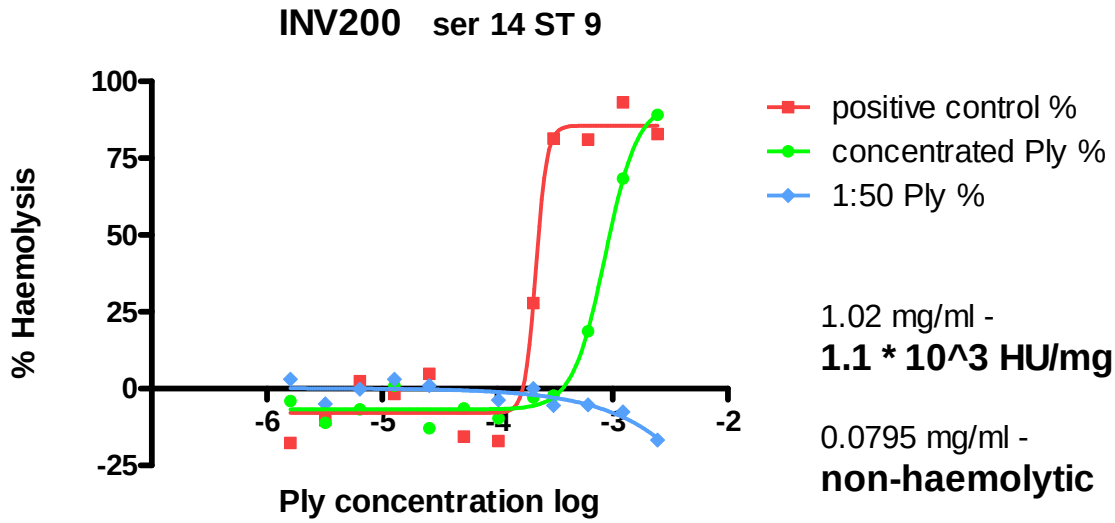
Graph 2. Haemolytic assay, protein concentrations and specific activities



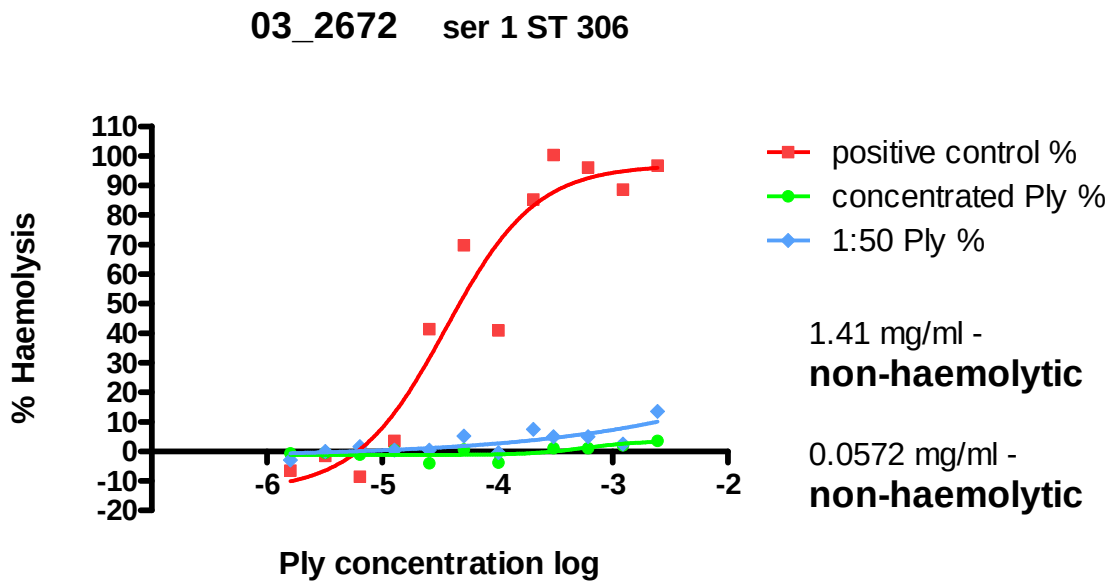
Graph 3. Haemolytic assay, protein concentrations and specific activities



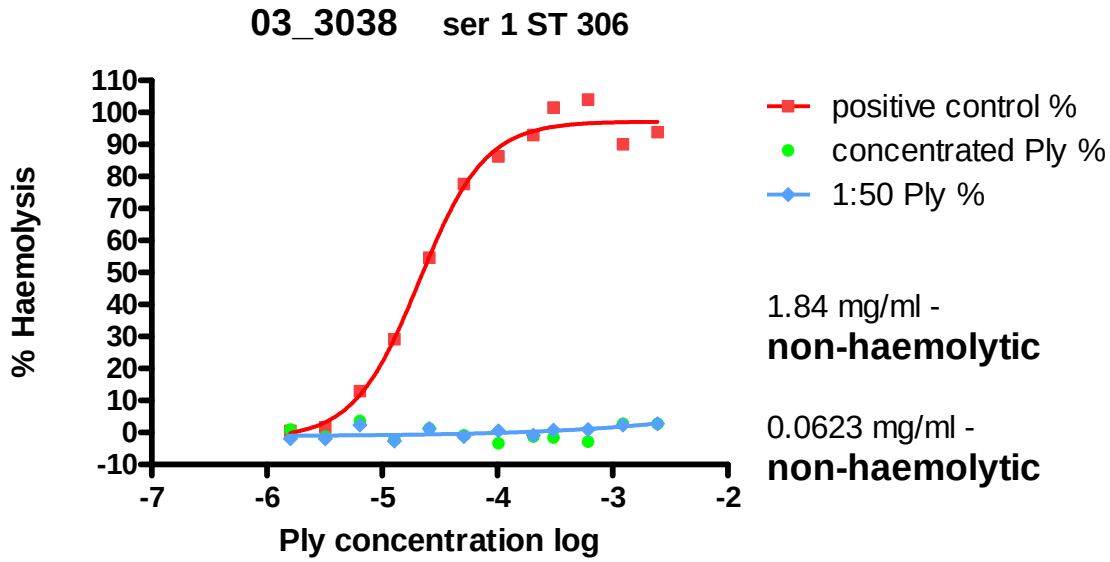
Graph 4. Haemolytic assay, protein concentrations and specific activities



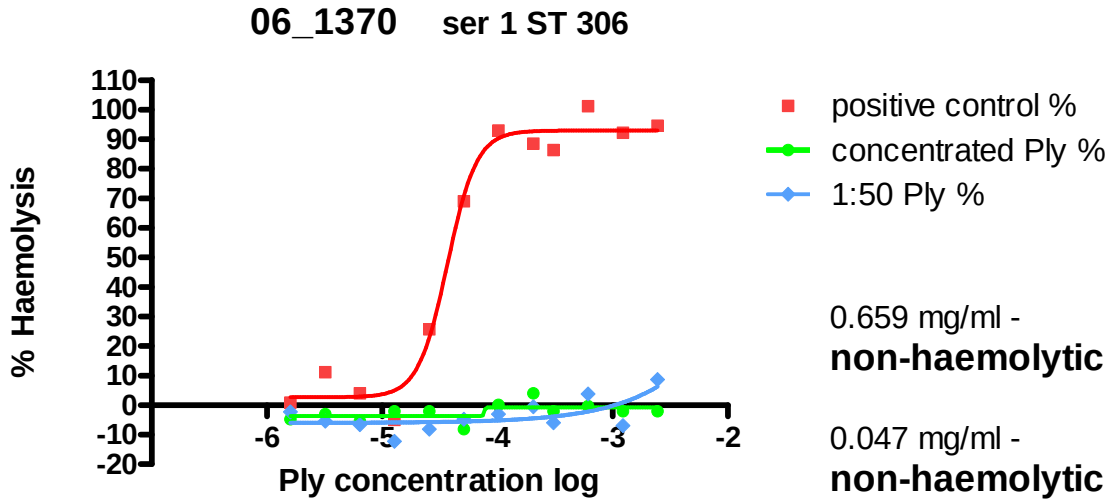
Graph 5. Haemolytic assay, protein concentrations and specific activities



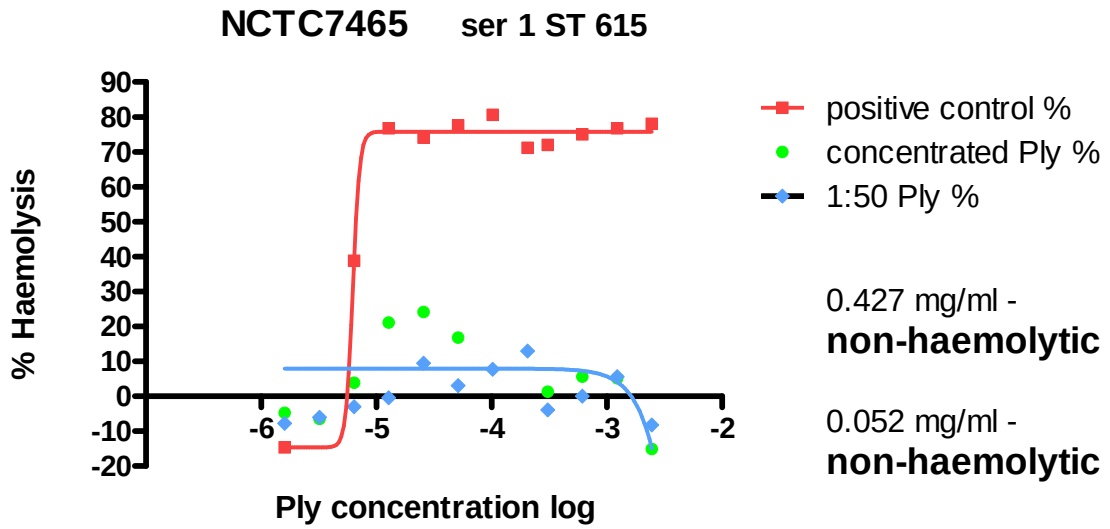
Graph 6. Haemolytic assay, protein concentrations and specific activities



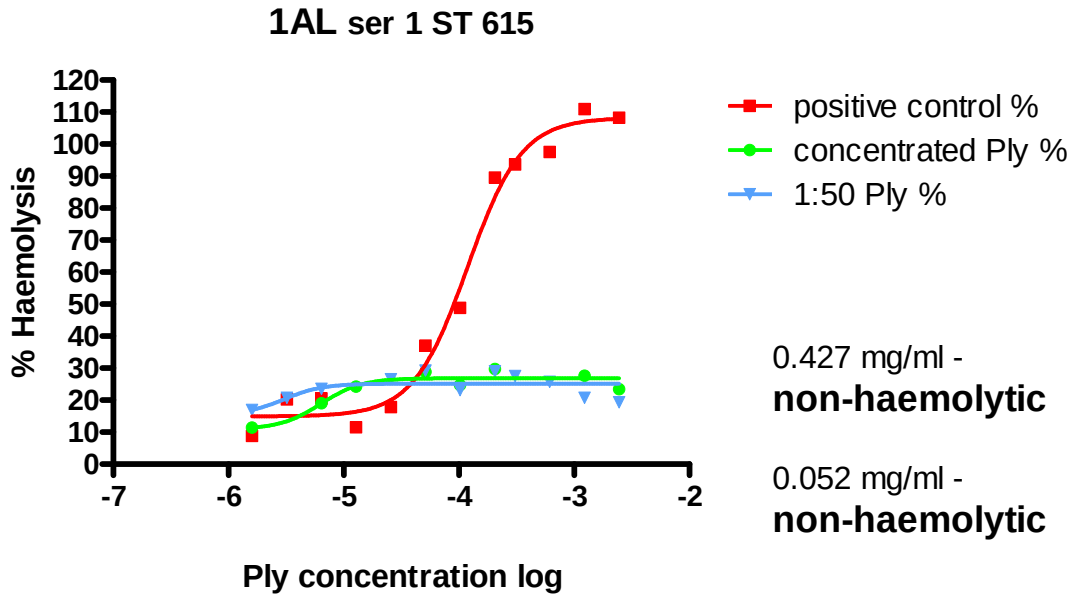
Graph 7. Haemolytic assay, protein concentrations and specific activities



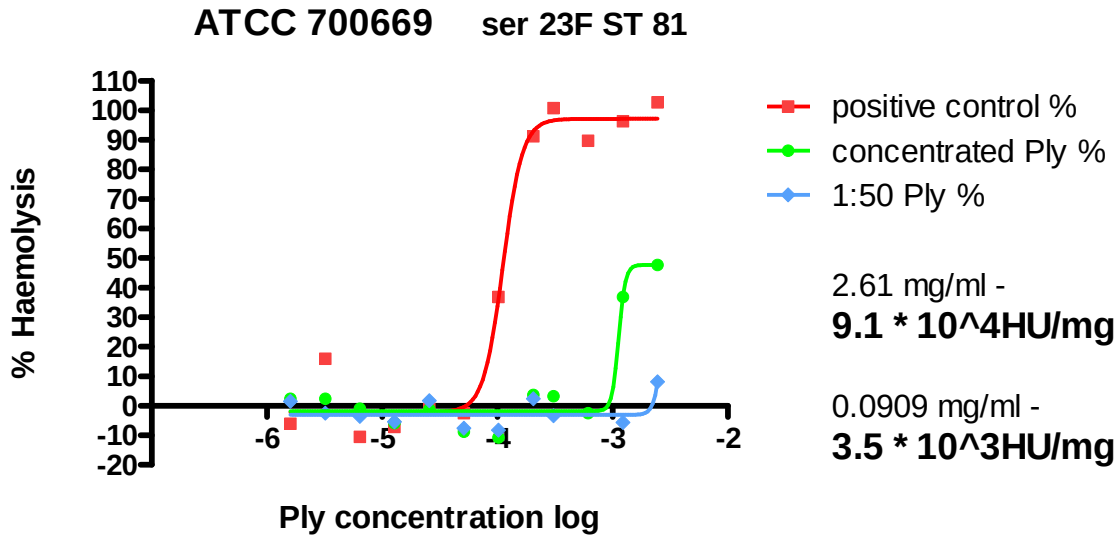
Graph 8. Haemolytic assay, protein concentrations and specific activities



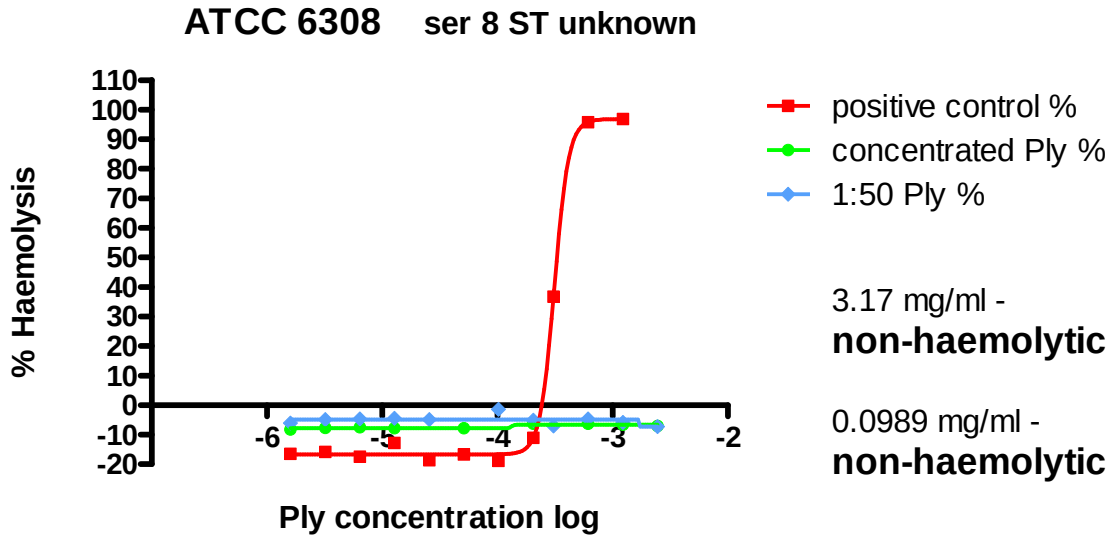
Graph 9. Haemolytic assay, protein concentrations and specific activities



Graph 10. Haemolytic assay, protein concentrations and specific activities



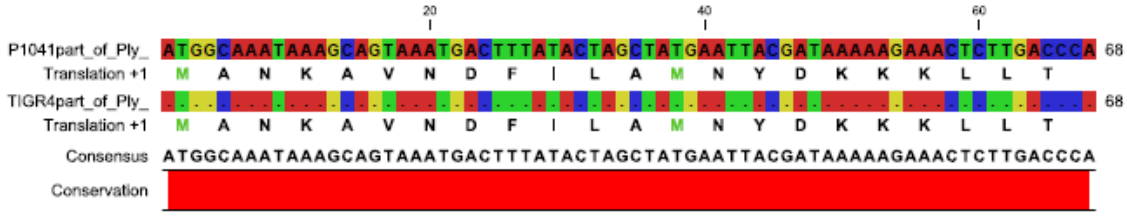
Graph 11. Haemolytic assay, protein concentrations and specific activities



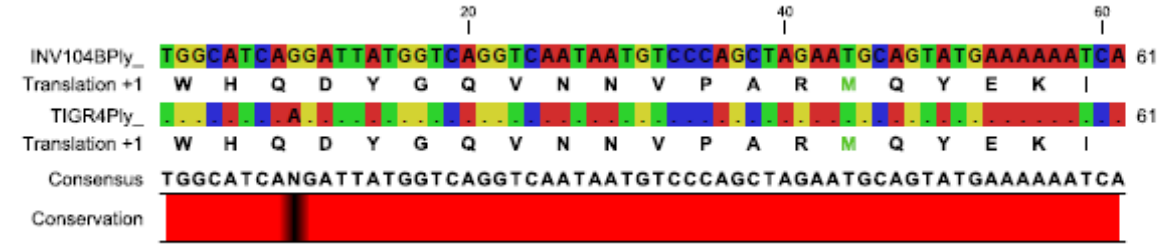
Graph 12. Haemolytic assay, protein concentrations and specific activities

Note: Data used for the graphs are the averages of duplicates.

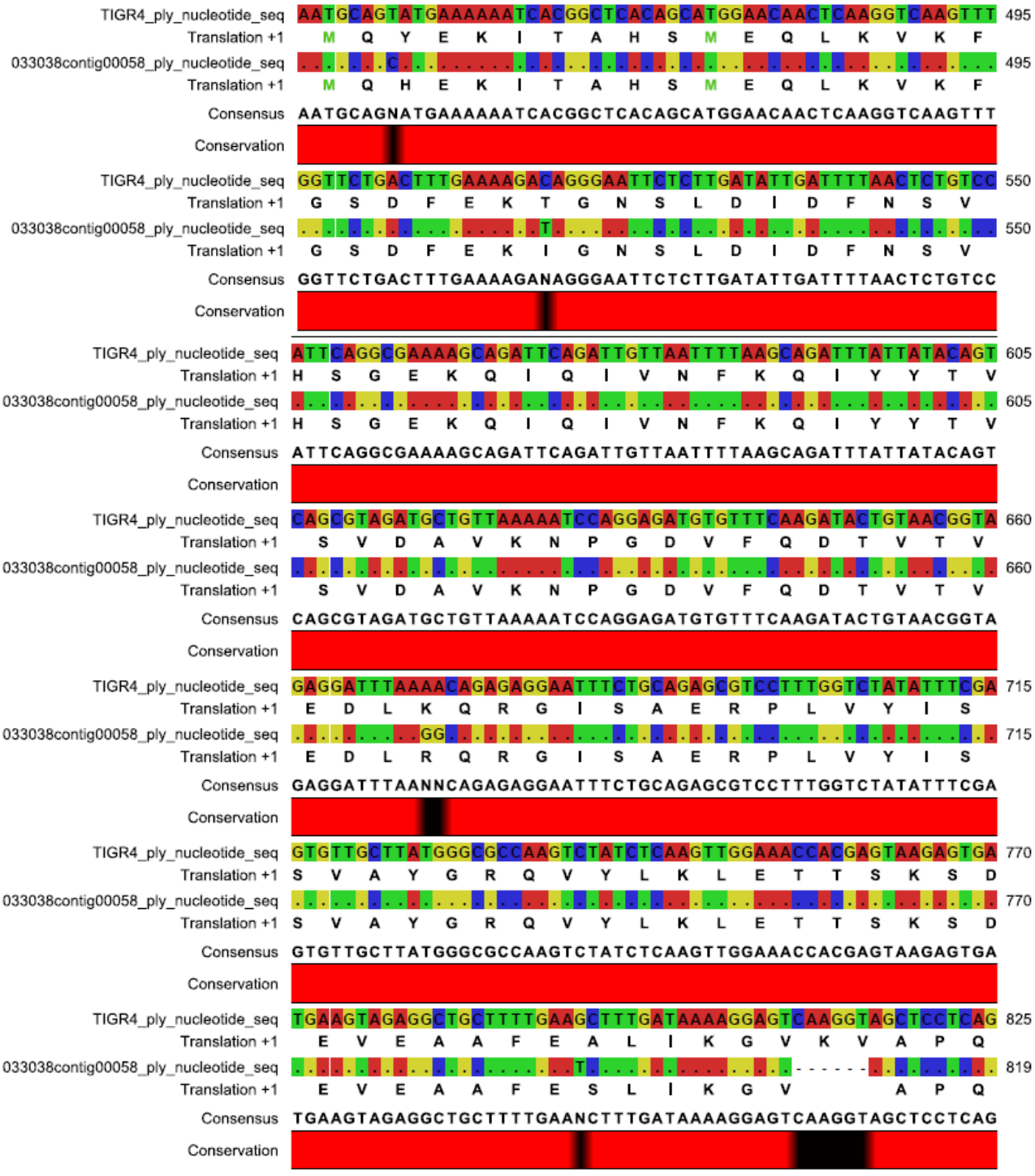
The reason for the loss of activity lies within the sequence for Ply. To investigate this, CLC Genomics Workbench programme was used. SNPs can be indentified by comparing at least two genomic regions so that the polymorphism is apparent. Alignments with the reference strain TIGR4 sequence for Ply revealed several SNPs that seem to be important and perhaps evolutionary beneficial to the pneumococcus. Picture 3 depicts a section taken from an alignment of Ply nucleotide sequences of TIGR4 and P1041. It is an example of an alignment of two completely same sequences. Picture 4 shows some variation at nucleotide sequence level which does not result in amino acid change (AA). Variation at nucleotide level means that INV104 has a Ply allele different from Ply allele of TIGR4. However, when describing proteins, only amino acid changes are relevant. ClustalW2 programme was used to assign numbers to Ply of the strains (see Ply types in Table 1). Few particular SNPs that are marked in Picture 5 and summarized in Table 2 have been identified.

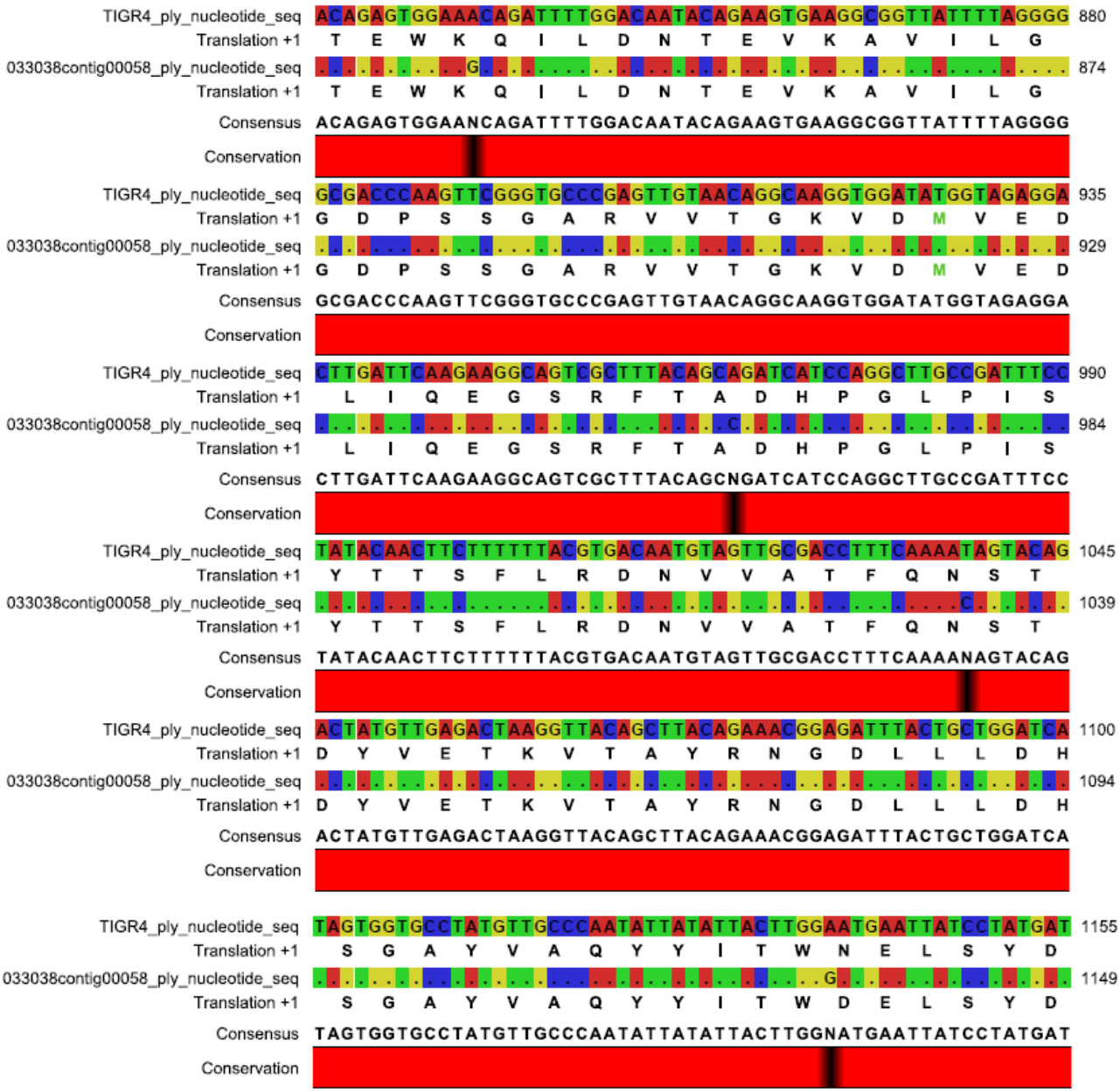


Picture 3. P1041 - TIGR4 Ply alignment – identical sequences



Picture 4. INV104B - TIGR4 Ply alignment – a variation (black) in nucleotides without change in AA





Picture 5. 03_3038 - TIGR4 Ply alignment – important SNPs (black)

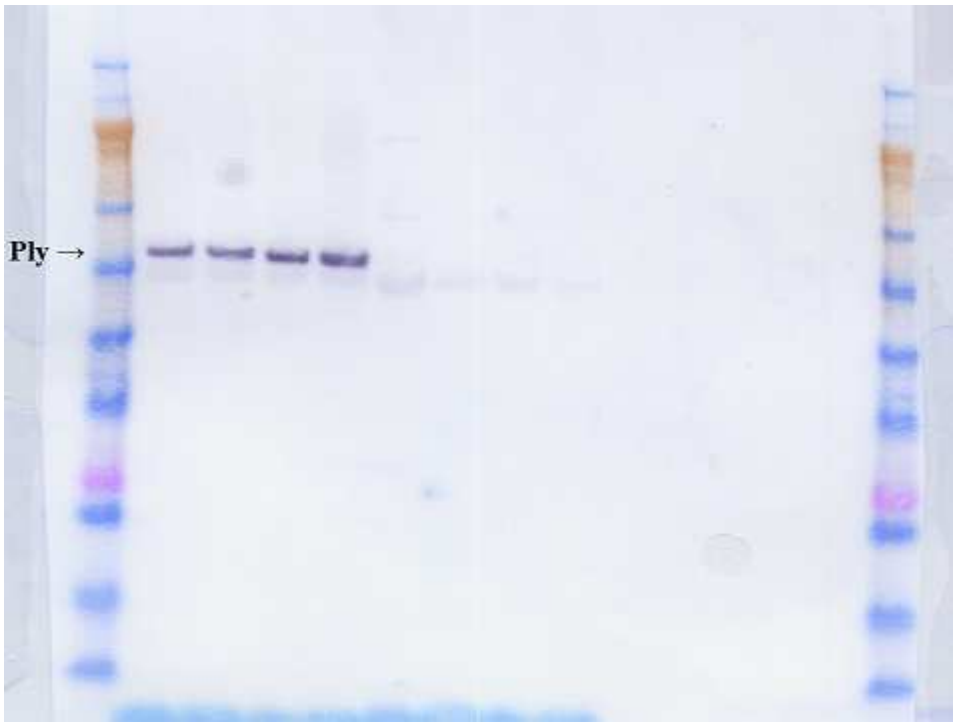
SNP POSITION	NUCLEOTIDE IN TIGR4	NUCLEOTIDE IN PLY5	AMINO ACID POSITION	AMINO ACID CHANGE
448	T	C	150	Tyr → His
515	C	T	172	Thr → Ile
671-2	AA	GG	224	Lys → Arg
793	G	T	265	Ala → Ser
810-15	CAAGGT	Deletion	270, 271	Lys, Val missing
1138	A	G	379	Asn → Asp

Table 2. SNPs and consequent changes in amino acids

These SNPs have been found in all strains of serotype 1 ST 306 and one strain of serotype 8 ST unknown. Change from Thr → Ile probably affects insertion of domain 3 into host cell membranes and therefore is responsible for the loss of Ply pore-forming function, while other SNPs contribute to overall decrease in haemolytic activity. SNPs are located in N-terminal end of the protein, a part that participates in pores oligomerization. The mutation localized at position 150 is unique to Ply type 5 and it is conserved among cholesterol-dependent cytolysins.

3. Pneumolysin protein might be missing in virulent strain

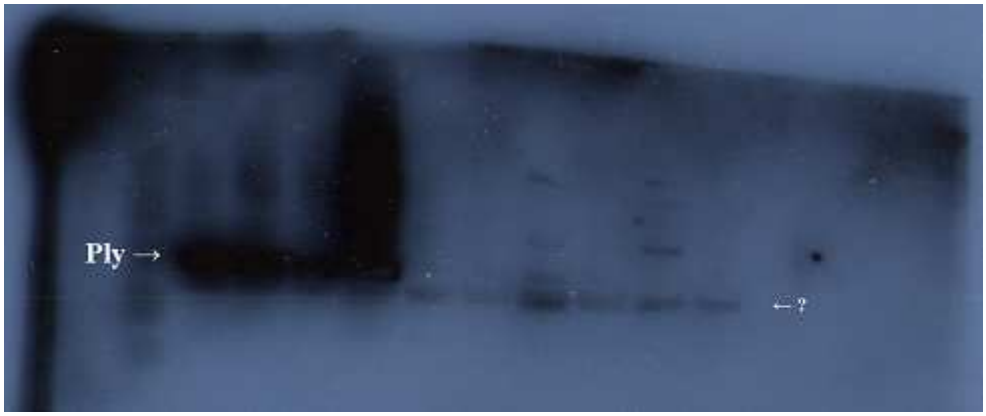
There are two more strains that showed no signs of haemolytic activity in the experiments; NCTC7465 and 1AL, both of serotype 1 ST 615. NCTC7465 is not haemolytic at all even though its Ply gene is exactly the same as TIGR4 Ply gene whose product is fully haemolytic. Sequence of 1AL Ply gene is not available. One possible explanation would be that Ply gene from NCTC7465 is not translated into protein, at least not under laboratory conditions. Western blot analysis was performed in order to confirm or deny this hypothesis (Picture 6).



Picture 6. Western blot for Ply. From the left: ladder, TIGR4 Ply (pellet), TIGR4 Ply (supernatant), ST 306 Ply (pellet), ST 306 Ply (supernatant), NCTC7465 Ply (pellet) 1, NCTC7465 Ply (supernatant) 1, NCTC7465 Ply (pellet) 2, NCTC7465 Ply (supernatant) 2, ladder

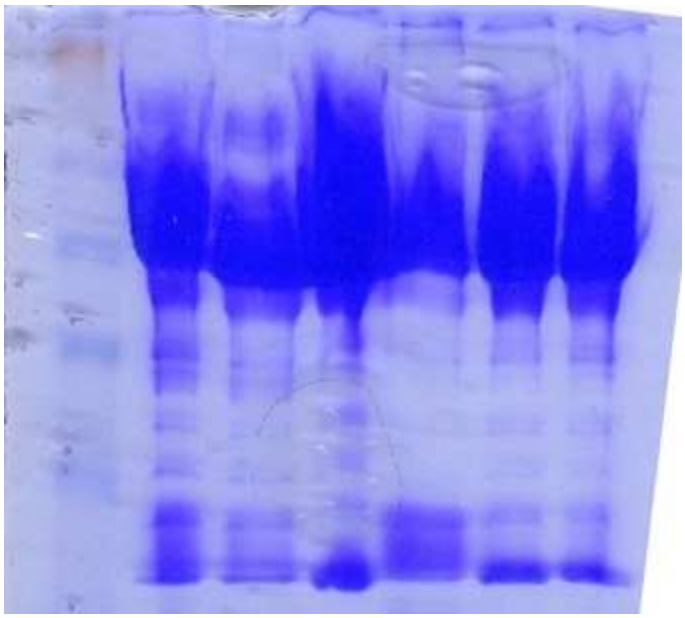
According to Western blot, ST 306 strain does translate its Ply gene into protein, which is present in both cell pellet and supernatant; however, NCTC7465 does not translate it or at very low level. In the next step, the quality of the samples was checked by introducing antibodies against PsaA protein (data not shown) and the sensitivity of this method was increased by using ECL kit so it would be possible to detect Ply if its

expression was reduced. Strain 1AL was included because it is also ST 615 like NCTC7465 and nonhaemolytic (Picture 7).

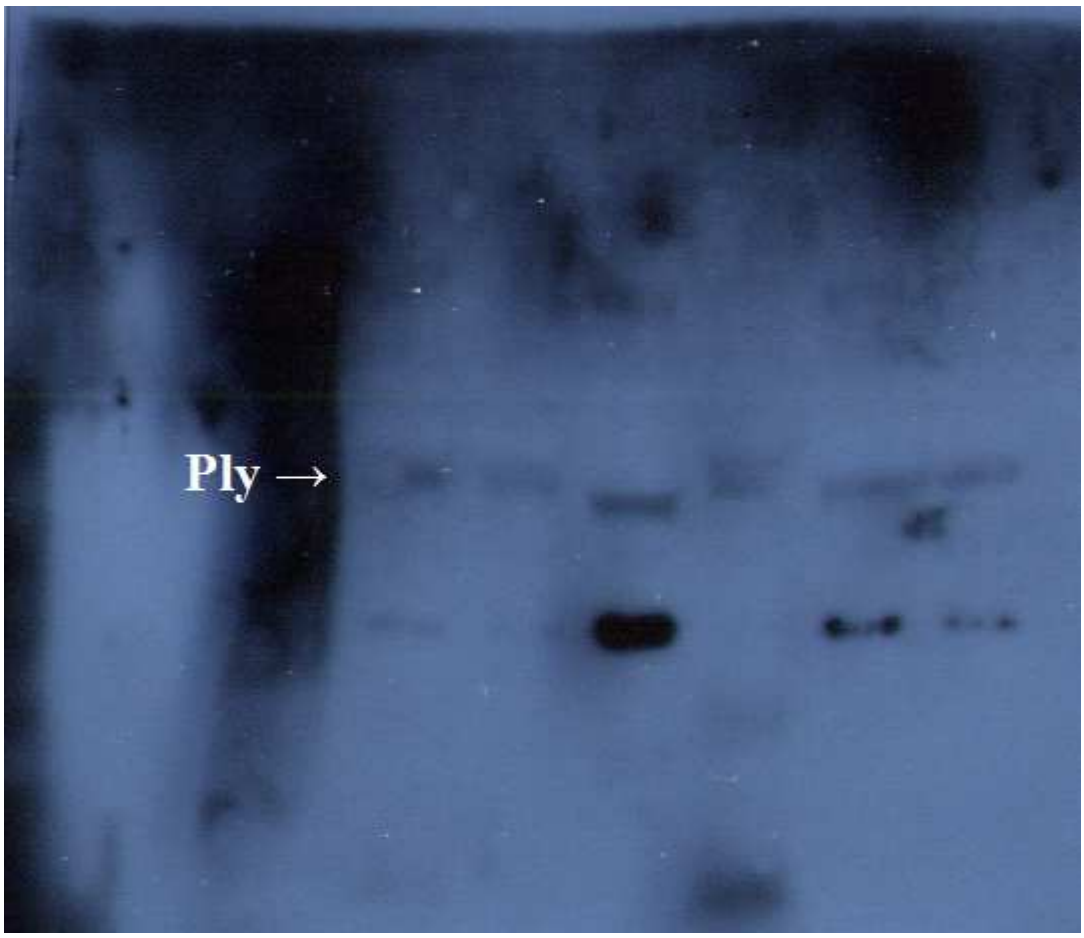


Picture 7. Western blot for Ply - ECL kit. From the left: ladder, TIGR4 Ply (pellet), TIGR4 Ply (supernatant), 03_3038 Ply (pellet), 03_3038 Ply (supernatant), NCTC7465 Ply (pellet) 1, NCTC7465 Ply (supernatant) 1, NCTC7465 Ply (pellet) 2, NCTC7465 Ply (supernatant) 2, 1AL Ply (pellet), 1AL Ply (supernatant)

Picture 7 shows that ST 615 strains do not express Ply. From an *in vivo* experiment carried out by Dr. A.M. Mitchell (Mitchell et al., in preparation), NCTC7465 surprisingly retains full virulence even though its Ply does not seem to be expressed. For that reason, the whole blood experiment was introduced in order to get as close as possible to *in vivo* conditions. When bacteremia was reached at 18h p.i., the whole blood was collected, double-spinned down and analysed. SDS page was done in order to check if there were any proteins in the samples at all and therefore it was worth carrying on with Western blotting (Pictures 8 and 9).



Picture 8. SDS page: protein-content from the whole blood samples. From the left: ladder, NCTC7465 mouse 1, NCTC7465 mouse 2, 03_3038 mouse 1, 03_3038 mouse 2, INV104B mouse 1, INV104B mouse 2

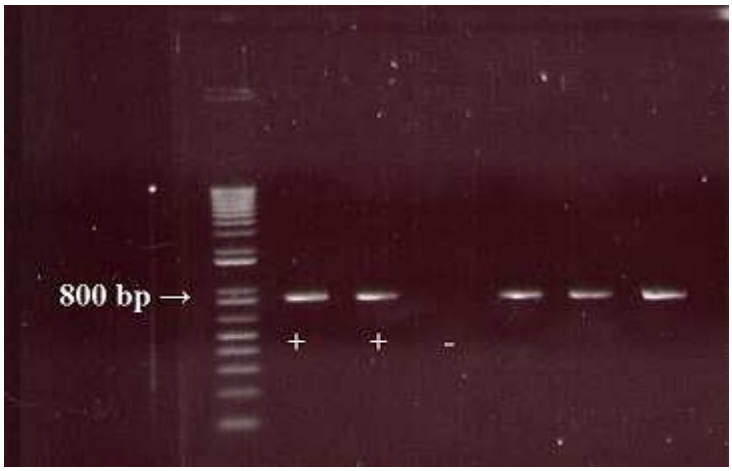


Picture 9. Western blot for Ply - ECL kit. The whole blood experiment. From the left: ladder, NCTC7465 Ply (mouse 1), NCTC7465 Ply (mouse 2), 03_3038 Ply (mouse 1), 03_3038 Ply (mouse 2), INV104B Ply (mouse 1), INV104B Ply (mouse 2)

Even though the Picture 9 is not very clear, it is possible to see Ply bands from ST 615 strain, which means that it does express Ply under *in vivo* conditions but no longer after it is grown *in vitro*.

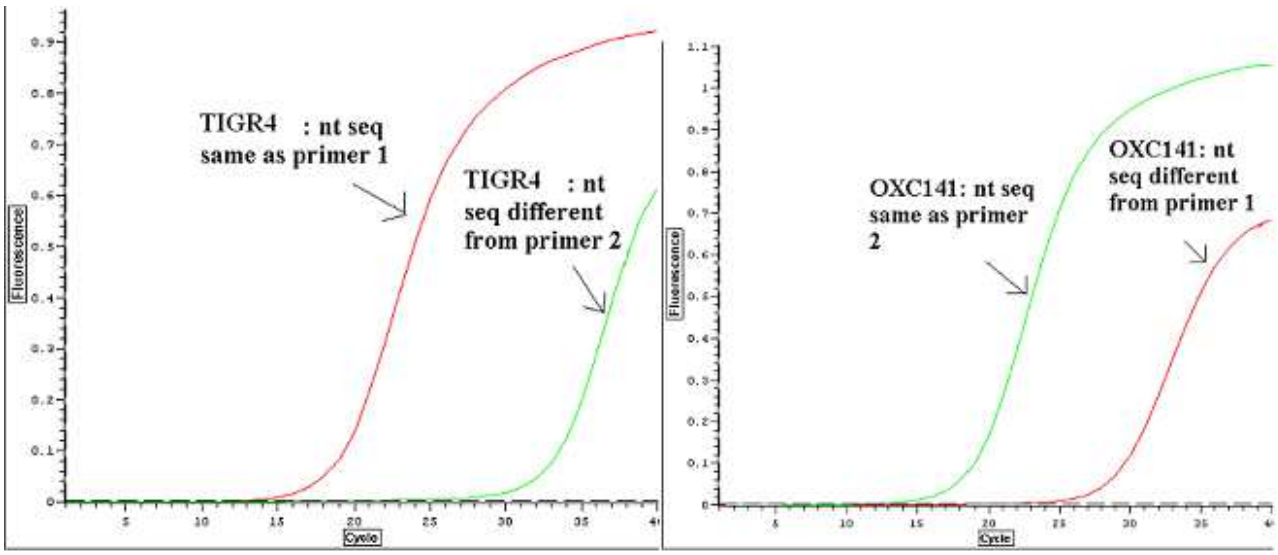
4. Hyaluronidase SNP genotyping

PCR confirmed presence and intactness of Hyl gene in all the strains used in this work. Picture 10 depicts amplified fragment of Hyl gene of a representative sample of the strains.



Picture 10. PCR amplification of Hyl gene fragment

Strain OXC141 (Oxford carriage isolate) carries a known SNP within its gene for Hyl which introduces a stop codon in Hyl mRNA and thereby abrogates its expression. OXC141 strain served as a control for mutant strains and TIGR4 strain was used as a control for wild type strains. Picture 11 shows the outcome of RT-PCR analysis for control strains and the results for all strains tested are shown in Table 3. This experiment ought to determine if there is any association between meningitis and Hyl activity.



Picture 11. RT-PCR control strains. Left: primer 1 (red curve) matches the TIGR4 Hyl gene wt sequence better than primer 2 (green curve). Right: primer 2 (green curve) matches the OXC141 Hyl gene mutated sequence better than primer 1 (red curve)

REFERENCE & SCOTTISH STRAINS	HYL GENE
TIGR4	Wild type
INV104B	Wild type
P1041	Wild type
OXC141	**Mutant**

INV200	Wild type
03_2672	Wild type
03_3038	Wild type
06_1370	Wild type
NCTC7465	Wild type
ATCC700669	Wild type
09_2458	Wild type
GSK3/43	Wild type
99_4038	**Mutant**
99_4039	**Mutant**
POLISH MENINGITIS STRAINS	HYL GENE
WA0001	**Mutant**
WA0002	Wild type
WA0003	Wild type
WA0004	Wild type
WA0005	Wild type
WA0006	Wild type
WA0007	Wild type
WA0008	Wild type
WA0009	**Mutant**
WA0010	Wild type
WA0011	**Mutant**
WA0012	Wild type
WA0013	Wild type
WA0014	Wild type
WA0015	Wild type
WA0016	Wild type
WA0017	Wild type
WA0019	Wild type
WA0020	Wild type
WA0021	Wild type
WA0022	Wild type
WA0023	Wild type
WA0024	Wild type
WA0025	Wild type

WA0027	**Mutant**
WA0028	Wild type
WA0030	Wild type
WA0031	Wild type
WA0032	Wild type
WA0033	Wild type
WA0034	Wild type
WA0035	Wild type
WA0036	Wild type
WA0037	Wild type
WA0038	Wild type
WA0039	Wild type
WA0042	Wild type
WA0046	Wild type
WA0047	Wild type
WA0048	Wild type
WA0051	Wild type
WA0052	Wild type
WA0053	Wild type
WA0054	Wild type
WA0055	Wild type
WA0056	Wild type
WA0057	Wild type
WA0058	Wild type
WA0059	Wild type
WA0060	Wild type
WA0061	Wild type
WA0062	Wild type
WA0063	Wild type
WA0064	Wild type
WA0065	Wild type
WA0067	**Mutant**
WA0068	Wild type
WA0070	Wild type
WA0071	Wild type
WA0072	Wild type
WA0075	**Mutant**

WA0076	Wild type
WA0077	Wild type
WA0078	Wild type
WA0079	Wild type
WA0080	**Mutant**
WA0081	Wild type
WA0082	Wild type
WA0083	Wild type
WA0084	Wild type
WA0085	Wild type
WA0086	**Mutant**
WA0087	Wild type
WA0089	Wild type
WA0090	Wild type
WA0091	Wild type
WA0092	Wild type
WA0093	Wild type
WA0094	**Mutant**
WA0095	Wild type
WA0096	Wild type
WA0097	Wild type
WA0098	Wild type
WA0099	Wild type
WA0100	Wild type
WA0101	Wild type
WA0103	Wild type
WA0104	Wild type
WA0105	Wild type
WA0106	Wild type
WA0107	Wild type
WA0109	Wild type
WA0110	Wild type
WA0112	Wild type
WA0113	Wild type
WA0115	Wild type
WA0116	Wild type
WA0117	Wild type

WA0118	Wild type
WA0120	Wild type
WA0121	**Mutant**
WA0124	Wild type
WA0128	Wild type
WA0129	Wild type
WA0130	Wild type

Table 3. Strains tested for Hyl SNP and their genotypes (wt, mut)

Out of 119 strains screened, 10 mutants were found among Polish meningitis isolates and 3 mutants in Scottish collection. Almost all mutant strains are of serotype 3 ST 180 or the same clonal group, only 1 mutant strain is of serotype 19F ST 423 suggesting that these strains with this particular genetic background might have come up with something to compensate mutated Hyl. This experiment is a constituent part of a complex study on allelic variation in virulence genes by SNP genotyping in a meningitis strain collection of *Streptococcus pneumoniae* (Mitchell *et al*., in preparation).

Discussion

1. Pneumolysin is present in all the strains

A great part of virulence is attributed to Ply. Virtually all pneumococcal clinical isolates produce Ply. PCR confirmed Ply gene presence in all the strains used in this work. There appears to be some differences in its activities among different strains, but it is generally highly conserved. This statement is supported by finding that pneumolysins from two strains isolated at literally opposite ends of the world at different times differ at only one amino acid and that is / methionine (Ile_{153}) (Met_{153}), respectively (Mitchell *et al*., 1990).

Streptococcus pneumoniae is known to be highly genetically variable even within serotypes. This work is partly focused on newly emerging invasive isolates. It appears that serotype 1 is on rise in European countries, but it is less frequently isolated from ill children in the USA, Finland, Canada or Australia (Hausdorff *el al*., 2000). Little is known about contribution of naturally occurring mutations in single genes to virulence.

Animal experiments done with strains representing lineage A (INV104B, 03_3038), lineage B (P1041), and lineage C (NCTC7465) revealed that strains from lineage A are avirulent whereas those from lineages B and C are highly virulent (Mitchell et al., in preparation). Genome sequencing of these clinical isolates has been performed to assess the genetic basis for this difference. Analysis of the genome sequences in combination with gene expression studies and *in vitro* assays has revealed possible causes for the large difference in virulence of these strains.

2. Pneumolysin genetic variability & haemolytic activity

The analysis confirms that a combination of specific SNPs located in Ply domains abolishes its binding, oligomerization and eventually lysis of erythrocytes, a finding in accordance with Jefferies and Kirkham studies (Jefferies *et al*., 2007), (Kirkham *et al*., 2006). One of the most successful invasive clones of serotype 1 ST 306 express a nonhaemolytic Ply, a virulence factor so far considered necessary for virulence. It seems that the presence of nonhaemolytic Ply correlates with specific serotypes and STs suggesting a mechanism to maintain this alternative Ply in the pneumococcal genome itself as if it would be somehow evolutionary beneficial to have nonhaemolytic Ply.

Serotype 1 ST 306 strains affected by haemolysis-decreasing SNPs are often associated with outbreaks. It is the presence of particular SNPs within gene for Ply in serotype 1 ST 306 strains that abolish Ply haemolytic activity. ST 306 strain with nonhaemolytic Ply is less virulent than ST 217 strain with wt Ply. These results points towards an assumption that maybe nonhaemolytic but still present Ply renders pneumococci an advantage insomuch as the bacteria are capable of multiplying without killing its host which is desirable. In this context, Ply deficiency appears more like an adaptation rather than a weakness. That raises the question whether Ply really is a virulence factor and to what extend it is needed to cause IPD. Other studies imply that nonhaemolytic Ply strains might be little disadvantaged in terms of virulence intensities, their haemolytic counterparts will better proliferate early in the blood (Harvey *et al*., 2011). A related haemolysin from *Listeria monocytogenes* may not be a factor of virulence in *sensu stricto*. Although

loss of haemolytic activity is accompanied by loss of virulence in experimental models, a direct proportional relationship between this activity and virulence was not observed. Not even an experimentally induced hyperhaemolytic haemolysin altered the original level of virulence of parental strain. More protein means greater haemolysis but not a greater virulence. Either the lysis of the erythrocytes is not itself the key factor in virulence or a saturation of the receptors for haemolysin (cholesterol in membranes in case of Ply) is brought about by low amounts of haemolysin. Furthermore, we do not know if *in vitro* production of haemolysin reflects the protein-generation *in vivo* (Kathariou *et al*., 1988).

3. Pneumolysin protein might be missing in a virulent strain

A great difference in virulence between ST 306 and ST 615 strains was observed. ST 306 strain does express Ply which is nonhaemolytic while ST 615 possesses wild type Ply and it was expected to be haemolytic but it was not. Western blot shown that the reason for this is that ST 615 strain Ply gene is not translated into protein after this strain is grown *in vitro*. ST 306 strain is able to cause bacteremia that persists at the same level without being eliminated or killing the animals, whereas ST 615 managed to kill all the animals within 30h p.i. (Mitchell et al., in preparation). The results show that ST 615 may express Ply only *in vivo* but not *in vitro.* There might be a regulatory mutation in this strain's genome switching Ply expression on and off according to environment. If Ply is a pneumococcal weapon against the host's immunity when it reaches the bloodstream, having it expressed on plates which resembling colonization conditions may be a luxury. There is growing evidence that pneumococci recovered from *in vivo* conditions are more virulent than those grown under *in vitro* conditions. This is consistent with results of Ply haemolytic activities; Ply released from *in vivo*-grown bacteria is more haemolytic than Ply released from *in vitro*-grown bacteria taking into account the same protein concentrations. However, this is true only for Ply that has been released from the cells and collected form the supernatant, cytoplasmic Ply obtained by sonication shows the same values of haemolytic activity (Kadioglu *et* al., 2004).

4. Hyaluronidase SNP genotyping

Virtually all strains of *S. pneumoniae* produce Hyl. PCR confirmed Hyl gene presence in the strains. Hyl contributes to pathogenesis by degradation of connective tissues thereby probably facilitating colonisation, and translocation between single compartments, i.e. from lungs to the vascular system and to the brain via bloodstream (Paton *et al*., 1997). Destruction of the normal connective tissue structure by Hyl may cause exposure to bacterial toxins and facilitates pneumococcal invasion of the bloodstream after colonisation of the upper respiratory tract (Polissi *et al*., 1998).

Out of all samples screened for SNP within Hyl gene, approximately 8% possessed mutated Hyl gene. If Hyl really is important for the pneumococcus to be able to cause meningitis, we would not find any or very few mutants carrying this SNP within Hyl (Kostyukova *et al*., 1995). This investigation of Hyl's importance needs continuation of screening another set of strains for Hyl SNP, this time non-meningitis isolates. Then statistics would be introduced to address the question.

5. The purpose of virulence factors

But what if a pathogen only wants to survive and not to destroy its host? Wouldn't it be more beneficial for it just to co-exist and thrive inside its host? Orihuela *et al* speculate whether the majority of virulence factors contribute positively to nasopharyngeal colonisation rather than to development of invasive disease because the pneumococcus has neither transmission factors nor environmental niche and progression to severe disease doesn't contribute to spread of bacteria to other hosts (Orihuela *et al,* 2004b). Successful infection is characterized by colonisation, multiplication, and transmission to a new host, death is a dead-end route for the pathogen therefore pathogenicity factors should be selected against according to Hava *et al*. The difficulty reaching the alveolus directly from the nasopharynx without binding anywhere in between and the need for a different set of surface proteins for adherence between nasopharynx and lung may partially explain why the incidence of pneumonia is so infrequent compared to the rate of carriage. Such a relatively low incidence of pneumococcal disease compared to its carriage is another evidence of its adaptation to humans (Malley *et al*., 2003). If we assume that *S. pneumoniae* have adapted primarily to colonize the human nasopharynx, with invasive infection representing "failed colonization" and a biological "dead-end," has Ply remained so highly conserved among pneumococci because it has critical functions during colonization? Next questions that should be addressed are based on Ply's presumed importance in pneumococcal infection: why is Ply not actively secreted? Is Ply released only during pneumococcal autolysis, and if so, does this represent "bacterial altruism," with some bacteria sacrificed to release factors necessary for the propagation of the whole bacterial population? Is Ply production regulated, and if so, what environmental factors stimulate its synthesis? Virulence factors may be initially there for colonisation and carriage, but they act as virulence factors once the invasion begins (Hava *et al*., 2003).

It is a well-known phenomenon that every time a pathogen encounters a new species, the meeting is thunderous. The burst of SARS (Severe Acute Respiratory Syndrome) in 2002 may serve as an example of such an interaction. Until that moment, the coronavirus was adapted to its natural host, pets, but than a new recombinant virus arose from a genetic shift and caused SARS in humans with quite high mortality (9.5%) (Rajčáni *et* Čiampor, 2006). Evolution drives organisms to adapt as best as they can. *Mycobacterium tuberculosis* is, in contrast to *Streptococcus pneumoniae*, sexually isolated, has a clonal lifestyle but still is a very successful pathogen of humans for centuries. Does it mean that this very pathogen managed to evolve into "perfect" pathogen which no longer needs to change itself in order to survive and thrive?

Virulence factors from *Salmonella enterica* subspecies 1 serovar Typhimurium are expressed in a bistable fashion, which means that two different phenotypes are expressed by isogenic organisms living in the same environment (Sturm *et al*., 2011). Such a regulation could come handy when expression of virulence factors is costly or imposes some kind of restriction but it is still needed since these are virulence factors. It would be interesting to perform a single cell analyses to find out whether any virulence factors from *Streptococcus pneumoniae* undergo the same regulation in order to increase its fitness. In the long run, the pneumococcus has met the human race quite recently, it is trying to get along with its new host and maybe one day it will fully become our commensal.

Summary

- Literature retrieval revealed that *Streptococcus pneumoniae* is a highly variable microorganism prone to variation. Opportunity to acquire new genes and mutations advantageous in given conditions increases this bacteria fitness and chances to survive.

- One of its most important virulence factors, pneumolysin, seems to be restricted in some of the newly emerging virulent pneumococcal clones or at least very strictly regulated. A fact, that SNPs abolishing haemolytic activity are maintained by invasive clones, points out that perhaps evolutionary force drives the pneumococcus towards adaption to its host in a less aggressive way.

- Quantitative PCR analysis is a useful tool for detecting even slight differences in genes; SNPs. The results suggest that hyaluronidase relates to development of pneumococcal meningitis, however, some pneumococcal clones may be capable of hyaluronidase substitution if it is missing and cause the disease without it.

- Combination of bioinformatics and laboratory work provides an opportunity to look into bacterial genomes while studying its behaviour in *in vitro* and *in vivo* assays. In other words, this means we can see direct effect of genotype on phenotype. Pneumococcal virulence is a complex mechanism resulting from combination of many factors such as serotype, sequence type, presence of mutations, host responses, and other external effects.

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