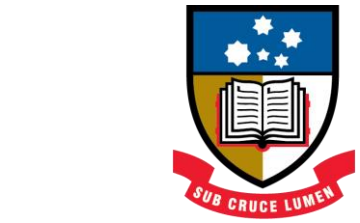
# Characterisation of *Streptococcus pneumoniae* Opacity Phase Variation




THE UNIVERSITY of ADELAIDE

**Melissa Hui Chieh Chai, BBiomedSc (Hon), MSc**

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy from the University of Adelaide

February 2016

Research Centre for Infectious Diseases
Department of Molecular and Cellular Biology
The University of Adelaide
Adelaide, S.A., Australia

# Table of Contents

# List of Figures

# List of Tables

## Abstract

*Streptococcus pneumoniae* (the pneumococcus) is a common nasopharyngeal commensal in humans that can invade deeper tissues causing a range of diseases, resulting in significant global morbidity and mortality. Any given strain of *S. pneumoniae* can undergo phase variation between two different colony phenotypes, termed opaque (O) and transparent (T), as determined by their morphology on clear, solid media. In animal models, the O form is more virulent and commonly isolated from normally sterile sites, such as the blood, lungs and brain, compared to its T counterpart, which is more likely to reside in the nasopharynx. To date, the mechanism involved in the switch between opacity phenotypes is not known and there is discordance in the literature in terms of the precise molecular differences between them. This project set out to comprehensively characterise phenomic (2D-DIGE), transcriptomic (DNA microarray) and genomic (IonTorrent sequencing) differences between opacity variants in three unrelated pneumococcal strains (D39, serotype 2; WCH16, serotype 6A; and WCH43, serotype 4).

Proteomic, transcriptomic and genomic analyses revealed strain-specific differences associated with phase variation, but no single difference was consistent across the three strains tested. Nevertheless, there were examples of proteins and genes belonging to the same putative functional groups that were regulated similarly in both the O and T phase between strains. One example was the upregulation of proteins and genes related to genetic competence in the O variants. However, mutagenesis of one such gene, encoding the competence stimulating peptide receptor (*comD*), did not alter opacity phenotype. There were also inconsistencies between genes identified as differentially expressed at an mRNA and protein level, such as genes involved in cell division, and amino acid biosynthesis and acquisition. These genes were upregulated at an mRNA level in the T variants, but no such upregulation in protein expression was identified by proteomic analysis. At a genomic level, all single nucleotide polymorphisms identified by IonTorent sequencing were independently verified. However, the insertion/deletions, particularly those associated with homopolymeric tracts were all found to be false call-outs, which is a limitation of this sequencing technology.

The role of epigenetic changes mediated by genetic rearrangements in a Type I restriction-modification (RM) system on opacity phenotype was also investigated. These rearrangements result in switching between six alternative DNA methylation site specificities impacting on genomic methylation patterns. Examination of six "locked" mutants (SpnD39IIIA-F) with monospecific DNA methylation patterns, indicated that there was an epigenetic impact on colony opacity phenotype and virulence. Importantly, these genetic rearrangements at the Type I RM locus also occurred during experimental infection. However, there were inconsistencies between the opacity phenotypes of locked mutants and the RM allele distribution in wild-type D39O and D39T. Thus, RM allele switching cannot fully account for colony opacity phase variation in pneumococci.

This study identified that pneumococcal phase variation is a complex, multifactorial phenomenon and different strains employ alternative mechanisms to attain opacity phenotypes. Furthermore, epigenetic changes impact pneumococcal opacity morphology and pathogenicity. Hence, the role of epigenetic factors in phase variation and pathogenesis should be investigated in future studies.

# Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

_____

Melissa Hui Chieh Chai

……/……/……

# Abbreviations

| | |
|---|---|
| °C | degrees Celsius |
| µg | microgram/s |
| µg/ml | microgram/milliliter |
| µl | microlitre/s |
| 2D-DIGE | two-dimensional differential gel electrophoresis |
| A | adenine |
| $A_{340}$ | absorbance at 340 nm |
| $A_{520}$ | absorbance at 520 nm |
| $A_{550}$ | absorbance at 550 nm |
| $A_{600}$ | absorbance at 600 nm |
| Adc | zinc ABC transporter |
| AdhE | alcohol dehydrogenase |
| AdoMet | S-adenosyl methionine |
| ADP | adenosine diphosphate |
| AGRF | Australian Genome Research Facility Ltd |
| AliA | an oligopeptide ABC transporter |
| *ami* | aminopterin resistance operon |
| APC | adelaide Proteomics Centre |
| APIA | acid-phenol:chloroform:isoamly alcohol |
| ATP | adenosine triphosphate |
| BA | blood agar |
| BBB | blood-brain barrier |
| Blp | bacteriocin protein |
| bp | base pairs |
| C | cytosine |
| C+Y | semi-synthetic medium |
| CAP | community-acquired pneumococcal (infections) |
| Cbp | choline-binding protein |
| $CD4^+$ | cluster of differentiation 4 |

| | |
|---|---|
| CFU | colony forming units |
| Cgl | competence protein |
| ChoP | pneumococcal cell-wall phosphorylcholine |
| CiaRH | two-component signal-transducing system |
| *cibA* | competence induced bacteriocin A gene |
| CNS | central nervous system |
| Com | competence protein |
| *comDE* | two-component signal-transducing system, competence |
| *cps* | capsular biosynthesis genes |
| CPS | capsular polysaccharide |
| CR | coding repeat |
| CreX | Cre tyrosine recombinase |
| CRP | C-reactive protein |
| CSF | cerebrospinal fluid |
| CSP | competence-stimulating peptide |
| Dam | deoxyadenosine methlytransferase |
| *dexB* | glucan 1,6-alpha-glucosidase |
| *divIVA* | cell division initiation gene |
| DNA | deoxyribonucleic acid |
| DnaJ | chaperone protein DnaJ |
| DnaJ/K | molecular chaperone |
| DOC | sodium deoxycholate |
| ECM | extracellular matrix |
| EDTA | ethylene-diamine-tetra-acetic-acid disodium salt |
| EF-G | elongation factor G |
| EF-Ts | elongation factor Ts |
| emPAI | exponentially modified protein abundance index |
| Erm/Ery | erythromycin |
| Fba | fructose-bisphosphate aldolase |
| FDR | false discovery rate |
| fH | factor H |

| | |
|---|---|
| FRR/EF-4 | ribosome-recycling factor |
| *fucK* | putative L-fuculose kinase |
| FusA | translation elongation factor G |
| g/L | grams per litre |
| G | guanine |
| g | relative centrifugal force |
| G-3-P | D-glyceraldehyde-3-phosphate |
| *galU* | UTP-Glc-1-phosphate uridylyltransferase |
| GAPDH | glycerolaldhyde-3-phosphate dehydrogenase |
| Gent | gentamicin |
| GlcNAc | N-acetyl-glucosamine |
| GlpO | Alpha-glycerophosphate oxidase |
| GMP reductase/*guaC* | guanosine 5'monophosphate oxidoreductase |
| Gor | glutathione reductase |
| GroEL | heat shock protein |
| GroES | heat shock protein |
| h | hour/s |
| hbMEC | human brain microvascular endothelial cells |
| Hic | factor H-binding inhibitor of complement |
| HIV | human immunodeficiency virus |
| Hyl | hylauronate lyase |
| *hsd* | host-specificity-determinant gene |
| *hsdM* | host-specificity-determinant gene for modification |
| *hsdR* | host-specificity-determinant gene for restriction |
| *hsdS* | host-specificity-determinant gene for sequence specificity |
| i.n. | intranasal |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| IEF | isoelectric focusing |
| IL-1 | interleukin-1 |
| IL-12 | interleukin-12 |

| | |
|---|---|
| *ileS* | isoleucyl-tRNA synthetase |
| IMP | inosine phosphate |
| INDEL | insertion/deletion |
| INF-γ | interferon γ |
| IPD | invasive pneumococcal disease |
| IPS | internal pool standard |
| IPTG | isopropyl-β-D-thio-galactopyranoside |
| ISP | Ion Sphere™ Particles |
| kb | kilobase/s |
| kDA | kilodalton/s |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| kg | kilogram/s |
| l | litre/s |
| LB | Luria Bertani broth |
| LC-ESI-IT MS | liquid chromatography electrospray ionisation ion-trap mass spectrometry |
| LctO | lactate oxidase |
| Ldh | lactate dehydrogenase |
| *lgtA* | beta-*N*-acetylglucosaminyltransferase |
| Liv | branched-chain amino acid ABC transporter |
| LPS | lipopolysaccharide |
| Lrp | leucine response regulatory protein |
| LuxS | S-ribosylhomocysteine lyase |
| *lysM* | lysin motif |
| LytA | N-acetylmuramoyl-L-alanine amidase |
| M | molar |
| Mal | maltose operon transcriptional protein |
| Mb | megabase/s |
| MDR | multi-drug-resistant |
| mg | milligram/s |
| mg/ml | milligram/millilitre |

| | |
|---|---|
| min | minute/s |
| ml | millilitre/s |
| mM | millimolar |
| Mod | methyltransferase |
| mRNA | messenger RNA |
| MurC | UDP-N-aceytylmuramate—alanine ligase |
| NAD | β-Nicotinamide adenine dinucleotide hydrate |
| NanA | neuraminidase A |
| ng | nanogram/s |
| ng/ml | nanogram/millilitre |
| nm | nanometers |
| nt | nucleotide/s |
| Nrd | anaerobic ribonucleoside triphosphate reductase |
| O | opaque variant |
| OD | optical density |
| OE-PCR | overlap-extension polymerase chain reaction |
| Opa | opacity outer-membrane proteins |
| ORF | open reading frame |
| p.s.i. | per square inch |
| PacBio | Pacific BioSciences |
| PAFr | platelet-activating factor receptor |
| PAGE | polyacrylamide gel electrophoresis |
| Pap | pyelonephritis-associated pilus |
| PbcA | C3-binding protein A |
| PBMC | human peripheral blood mononuclear cells |
| PBP2B | penicillin-binding protein 2B |
| PBS | phosphate buffered saline |
| PcpA | pneumococcal choline-binding protein A |
| PCR | polymerase chain reaction |
| PCV13 | pneumococcal conjugate vaccine, 13 serotypes |
| PCV7 | pneumococcal conjugate vaccine, 7 serotypes |

| | |
|---|---|
| PECAM-1 | platelet endothelial cell adhesion molecule-1 |
| Pfl | pyruvate formate lyase/formate acetyltransferase |
| PGM | personal genome machine |
| *pgm* | phosphoglucomutase |
| Pht | polyhistidine triad |
| PiaA | pneumococcal iron uptake |
| PIgR | polymeric Ig receptor |
| Pit | pneumococcal iron transport |
| PiuA | pneumococcal iron uptake |
| PlgR | polymeric Ig receptor |
| Ply | pneumolysin |
| PMT | photomultiplier |
| PNSP | penicillin-non-susceptible *S. pneumoniae* |
| PotD | spermidine/putrecine ABC transporter |
| Ppc | phosphoenolypyruvate carboxylase |
| PPI-1 | pneumococcal pathogenicity island 1 |
| PpmA | putative proteinase maturation protein A |
| PPSV23 | pneumococcal polysaccharide vaccine, 23-valent |
| PrsP | pneumococcal serine-rich protein |
| PsaA | pneumococcal surface adhesin A |
| PspC | pneumococcal surface protein C |
| PurA | adenylosuccinate synthetase |
| Pyk | pyruvate kinase |
| qRT-PCR | quantitative real-time PCR |
| qRT-PCR | real-time reverse transcription polymerase chain reaction |
| *radC* | DNA repair gene |
| RE | restriction endonuclease |
| RM | restriction-modification |
| RNA | ribonucleic acid |
| *rpsA* | ribosomal protein S1 gene |
| RT-PCR | reverse transcription polymerase chain reaction |

| | |
|---|---|
| s | second/s |
| SB | serum broth |
| SDH | streptococcal surface dehydrogenase |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyarcylamide gel electrophoresis |
| *sIgA* | secretory component of immunoglobulin A |
| SMRT | single-molecule real-time |
| SNPs | single nucleotide polymorphisms |
| SpsA | secretory IgA-binding protein |
| SpxB | pyruvate oxidase |
| sRNA | short non-coding RNA |
| SsbB | single-stranded DNA-binding protein |
| SSM | slipped-strand mispairing |
| T | transparent variant |
| T | thymine |
| TA | teichoic acid |
| TAE | tris-acetate EDTA buffer |
| TBE | tris, borate and EDTA |
| THY | todd-Hewitt broth supplemented with yeast extract |
| THY+catalase | THY supplemented with catalase |
| TLR | toll-like receptor |
| TNF | tumour necrosis factor |
| TRD | target recognition domain |
| TSB | tryptic soy broth |
| TTBS | tris-buffered saline containing Tween 20 |
| U | unit |
| U/ml | unit/millilitre |
| UDP-Glc | UDP-glucose |
| v/v | volume per volume |
| w/v | weight per volume |
| WT | wild-type |

| | |
|---|---|
| *wzy* | capsular biosynthesis gene |
| ZMW | zero-mode waveguide |

## Acknowledgements

I would like to firstly thank my principal supervisor, Professor James Paton for the opportunity to work on this project under his supervision and guidance. Thank you for the support, patience and advice throughout the course of my studies. Thank you also to Dr. David Ogunniyi for his guidance and helping me brainstorm ideas pertaining to my project, especially during the crucial first few months of my studies. I would also like to extend my gratitude to both past and present members of the Paton Laboratory, in particular, Dr. Richard Harvey, Dr. Lauren McAllister and Dr. Claudia Trappetti for your constant encouragement and letting me pick their brains to get through the last leg of my journey into finishing up the write-up. To Dr. Danny Wilson, Dr. Adrienne Paton, Cathy Scougall, Zarina Amin, Dr. Layla Mahdi, Dr. Melanie Higgins, Dr. Austen Chen, Dr. Charlie Plumptre, Dr. Catherine Hughes, Dr. Trisha Rogers, Dr. Hui Wang, Dr. Adam Potter, Stephanie Philp, Sarah Sims and Rory Leahy for contributing to the vibrancy of the place throughout my time in the Paton Laboratory. Also, to Dr. Florian Weiland, Mark Van der Hoek and Rosalie Kenyon for their advice and technical skills relating to certain areas of my project.

Thank you to the badminton folks, especially, Michael, Des and Paul, who have taken me in when I first arrived in Adelaide. Thank you for all the laughter, fun, pain but most of all, your company both on and off court. You have all made settling into Adelaide a much easier transition. To Agnes and Catherine, even though we are miles apart, I will always appreciate the friendship and chats (and the reminder about all things Kiwiana) about anything and everything. I look forward to many more adventures together.

A huge and special thanks goes out to my family, for their constant encouragement, support and someone to talk to, when I needed it most, no matter the time. Also, lots of love goes out to Rebecca and her family, especially the little girlies and their random FaceTime calls. I guess being a night owl helps with the time difference! Love you all lots.

Finally, to anyone that I may have not named (you know who you are), thank you for being part of my journey and making it such an enjoyable one.