Differential Expression of *Streptococcus Pneumoniae* Genes during Pathogenesis

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Chapter Two: Materials and Methods

Chapter Three: Optimisation of a mouse model of invasive pneumococcal disease and methods for extraction of RNA from *in vivo*-derived pneumococci

Abstract

Streptococcus pneumoniae is a nasopharyngeal commensal in most healthy individuals. However, it can translocate from this niche to deeper tissues, causing diseases such as otitis media, meningitis, sepsis and pneumonia, which are responsible for significant morbidity and mortality worldwide. At the commencement of this work, inherent difficulties in harvesting sufficient bacterial numbers from experimental animals restricted the examination of pneumococcal gene expression during pathogenesis, and thus virulence gene transcription patterns were largely unknown outside of an *in vitro* environment. This thesis aimed to investigate such transcriptional patterns *in vivo*, and to hence gain a better understanding of pneumococcal behaviour during colonisation and disease.

This work describes refinement of an intranasal *S. pneumoniae* infection model in CD-1 mice that enables pneumococci to be harvested from multiple niches with low contamination by nasopharyngeal microflora or host tissue, and minimal cross-contamination with circulating pneumococci in the vascular system. The challenge route simulates the acquisition of *S. pneumoniae* in the human population, and progression to IPD occurs naturally. RNA extraction, enrichment and linear amplification procedures were optimised so that RNA could be obtained from *in vivo* site in sufficient quantities and with sufficient integrity to be used in semi-quantitative assays. Linear amplification allowed the examination of gene expression in niches where low bacterial numbers had previously prevented such analyses.

Real-time RT-PCR and microarray analyses were used to examine bacterial RNA samples recovered from the nasopharynx, lungs, blood and brains of CD-1 mice, providing the first comparative transcriptional data for pneumococci during carriage and disease, within the same animal model. Two pneumococcal serotypes were examined; a type 2

(D39) and a type 6A (WCH16) strain. CbpA, Ply, and SpxB were shown to be important for carriage in both strains, with pneumococci up-regulating the expression of the genes encoding these virulence proteins in the nasopharynx. This provides *in vivo* evidence supporting the ascribed roles of these proteins in reducing the level of competing microflora and promoting nasopharyngeal adherence. Similarly, D39 *nanA* and *pspA* transcription levels were up-regulated in the nasopharynx. The level of *pspA* mRNA was also higher in the blood than the lungs, suggesting an increased requirement in the bloodstream, where PspA is involved in reducing complement-mediated opsonisation. Despite the anti-phagocytic role of the pneumococcal polysaccharide capsule in the bloodstream, D39 *cpsA* mRNA was present in similar quantities in the nasopharynx, lungs and blood, which may support previous studies indicating post-transcriptional regulation of capsule expression. However, *cpsA* expression was up-regulated in the blood for WCH16. These results may indicate the existence of strain-specific differences in virulence gene regulation.

Microarray analysis of *in vivo*-harvested *S. pneumoniae* D39 found that mRNAs encoding components of phosphotransferase systems, CbpA, a putative neuraminidase, and v-type sodium ATP synthase subunits were significantly higher in bacteria involved in carriage than bacteraemia. Conversely, the expression of genes involved in competence, and *dinF* (present on a competence-induced operon), were up-regulated in the blood compared to the nasopharynx, providing evidence that competence is induced during bacteraemia. Pneumococci also showed increased expression of genes involved in fatty acid metabolism, *pgdA*, *lytB* and *cbpG* in the blood compared to the nasopharynx. This study used a single pneumococcal strain and infection model and, therefore, overcomes inherent issues of serotype/strain- and animal model- specific gene expression that may have complicated interpretation of data in previous studies.

This thesis reports some of the first *in vivo* pneumococcal gene expression data gained using a single animal model and pneumococcal strain. The data reinforce the

putative roles of several virulence factors, and provides novel transcription data for pneumococci during carriage. Results suggest the existence of core genes that are essential for infection in multiple pneumococcal serotypes, whereas other genes appear to have strain-specific roles.

Declaration

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Published work included in this thesis:

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List of abbreviations

Abbreviations acceptable to the American Society for Microbiology are used without definition in this thesis. Additional and frequently used abbreviations are defined when first used in the text, and are listed below.

A_{260}, A_{280}, A_{600}	Absorbance at 260nm, 280 nm, or 600nm respectively
BA	Blood agar
BBB	Blood-brain barrier
BMEC	Brain microvascular endothelial cells
C3b	Complement component 3b
CbpA	Choline binding protein A
CBP	Choline binding protein
CBR	Choline binding region
CFU	Colony forming units
ChoP	Phosphorylcholine
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
CSP-1	Competence stimulating peptide 1
CSP-2	Competence stimulating peptide 2
C-terminus	Carboxy terminus
Ct	Cycle threshold
DFI	Differential fluorescence induction
DIG	Digoxigenin
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
Ery	Erythromycin
g	Gravity units
hr	Hour(s)
Ig	Immunoglobulin
IN	Intranasal
IP	Intraperitoneal
IPD	Invasive pneumococcal disease
Ig	Immunoglobulin
kDa	Kilodalton(s)
LB	Luria Bertani broth

LytA	Autolysin A
M	Molar
min	Minute(s)
mRNA	Messenger ribonucleic acid
NanA	Neuraminidase A
N-terminus	Amino terminus
O/N	Overnight
ORF	Open reading frame
PAF	Platelet activating factor
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PiaA	Pneumococcal iron acquisition A
pIgR	Polymeric immunoglobulin receptor
Ply	Pneumolysin
PS	Polysaccharide
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PTS	Phosphotransferase systems
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Second(s)
SEM	Standard error of the means
STM	Signature-tagged mutagenesis
THY	Todd-Hewitt broth supplemented with yeast extract
TLR	Toll-like receptor
WCL	Whole cell lysate
WT	Wild-type