

**Molecular analysis of the role of
polynucleotide phosphorylase
in the virulence of
*Dichelobacter nodosus***

Suresh Kumar Athiappan Palanisamy

B.Sc., (University of Madras),

M.Sc.,(Bharathidasan University)

A thesis submitted for the degree of Doctor of Philosophy of
the University of New England

June 2007

**“this is not the end;
this is not even the beginning of the end,
but this is perhaps the end of the
beginning”**

WINSTON CHURCHILL.

Preface

This thesis describes research undertaken at the University of New England, Armidale, under the supervision of Associate Professor Brian F. Cheetham. I certify that the substance of this thesis has not been submitted as a part of another degree, and is not currently being submitted for any other degree or qualification. I further declare that all sources cited and any help received in preparing this thesis have been acknowledged.

..... 

Suresh Kumar Athiappan Palanisamy

Acknowledgments

It is a great privilege for me to express my profound regard and deep sense of gratitude to my supervisor, Associate Professor Brian Cheetham, Molecular and Cellular Biology, University of New England, Armidale, NSW, Australia. His sagacity has impressed and enlightened me, and from the very first moment he made me welcome in his laboratory and allowed me to choose a topic for my dissertation and followed me through to its completion. His invaluable guidance, encouragement and constructive criticism throughout the project as well as his patience, have helped me considerably for which I express my gratitude to him.

I owe my sincere thanks to Associate Professor Margaret Katz, Molecular and Cellular Biology, University of New England, Armidale, NSW, Australia, for her suggestions and encouragement and for the use of her laboratory to complete part of my project presented in this thesis.

Very special thanks to Megan Sutherland and Jenifer Druitt for their support and technical assistance in the laboratory and encouragement in the preparation of my thesis. I also thank Dr. Heather Nonhebel, Dr. Paolo Tortora, Stella, Paul, Sue, Katrin, Kim and Kannan for their technical support and advice in the course of my study.

I am very appreciative of the services provided by the Dixon Library, University of New England, Armidale. I am also very thankful to all my friends for their assistance in the

collection reference articles and encouragement. I owe my thanks to the teaching and non-teaching staff in the McClymont Building and UNE Research Services for providing a friendly and helpful environment.

I also thank the University of New England for providing me with a UNERA-India scholarship, without which I would not have been able to pursue my PhD in Australia.

Above all, I am heavily indebted to my parents and my sister for giving me their sustained support and encouragement in completion of this work successfully.

Abbreviations

µg	microgram
µl	microliter
aa	amino acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	base pair/s
CTAB	cetratri methyl ammonium bromide
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxyriboculeotide triphosphate
dsDNA	double stranded DNA
EDTA	ethylene diamine tetra acetic acid
G6PD	glucose 6 phosphate dehydrogenase
GTP	guanosine triphosphate
IS	Insertion sequence
int	integrase
<i>kan^R</i>	kanamycin R
kbp	kilobasepair
MW	molecular weight
ng	nanogram
NSW	New South Wales
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PEP	Phosphoenol pyruvate
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
ser	serine
spp.	species
<i>tetM</i>	tetracycline M
tRNA	transfer RNA
UV	ultra violet
<i>vap</i>	virulence associated protein
<i>vrl</i>	virulence related locus
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Summary

Dichelobacter nodosus is the key causative agent of ovine footrot. *D. nodosus* strains display a range of virulence, from benign to virulent. In general, virulent strains secrete higher levels of thermostable proteases and have increased twitching motility compared to benign strains. Four integrated genetic elements have been identified, the *intA*, *intB*, *intC* and *intD* elements that integrate into *tRNA-ser* genes next to *pnpA* (polynucleotide phosphorylase) and *glpA* in *D. nodosus*.

PnpA acts as a global regulator of virulence in *Salmonella enterica* and GlpA is closely related to CsrA and RsmA, which act as global repressors of virulence in *Helicobacter pylori* and *Erwinia carotovora*. Whittle *et al.* (1999) proposed that genetic elements next to *glpA* and *pnpA* alter the expression of *glpA* and *pnpA*, thus regulating virulence in *D. nodosus*. The hypothesis that PnpA is a virulence repressor in *D. nodosus* was investigated by using a suicide plasmid containing two segments from *pnpA* flanking the *tetM* gene to produce *pnpA* knockouts in virulent and benign strains. Benign strains with *pnpA* knockouts had a significant increase in twitching motility, supporting the hypothesis that PnpA is a virulence repressor in *D. nodosus*. Complementation experiments confirmed that the increase in twitching motility was due to the *pnpA* knockout. Secreted proteases from virulent strains were found to be considerably more thermostable than proteases from benign strains, but the *pnpA* knockout in benign strains did not increase protease thermostability. A PnpA specific phosphorolytic activity assay showed that the virulent strains had a slightly lower level of PNPase activity than benign strains.

A qPCR assay was developed to measure mRNA levels from *pnpA* and *aprV2*, which encodes a thermostable protease. Differences in mRNA levels between strains were not large, and there was no correlation with virulence. However, there may be post-transcriptional regulation of *pnpA* and *aprV2* expression. Amino acid differences between both PnpA and AprV2 in benign and virulent strains were identified. Although these changes do not appear likely to alter the functions of PnpA and AprV2, their role in virulence should be investigated further.

Table of contents

Preface	iii
Acknowledgments	iv
Abbreviations	vi
Summary	vii
Table of contents	ix
Publications associated with this thesis	xvi
Chapter 1. Introduction	1
1.1. Ovine footrot.....	1
1.2. Pathology.....	3
1.3. Clinical signs.....	3
1.3.1. Virulent footrot (VFR)	4
1.3.2. Benign footrot (BFR).....	4
1.4. Characteristics of growth	5
1.5. Epidemiology and transmission	6
1.6. Virulence determinants of <i>D. nodosus</i>	6
1.6.1. Fimbriae	6
1.6.2. Extracellular proteases	8
1.7. Disease treatment and control.....	9
1.7.1. Foot-bathing	10
1.7.2. Antibiotic treatment	10
1.7.3. Vaccination	11

1.8.	Diagnosis and testing	13
1.8.1.	Foot scoring	13
1.8.2.	Clinical pathology.....	14
1.8.3.	PCR methods to detect ribosomal RNA	15
1.8.4.	Gelatin gel tests (Protease thermostability assay)	15
1.8.5.	Elastase test.....	16
1.8.6.	Zymograms.....	16
1.8.7.	Gene probing.....	17
1.8.8.	Conclusions on diagnosis	17
1.9.	Genomic islands in <i>D. nodosus</i>	17
1.9.1.	The <i>intA</i> element.....	17
1.9.2.	The <i>intB</i> element	19
1.9.3.	The <i>intC</i> element.....	19
1.9.4.	The <i>intD</i> element.....	20
1.9.5.	The <i>vrl</i> (Virulence-related locus)	20
1.9.6.	Bacteriophage DinoHI.....	21
1.10.	Putative global regulators of virulence in <i>D. nodosus</i>	22
1.10.1.	Polynucleotide Phosphorylase (PNPase).....	22
1.10.1.1.	Role of PNPase	22
1.10.1.2.	PNPase structural studies	23
1.10.1.3.	Other ribonucleases which perform similar functions to PNPase.....	26
1.10.1.4.	PNPase as a global regulator of virulence	27
1.10.2.	<i>glpA</i>	28
1.10.2.1.	Role of CsrA.....	28
1.10.2.2.	Structural studies of CsrA.....	29
1.10.2.3.	CsrA as global regulator of virulence.....	30
1.10.2.4.	RsmA	30
1.10.3.	<i>ssrA</i> (small stable RNA)	32
1.11.	Model for virulence.....	33

1.12.	Proposed Project.....	35
Chapter 2. Materials and Methods.....		36
2.1.	Sources of chemicals.....	36
2.1.1.	Commercial kits used.....	38
2.2.	Bacterial strains and culture.....	39
2.2.1.	<i>Dichelobacter nodosus</i> strains.....	39
2.2.2.	<i>Escherichia coli</i> strains and media.....	40
2.3.	Preparation of DNA.....	40
2.3.1.	Preparation of <i>D. nodosus</i> genomic DNA.....	40
2.3.2.	Preparation of plasmid DNA.....	41
2.4.	Construction of plasmids.....	42
2.5.	Bacterial transformation.....	43
2.5.1.	Transformation of <i>E. coli</i> strain DH5 α	43
2.5.2.	Transformation of <i>D. nodosus</i> strains.....	43
2.6.	Agarose gel electrophoresis.....	44
2.7.	Southern blotting.....	45
2.7.1.	DNA Probe preparation.....	45
2.7.2.	Southern blot preparation.....	45
2.8.	Design of primers for PCR and DNA sequencing.....	47
2.9.	DNA sequencing.....	47
2.10.	Polymerase chain reaction (PCR).....	47
2.11.	Preparation of dialysis tubing.....	48
2.12.	PNPase assay.....	48
2.13.	<i>D. nodosus</i> protease thermostability assay.....	49
2.14.	Twitching motility assay.....	50

2.15.	RNA isolation and RNA gels.....	51
2.15.1.	RNA isolation and analysis	51
2.15.2.	RNA gels	51
2.16.	Real time PCR.....	52
2.16.1.	Construction of standard plasmids and calculation of plasmid copy number	52
2.16.2.	Real time PCR conditions	53
 Chapter 3. Producing <i>pnpA</i> knockouts in <i>D. nodosus</i>		54
3.1.	Introduction	54
3.1.1.	Construction of gene knockouts in <i>D. nodosus</i>	54
3.1.2.	Prior work.....	55
3.1.3.	Strains chosen for the knockout experiments	56
3.2.	Results.....	61
3.2.1.	<i>pnpA</i> structure prediction	61
3.2.2.	Sequencing of pCF5.....	62
3.2.3.	Identification of <i>D. nodosus</i> strains by Southern blotting	65
3.2.4.	Transformation of <i>D. nodosus</i> strains A198, C305 and 819 with pCF5	67
3.2.5.	Transformation of <i>D. nodosus</i> strain 1493 with pCF5.....	72
3.2.6.	Transformation of <i>D. nodosus</i> strain 2483 with pCF5.....	74
3.2.7.	Transformation of <i>D. nodosus</i> strains UNE61 and UNE64 with pCF5.....	76
3.2.8.	Summary of the transformation results.....	77
3.3.	Discussion	78
 Chapter 4. Analysis of PNPase, extracellular proteases and twitching motility in <i>D. nodosus</i>		80
4.1.	Introduction	80

4.2.	Results.....	80
4.2.1.	PNPase assay	80
4.2.1.1.	PNPase assay using a commercial enzyme	80
4.2.1.2.	Development of PNPase activity for <i>D. nodosus</i> extracts	82
4.2.1.3.	PNPase activity of virulent and benign strains	83
4.2.1.4.	PNPase activity in <i>pnpA</i> knockout strains	84
4.2.2.	Protease thermostability assay	87
4.2.3.	Twitching motility assay	92
4.3.	Discussion	95
4.3.1.	PNPase assays	95
4.3.2.	Protease thermostability assays	96
4.3.3.	Twitching motility assays	97
4.3.4.	Conclusion	97

Chapter 5. Complementation of the *D. nodosus* strain 2483 *pnpA*

knockout.....	98	
5.1.	Introduction	98
5.2.	Results.....	98
5.2.1.	Construction of a <i>pnpA</i> complementation plasmid, pSK81	98
5.2.2.	Sequence analysis of the complementation plasmid (pSK81)	100
5.2.3.	Sequencing analysis of part of the <i>pnpA</i> gene in benign and virulent <i>D. nodosus</i> strains.....	103
5.2.4.	Transformation and Southern blot analysis of 2483 <i>pnpA</i> knockout strains with pSK81.....	106
5.2.5.	Twitching motility assays	111
5.3.	Discussion	113
5.3.1.	Effect of <i>pnpA</i> knockouts on competence	113

5.3.2. Significance of variation in PnpA sequence	114
---	-----

Chapter 6. Development of a quantitative PCR assay for *pnpA* and *aprV2* mRNA in *D. nodosus* 116

6.1. Introduction	116
6.1.1. Reverse-transcriptase quantitative PCR (RT-qPCR)	117
6.2. Results.....	118
6.2.1. Construction of an internal standard (pSKrR1 - 16S rRNA plasmid) for the standard curve.....	118
6.2.2. Plasmid standards for <i>pnpA</i> and <i>aprV2</i>	120
6.2.3. Design of probes and primers for RT-qPCR	120
6.2.4. Standard curve analysis for 16S rRNA	121
6.2.5. RNA isolation and quantitation.....	125
6.2.6. Expression analysis of 16S rRNA in the experimental strains.....	126
6.2.7. Expression analysis of <i>pnpA</i>	128
6.2.8. Measurement of <i>aprV2</i> levels in <i>D. nodosus</i>	130
6.2.9. Sequence analysis of <i>aprV2</i> and <i>aprB2</i> genes from virulent and benign experimental strains.....	133
6.2.10. Protein structure prediction analysis of <i>aprV2</i> and <i>aprB2</i> protein from virulent and benign strains.....	134
6.3. Discussion	138

Chapter 7. Discussion and future directions..... 140

7.1. Model for virulence.....	140
7.2. Differences in PNPase activity between strains.....	140
7.3. Effect of <i>pnpA</i> knockout on twitching motility	141

7.4.	Effect of <i>pnpA</i> knockout on protease thermostability	142
7.5.	Expression of <i>pnpA</i> mRNA	143
7.6.	Expression of <i>aprV2</i> mRNA	144
7.7.	Mutations in PnpA.....	144
7.8.	Mutations in AprV2.....	145
7.9.	Lineage of benign strains	145
7.10.	Modification of integrated elements in strains	146
7.11.	Conclusions.....	146
References.....		148
Appendix A - Oligonucleotides.....		174
Appendix B - qPCR data		176

Publications associated with this thesis

Manuscript in preparation

Suresh Kumar, A. P., Fletcher. C., Hyman, J., Katz, M. E., and Cheetham, B. F. The C-terminal deletion of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of *Dichelobacter nodosus*, the causative agent of ovine footrot.

Conference abstracts

Suresh Kumar Athiappan Palanisamy, Carrie Fletcher, Jessica Hyman, Margaret E Katz, and Brian F Cheetham. The putative global regulator gene of virulence, polynucleotide phosphorylase regulates the twitching motility: one of the virulence characteristics of *Dichelobacter nodosus*. Australian Society for Microbiology, Adelaide, South Australia. 2007.

Brian F Cheetham, Suresh K. A. Palanisamy, Carrie Fletcher, Dale Kenny, Warwick Locke, Jessica Hyman and Margaret E. Katz. Regulation of virulence gene networks by integration of mobile genetic elements. European Science Foundation – EMBO Symposium, Costa Brava, Spain. 2006.

Suresh Kumar Athiappan Palanisamy, Carrie Fletcher, Margaret E Katz, and Brian F Cheetham. The effect of polynucleotide phosphorylase knockouts on virulence characteristics of *Dichelobacter nodosus*. Australian Society for Biochemistry and Molecular Biology, Brisbane, Queensland. 2006.

Suresh Kumar Athiappan Palanisamy, Carrie Fletcher, Margaret E Katz, and Brian F Cheetham. The role of polynucleotide phosphorylase in the virulence of *Dichelobacter nodosus* the causative agent of footrot. Australian Society for Microbiology, Canberra, ACT, Australia. 2005.