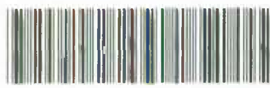
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Molecular analysis of the virulence-associated protein regions and other genetic elements in the genome of *Dichelobacter nodosus*

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Preface

This thesis describes research undertaken at the University of New England, Armidale, under the supervision of Dr 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.

. . . .

Gabrielle Whittle

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Abbreviations

aa	amino acids
ABC	ATP-binding cassette
AHL	acyl-homoserine-lactone
askA	aspartokinase A
ATP	adenosine triphosphate
att	bacteriophage attachment site
BPD	binding-protein dependent
bp	base pairs
cDNA	complimentary DNA
CSPD®	Disodium 3-(4-Methoxyspiro{1,2-dioxetane-3,2-(5-chloro)tricyclo [3.3.1.1 3,7] decan}-4-yl) phenyl phosphate
CTAB	cetyltrimethylammoniumbromide
dap	*intD* element-associated protein
dATP	deoxyadenosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
gep	genetic element-associated protein
glpA	global repressor protein A
HSL	homoserine lactone
int	integrase
IS	insertion sequence
KAc	potassium acetate
kb	kilobases
LPS	lipopolysaccharide
MW	molecular weight
nt	nucleotides
OMP	outer membrane protein
ORF	open reading frame
Pais	pathogenicity islands
PCR	polymerase chain reaction
PEG8000	polyethylene glycol 8000
pfu	plaque forming units
pnpA	polynucleotide phosphorylase
RNA	ribonucleic acid
RNase A	ribonuclease A
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
spp.	species
sMQH_2O	sterile millique water
TE	tris-EDTA
TEMED	*N, N, N, N*-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
Tween-20	polyoxyethylenesorbitan mono-laurate
UV	ultraviolet
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside
vap	virulence-associated protein
vrl	virulence-related locus

Glossary

Bacteriophages (phages) are viruses that infect bacterial hosts. Bacteriophage have a wide variety of sizes, shapes and lifestyles. Some bacteriophage contain small single stranded genomes (either DNA or RNA), some have a few as four genes whilst others like have more than 200 genes. Although some are completely virulent and result in the lysis of all cells they invade (*E. coli* bacteriopnage T4), others are temperate (*E. coli* bacteriophage λ)and integrate into the host genome. Temperate bacteriophage are known to affect the antigenicity and pathogenicity of hosts they lysogenize (Campbell, 1996).

Conjugative transposons are discrete DNA segments that in general encode antibiotic resistance determinants, and have the ability to translocate between the chromosomes of different cells without the need of another genetic element such as a plasmid (Clewell *et al.*, 1995; Salyers *et al.*, 1995a; Salyers *et al*, 1995b). These elements encode functions involved in their excision, conjugal transfer and integration into the target chromosome, and occur in both Gram positive and Gram negative bacteria. In addition, conjugative transposons are known to be able to mobilise co-resident plasmids and other unlinked genetic elements in *cis* and/or *trans*. They are transposon-like in that they excise and insert into DNA, but are plasmid like in that they form a covalently closed circular transfer intermediate, which is not thought to undergo replication prior to transfer (Clewell *et al.*, 1995; Salyers *et al.*, 1995a; Salyers *et al*, 1995b).

Integrons consist of a 5'-conserved region (1.4 kb), a central variable region and a 3' -conserved region (Stokes & Hall, 1989). The integrase genes that are carried by these elements are located in the 5'-conserved region and are responsible for the insertion, deletion and rearrangement of the variable region, which typically contains one or more genes, which share the same orientation, and are often antibiotic resistance genes. (Hall, *et al.*, 1991; Collis & Hall, 1992). Integrons are often associated with the antibiotic resistance genes commonly found on both plasmids and transposons of Gram negative bacteria (Stokes & Hall, 1989).

Plasmids are autonomously replicating extrachromosomal molecules of less than chromosomal size (Birge, 1994). Although most of the plasmids that have been discovered are covalently closed circular molecules, there are also plasmids that are linear and have telomeric structures analogous to eukaryotic chromosomes (Dorman, 1994). Some plasmids contain transfer (*tra*)genes which encode proteins responsible for sex-pilus mediated conjugative transfer from one bacterial host cell to another, and are hence referred to as self-transmissible or conjugative plasmids . Other plasmids are not self-transmissible, but instead encode mobilisation (*mob*) genes which allow the *mob* encoding plasmid to be transferred if an appropriate conjugative element is present in the same host cell. Plasmids have been at the forefront of microbiological research for many years and this is primarily attributable to their ability to carry and disseminate genes specifying resistance to antibiotics, and genes which have a role in virulence. however there are many other mobile genetic elements which also contribute to the spread of antibiotic resistance and pathogenesis including bacteriophage, transposons, conjugative transposons, integrons and pathogenicity islands.

Pathogenicity islands (Pais) are distinct genetic units that are present in pathogenic strains but absent or rarely present in non-pathogenic variants of the same or related species (Hacker *et al.,* 1997). Pais tend to be large (up to 200 kb in size) and often carry more than one gene encoding a virulence factor and tend to have a G + C content that differs from that of the host organism which is thought to be related to their horizontal acquisition. Pais are often integrated into the 3'-end of tRNA genes, carry an integrase gene, and are often flanked by direct repeats or insertion sequences. Most but not all of these regions tend to be unstable (Hacker et al, 1997; Mescas et al., 1996).

Transposons are discrete genetic units which typically carry genes that encode transposase functions, which catalyse their movement between non-homologous positions in the bacterial genome, although the details of their structures and mechanisms of transposition vary greatly. The contributions of transposons to the rearrangement of bacterial genomes has been well documented (Dorman, 1994) as is their association with antibiotic resistance genes found on plasmids and on other prokaryotic genetic elements (Craig, 1996).

Summary

Dichelobacter nodosus is the principal causative agent of ovine footrot, a mixed bacterial infection of the hoof. *D. nodosus* strains are classified as benign, intermediate or virulent depending upon the severity of disease they cause in sheep. Previous work has led to the identification of a virulence-associated DNA region in *D. nodosus* virulent strain A198, called the *vap* element which may have a role in virulence. However, there is no transformation system for *D. nodosus* which precludes direct testing of the role of these and other genes in virulence. In this work, a native *D. nodosus* plasmid pDN1 was isolated, sequenced, characterised and modified to contain appropriate antibiotic resistance markers and a multiple cloning site. Derivatives of pDN1 were subsequently used in transformation experiments in an effort to develop a transformation system for *D. nodosus*.

In the absence of a transformation system, more indirect methods were utilised to determine whether the *vap* genes of *D. nodosus* have a role in virulence. Past investigations have concentrated on analysis of the *vap* genes at the DNA level. In this work, northern blot experiments were undertaken to determine whether the *vap* genes are expressed at the RNA level in the virulent and/or benign strains in which they are present. Results indicated that in general, the *vap* genes are expressed in the virulent and benign strains in which they are present, and although differential expression of the *vap* genes was observed, the differences observed were not related to the virulence of the isolates.

In addition to the *vap* element, part of an *intB* element was previously identified. In an effort to further characterise the *intB* element, chromosome walking 4.2 kb downstream of regions which were determined previously was undertaken. Results suggest that these downstream sequences, which include genes *gepC-gepG*, are not part of an integrated genetic element.

In addition, Southern blot analysis was utilised in order to determine the prevalence, arrangement and the integrity of the *intB* element in seventeen strains of *D. nodosus*.

A sequence previously separating the *pnpA* gene and $intB_N$ was lost from, or moved position in, the original laboratory strain of C305 (C3051), to generate the current laboratory strain of C305 (C3052). In order to investigate the nature of these sequences that separated *pnpA* and $intB_N$ in strain C3051, a sequence of 3.7 kb immediately upstream of $intB_N$ in strain C3051 was determined, and led to the identification of a mobilisable and possibly conjugative plasmid or transposon, that has been called the *intD* element. The presence of these *intD* element sequences in seventeen different strains of *D. nodosus* was determined.

The presence of a new genetic element, called the *intC* element, was postulated previously. In this work, Southern blot analyses and PCR experiments were used to confirm that *intC*, *orf242*, *orf171*, *vapG"* and *vapH"* are part of an *intC* element, and to examine the prevalence of *intC* element sequences in seventeen different strains of *D. nodosus*. The spontaneous loss of the *intC* and the *intD* element in one strain of *D. nodosus* was observed to result in the loss of protease thermostability, a virulence factor in *D. nodosus*. Consequently, the integration sites for the *vap*, *intB* and *intC* elements were investigated in seventeen strains of *D. nodosus*. These results suggest that, in *D. nodosus*, virulence may be modulated by the site-specific integration of genetic elements.

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