BACTERIOPHAGE OF *Burkholderia pseudomallei*; FRIEND OR FOE?

Thesis submitted by

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Bachelor of Science (Hons) (JCU)
Graduate Diploma of Biomedical Sciences (JCU)
In November 2006

for the degree of Doctor of Philosophy
in the School of Veterinary and Biomedical Sciences
at James Cook University

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), The James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001), and the James cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology recieved clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A526 and A978).

J. Elliman   Date

ACKNOWLEDGEMENTS

Thanks must firstly go to Robert Hirst and Brenda Govan for starting me off on my PhD and supervising me through a rapid learning curve. Thanks must also go to Jane Oakey for suggesting I look for bacteriophage when my initial work hit a brick wall, for being so interested in my work and for assistance with various lab protocols. Great thanks must go to my current supervisor Leigh Owens, who took me on as a student midstream following Robert's retirement, found me paid work to support myself and was always available to impart the wisdom of his years. Leigh, you saved my bacon in so many ways (and no, you're not old, even if we students have made you lose your hair). Thanks also to Jan Smith for employing me, thus keeping me financially afloat.

Thanks to the multitude of students that came and with whom I shared an office, as well as those in other offices. I'd particularly like to thank Ray for his advice in the field of molecular biology. I'd also like to thank Kjersti, for her many distractions when chapter 4 was getting overwhelming. Thank-you to Ryutaro Ueda for helping me with processing of large amounts of PCR and sequencing. Also thanks to all the academics, students, techies and office staff for their interest and help through the convoluted administrative and organisational mazes of academia.

I'd also like to thank my friends who gave me emotional support through my years of study; Kris and Simon, who fed me, listened to me babble on about things they didn't understand and made me do physical exercise; Ros who went shopping with me (lots); Brad, who proofread my thesis; and Amanda, Chris and Andrea, who not only asked me what I was doing, but took me on holidays with them.

Finally I'd like to thank my family, especially my Uncle Des & Aunty Marian, (if you don't know why, I'll tell you later). Most importantly my parents who have supported me through my whole PhD - prayerfully, emotionally, and by giving me somewhere to stay rent free! Lastly I'd like to thank Paul and Doug for encouraging me to strive by constantly asking whether I was finished yet. Thankyou all so much.

A straight line may be the shortest distance between two points, but it is by no means the most interesting - Dr Who, from 'The Time Warrior'

ABSTRACT

Lysogenic bacteriophage carrying virulence determinants have been demonstrated to be responsible for the pathogenicity of many bacteria. Bacteriophage, or components of bacteriophage, have also been successfully used in the treatment of bacterial infections. *Burkholderia pseudomallei* is the causative agent of melioidosis and has been shown to carry bacteriophage. The role of bacteriophage in virulence of *B. pseudomallei* isolates has not yet been determined, nor have bacteriophage been examined for their potential in treatment of melioidosis.

A screen for identification of bacterial isolates of interest was developed and 50 isolates were examined. Thirty-one selected isolates were then examined for bacteriophage using techniques including; transmission electron microscopy (T.E.M), mitomycin C assay, UV assay, plaque assay and restriction digestion assay. A combination of mitomycin C assay and either plaque assay or restriction digestion assay were determined to be 96.77% accurate for testing for bacteriophage in *B. pseudomallei* isolates. Five techniques for the concentration of bacteriophage (commercial Qiagen kit, magnesium hydroxide precipitation, PEG precipitation, zinc chloride precipitation, ultracentrifugation) were examined and ultracentrifugation determined to be the best. Two methods of DNA extraction (commercial nucleobond AX kit, phenol chloroform extraction) were compared and a phenol chloroform extraction was modified for use.

A bacteriophage amplification system involving inoculation of bacteriophage into a broth of host *B. pseudomallei*, followed by lysis, was developed and optimised for production of lysogenic bacteriophage of *B. pseudomallei*. Addition of a 1:1 dose of bacteriophage to bacteria at an $O.D._{600nm}$ of 0.1 in 10-100ml of broth resulted in the production of $1x10^{11}$plaque forming units (pfu)/ml of media upon lysis at 7.5 hours post-inoculation.

Lysogenic bacteriophage extracted from highly virulent *B. pseudomallei* isolate NCTC 13178 was given the name BupsΦ1 and was characterised as being from the

family *Myoviridae* with a genome 55.1kb long. This bacteriophage was then used for infection assays and molecular analysis to determine whether it played a role in virulence. Endolysin of this bacteriophage was also extracted to determine its potential for use in therapy.

Four *B. pseudomallei* isolates tested negative for the presence of bacteriophage (#13, #69, #83, E4) and one isolate of particular interest (NAFC), were infected with BupsΦ1. Bacteriophage infection was found to alter colonial morphology on Ashdown agar. Infection assays in a BALB/c mouse model were carried out and no clear relationship between addition of bacteriophage BupsΦ1 and virulence was found. One experiment with NAFC resulted in greatly increased virulence, but this could not be repeated. All other experiments where infection with bacteriophage was successful resulted in minor upregulation or downregulation of virulence. Examination of plaque production of infected and control isolates indicated that prophage stability may play a role in survival of *B. pseudomallei* as addition of bacteriophage from NCTC13178 restored lysogenic stability to NAFC in several cases.

Of the expected 55.1kb genome size from BupsΦ1, 51.3kb was sequenced with 40.9kb of this confirmed as bacteriophage. The open reading frames were determined using ORF finder and direct analysis. These open reading frames were analysed by BLASTx for putative function and several potential virulence genes were identified, as were structural, replication and lysogeny genes.

Possible virulence genes include putative anaerobic dehydrogenase and oxidoreductase genes. Putative structural genes included the terminase large subunit, portal protein, head morphogenesis, tail assembly and tail fibre genes. Putative replication and lysogeny genes included transposases, insertion elements and integrase, an RNA polymerase sigma subunit, DNA cytosine methylase, Holliday junction resolvase, repressor protein, and a weak match to *cro*, the gene responsible for triggering lysis.

Two genes of interest, the endolysin gene and a possible ADP-ribosyltransferase gene (a gene often involved in virulence) were not identified by BLASTx analysis. Techniques designed to identify genes with limited amino acid homology across species, such as identification of conserved amino acid pattern, chemo-physical comparison and phylogenetic tree analysis including bootstrap scoring, were then used to identify several open reading frames which were possible matches to these previously unidentified genes.

The endolysin of BupsΦ1 was extracted under nine combinations of conditions from literature , using a natural host system (*B. pseudomallei* #4). EDTA was found to aid lysis, while chloroform was found to have no effect. Extracts were concentrated using Centricons™ and both neat and concentrated extracts were tested for their ability to lyse both killed and live *B. pseudomallei* #4 in broth and plate format.

Neither the extracted endolysin nor its concentrate was found to lyse any of the *B. pseudomallei* in a form not attributable to live bacteriophage. Hence endolysin was determined not to function "from without" against *B. pseudomallei*. As such, this possibility for treatment of *B. pseudomallei* was eliminated.

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LIST OF ABBREVIATIONS

aa	amino acid
ADP	adenosine diphosphate
ADP-RT	ADP- ribosyltransferase
BHIB	brain heart infusion broth
bp	base pair
BupsΦ1	*Burkholderia pseudomallei* bacteriophage 1, from isolate NCTC 13178
BV	bacterial vaginosis
C14-NAD	carbon-14 nicotinamide adenine dinucleotide
cos	cohesive
cfu	colony forming unit
CIAP	calf intestinal alkaline phosphatase
CTAB	hexadecyltrimethyl ammonium bromide
ddH_2O	deionised water
DNA	deoxyribonucleic acid
DNase 1	deoxyribonuclease 1
dNTP	PCR nucleotide mix (deoxynucleotide triphosphate)
DTT	dithiothreitol
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EF-2	elongation factor two
EHEC	enterohaemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
g	gravity
GI	genetic island
ICTVdB	The Universal Database of the International Committee on Taxonomy of Viruses
ID_{50}	50% endpoints of infectious dose
int	integrase
IPTG	isopropyl-β-D-thiogalactopyranoside
JCU	James Cook University
kb	kilobase
LB	Luria Bertani
Mb	megabase
mwt	molecular weight
N.P.V.	negative predictive value
O.D.	optical density

ORF	open reading frame
pac	packaging
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PC3	physical containment level three
PEG	polyethylene glycol
PFGE	pulse field gel electrophoresis
pfu	plaque forming unit
PI	pathogenicity island
P.P.V.	positive predictive value
rpm	revolutions per minute
RNA	ribonucleic acid
RNase A	ribonuclease A
SBA	sheep blood agar
SDS	sodium dodecyl sulphate
SLT	shiga-like toxin
STS	serine-threonine-serine
TAE	tris acetate
TBE	tris borate
T.E.M.	transmission electron microscopy
Tm	melting temperature
tRNA	transfer ribonucleic acid
TSB	tryptone soya broth
TTSS	type three secretion system
UV	ultraviolet
UV-C	ultraviolet radiation at 254nm
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Xis	excisase