

# **INTEGRONS IN PSEUDOMONADS ARE ASSOCIATED WITH HOTSPOTS OF GENOMIC DIVERSITY.**

Neil Wilson BSc. (Hons)

Department of Biological Sciences,  
Macquarie University, Australia.



Thesis submitted: August 2007

A thesis submitted for fulfilment of the requirements of the degree  
of Doctor of Philosophy

# TABLE OF CONTENTS

<b>SYNOPSIS.....</b>	<b>VII</b>
<b>STATEMENT OF CANDIDATE .....</b>	<b>IX</b>
<b>ACKNOWLEDGMENTS .....</b>	<b>X</b>
<b>ABBREVIATIONS .....</b>	<b>XIII</b>
<b>CHAPTER 1 – LITERATURE REVIEW .....</b>	<b>- 1 -</b>
<b>1.1 – Bacterial Diversity .....</b>	<b>- 1 -</b>
<b>1.2 - The nature of genetic organisation .....</b>	<b>- 2 -</b>
<b>1.3 – Origins of Genetic Novelty.....</b>	<b>- 3 -</b>
<b>1.4 – Genetic modularisation .....</b>	<b>- 6 -</b>
<b>1.5 – Operons .....</b>	<b>- 7 -</b>
1.5.1 – Operon Evolution .....	- 8 -
<b>1.6 – Integrons.....</b>	<b>- 11 -</b>
1.6.1 – Background .....	- 11 -
1.6.2 – Core Integron Distribution and Diversity .....	- 16 -
1.6.3 – Integrons and Genomic Context .....	- 27 -
<b>1.7 – The mobile integron paradigm .....</b>	<b>- 31 -</b>
<b>1.8 – The generalised chromosomal integron .....</b>	<b>- 34 -</b>
1.8.1 – Limitations of the generalised CI concept.....	- 38 -
<b>1.9 - The need for model organisms to characterise CIs .....</b>	<b>- 40 -</b>
1.9.1 – <i>Pseudomonas</i> spp. as a model organism .....	- 40 -
<b>1.10 – The genus <i>Pseudomonas</i> .....</b>	<b>- 42 -</b>
1.10.1 – A brief history of genus <i>Pseudomonas</i> .....	- 42 -
1.10.2 – The <i>Ps. stutzeri</i> species complex.....	- 42 -
1.10.3 – Relationships between <i>Ps. stutzeri</i> genomovars .....	- 44 -
1.10.4 – <i>Ps. stutzeri</i> distribution and ecology .....	- 45 -

1.10.5 - By what mechanisms do genomovars diverge?.....	46 -
1.10.6 - A role for integrons in the diversification of <i>Ps. stutzeri</i> genomovars.....	46 -
<b>1.11 – Aims of the present study .....</b>	<b>48 -</b>
<b>CHAPTER 2 – GENERAL MATERIALS AND METHODS.....</b>	<b>50 -</b>
<b>2.1 – Bacterial Strains .....</b>	<b>50 -</b>
<b>2.2 – DNA Extraction.....</b>	<b>52 -</b>
<b>2.3 – PCR.....</b>	<b>52 -</b>
<b>2.4 – Agarose Gel Electrophoresis.....</b>	<b>53 -</b>
<b>2.5 – Southern Hybridisation .....</b>	<b>53 -</b>
<b>2.6 – Preparation of PCR Products and plasmid DNA for Sequencing .....</b>	<b>54 -</b>
<b>2.7 – Sequence Analysis .....</b>	<b>55 -</b>
<b>CHAPTER 3 – CHARACTERISATION OF STRAIN COLLECTION.....</b>	<b>57 -</b>
<b>3.1 – Introduction .....</b>	<b>57 -</b>
<b>3.2 – Materials and methods .....</b>	<b>64 -</b>
3.2.1 – BOX-PCR.....	64 -
3.2.2 – Analysis of BOX-PCR profiles .....	64 -
3.2.3 – 16S rDNA and 16S-23S IGS PCR.....	65 -
3.2.4 – RFLP analysis of 16S rDNA and IGS1 sequences.....	65 -
3.2.5 – Sequence Analysis.....	66 -
<b>3.3 – Results .....</b>	<b>69 -</b>
3.3.1 – BOX-PCR .....	69 -
3.3.2 – Analysis of 16S rRNA gene RFLP profiles.....	72 -
3.3.3 – Analysis of IGS1 RFLP profiles .....	74 -
3.3.4 – Confirmation of strain independence and provenance .....	76 -
3.3.5 – 16S rRNA gene phylogeny .....	78 -
3.3.6 – 16S-23S IGS phylogeny.....	82 -
<b>3.4 – Conclusions .....</b>	<b>86 -</b>
<b>CHAPTER 4 – DISTRIBUTION OF INTEGRONS AND GENE CASSETTES IN <i>PSEUDOMONAS</i> .....</b>	<b>89 -</b>

<b>4.1 - Introduction.....</b>	<b>- 89 -</b>
<b>4.1 - Materials and methods .....</b>	<b>- 95 -</b>
4.2.1 - PCR screening for integrons.....	- 95 -
4.2.2 – Probe design for integron detection .....	- 98 -
4.2.3 – Southern hybridization .....	- 100 -
4.2.4 – Sequence Analysis.....	- 101 -
4.2.5 – Public Database Searches .....	- 101 -
<b>4.3 – Results .....</b>	<b>- 103 -</b>
4.3.1 – PCR detection of integrons .....	- 103 -
4.3.2 – Southern hybridisation integron detection – Core Integron .....	- 106 -
4.3.3 – Southern hybridisation integron detection – 59-be .....	- 110 -
4.3.4 – Analysis of Recovered integrons .....	- 114 -
4.3.4.1 – Analysis of recovered <i>intl</i> genes.....	- 114 -
4.3.4.2 – Analysis of recovered <i>attI</i> regions .....	- 118 -
4.3.4.3 – Putative identification of $P_c$ .....	- 120 -
4.3.4.4 - Putative identification of <i>intl</i> regulatory sequences .....	- 122 -
4.3.4.5 – Analysis of Gene Cassettes.....	- 124 -
4.3.5 – <i>in silico</i> analysis of <i>Pseudomonas</i> family integron distribution .....	- 124 -
<b>4.4 – Conclusions .....</b>	<b>- 126 -</b>
 <b>CHAPTER 5 – GENOMIC CONTEXT OF <i>PSEUDOMONAS</i> INTEGRONS .....</b>	 <b>- 131 -</b>
<b>5.1 - Introduction.....</b>	<b>- 131 -</b>
<b>5.2 - Materials and methods .....</b>	<b>- 135 -</b>
5.2.1 – Summary of cloning, detection and sequencing strategy.....	- 135 -
5.2.2 - Analysis of gene cassettes .....	- 136 -
5.2.3 - Sequence Analysis.....	- 136 -
5.2.4 - Phylogenetic Analysis .....	- 138 -
<b>5.3 – Results .....</b>	<b>- 139 -</b>
5.3.1 – Recovery of addition integron sequences.....	- 139 -
5.3.1.1 – Extension of existing integron sequences .....	- 139 -
5.3.1.2 – Characterisation of <i>Ps. stutzeri</i> 19SMN4 (4A) and DNSP21 (5A) integrons.....	- 140 -
5.3.1.3 – Characterisation of <i>Ps. stutzeri</i> Gv.2 integrons .....	- 144 -
5.3.1.4 - Characterisation of additional integrons / cassette arrays .....	- 144 -
5.3.1.5 – Analysis of recovered gene cassettes.....	- 148 -
5.3.2 - Recovery and analysis of integron flanking genes .....	- 152 -
5.3.2.1 – Characterisation of integrons at locus 1 .....	- 158 -

5.3.2.2 – Characterisation of integrons at locus 2 .....	- 159 -
5.3.3 - Phylogenetic analysis of integron flanking genes .....	- 164 -
5.3.4 - Phylogenetic analysis of <i>intl</i> relative to genomic context .....	- 166 -
5.3.5 - Synteny and ancestry of integron loci across <i>Pseudomonas</i> spp. ....	- 172 -
5.3.6 - Analysis of integron boundaries .....	- 178 -
5.3.7 - Detection of a CI in the genome sequence of <i>Ps. mendocina</i> YMP .....	- 183 -
<b>5.4 - Conclusions .....</b>	<b>- 192 -</b>
 <b>CHAPTER 6 - EVOLUTIONARY ANALYSIS OF <i>PSEUDOMONAS</i> SPP. INTEGRONS-</b>	<b>199 -</b>
<b>6.2 - Materials and methods .....</b>	<b>- 202 -</b>
6.2.1 – Genomic Analyses and Codon Usage Analyses .....	- 202 -
6.2.2 – Statistical analysis of codon usage data .....	- 202 -
<b>6.3 – Results .....</b>	<b>- 203 -</b>
6.3.1 – G + C content analysis .....	- 203 -
6.3.2 – Codon Usage Analysis .....	- 206 -
6.3.2.1 – Analysis of relative codon usage data using PCA and CA .....	- 208 -
6.3.2.2 – Assessment of <i>Ps. aeruginosa</i> as a surrogate for codon usage in <i>Ps. stutzeri</i> .....	- 209 -
6.3.2.3 – Codon usage of <i>Pseudomonas</i> spp. CIs relative to chromosomal genes .....	- 212 -
<b>6.4 – Conclusions .....</b>	<b>- 215 -</b>
 <b>CHAPTER 7 – FINAL DISCUSSION .....</b>	<b>- 218 -</b>
7.1 – Integron acquisition and loss in <i>Pseudomonas</i> spp.....	- 219 -
7.2 – Integrons are associated with hotspots for recombination .....	- 222 -
7.3 – Implications for the concept of the generalised CI.....	- 224 -
7.4 – Future Work .....	- 231 -
 <b>APPENDIX 1 – FOSMID LIBRARY CONSTRUCTION AND SCREENING .....</b>	<b>- 233 -</b>
<b>A.1 - Introduction .....</b>	<b>- 233 -</b>
<b>A.2 - Materials and methods.....</b>	<b>- 233 -</b>
A.2.1 - Fosmid library construction.....	- 233 -
A.2.2 - Estimation of library coverage.....	- 233 -
A.2.3 - Colony hybridisation.....	- 234 -
A.2.4 - Fosmid purification.....	- 235 -
A.2.5 - Sequencing of fosmid clones .....	- 236 -

A.2.6 - PCR recovery of additional integron boundary sequences .....	- 238 -
A.2.7 - Hybridisation screening for integron flanking genes.....	- 239 -
<b>A.3 - Results.....</b>	<b>- 241 -</b>
A.3.1 - Construction of fosmid libraries and estimation of genomic coverage.....	- 241 -
A.3.2 - Screening fosmid clone libraries for integron sequences.....	- 244 -
A.3.3 - Recovery of complete integrons and flanking sequences .....	- 248 -
A.3.4 - PCR recovery of additional integron boundary regions .....	- 249 -
A.3.4.1 - Recovery of 5' boundary regions .....	- 249 -
A.3.4.2 - Recovery of 3' boundary from <i>Ps. stutzeri</i> BAM17 .....	- 250 -
A.3.5 - Screening strain collection for integron flanking genes .....	- 251 -
A.3.6 – Screening fosmid libraries for integron flanking genes .....	- 254 -
<b>A.4 - Conclusions .....</b>	<b>- 255 -</b>
<b>REFERENCES.....</b>	<b>- 257 -</b>

## SYNOPSIS

Integrans associated with mobile genetic elements have played a central role in the emergence and spread of multiple antibiotic resistance in many pathogenic bacteria. However, the discovery of integrans in the chromosomes of diverse, non-pathogenic bacteria suggests that integrans have a broader role in bacterial evolution. The *Pseudomonas stutzeri* species complex is a well studied model for bacterial diversity. Members of the complex are genetically closely related, but sub-taxa are not able to be defined by exclusively shared sets of phenotypic characters. Rather, on the basis of total DNA:DNA similarity, *Ps. stutzeri* strains have been divided into 17 different groups (termed genomovars). Two *Ps. stutzeri* strains have been found to contain Chromosomal Integrans (CIs). This thesis involved exploration of the hypothesis that a CI was present in the common ancestor of the *Ps. stutzeri* species complex and assessed the impact of integrans on diversity across all Pseudomonads. The history and significance of integrans is discussed in Chapter 1 as part of a literature review, and general materials and methods are provided in Chapter 2. Chapters 3 – 6 comprise the sections in which data generated during my PhD project are presented. A comprehensive analysis of the relationships between the strains being analysed is presented in Chapter 3. In Chapter 4, results of PCR and hybridisation screening for integrans across the strain collection are presented. In Chapter 5 the recovery of additional integrans and in depth sequence analysis of the recovered integrans are described. Finally, Chapter 6 contains statistical analyses of integron-associated genes and Chapter 7 contains a final discussion the most significant findings. Twenty-three *Pseudomonas* spp. strains were screened for the presence of integrans. All but three were found to contain integron-like sequences; however, most integron sequences recovered contained inactivated core integrans.

Despite having a chromosomal locus, integrons in *Pseudomonas* were found to have properties indicative of frequent horizontal transfer. Evidence was also obtained which suggests that integrons have been acquired at the same locus on multiple independent occasions. This has not been observed in other families of chromosomal integrons and suggests that the loci at which integrons in *Pseudomonas* are found are hotspots for recombination.



## **STATEMENT OF CANDIDATE**

This work is original and has not been submitted for a higher degree to any other university or institution. The work of others, when drawn upon, is referenced fully.

Approximately 50% of the DNA sequencing performed in Chapter 5 was performed by another researcher. All analyses of DNA sequence data presented in this thesis were performed by the author.

Signed

Neil Wilson

Date

## **ACKNOWLEDGMENTS**

To my beautiful girlfriend Linh, thanks most of all just for being there for me (and generally putting up with me!). Thanks for your patience while I was writing-up. Thanks for listening and being interested in my nerdy stories and for getting excited when I get a good result, even if it is just a band on a gel! Thanks for sharing the good times and for listening to the whinges in the not so good times. It is great to have a partner that shares my passions. It's been great to be able share my life and my passion for science with you.

To my parents, Frank and Lesley, before anyone else I would not have been able to accomplish this without you both. I can't thank you enough for all the support you have given me. From making sure I received a good education to the unwavering moral and financial support you provided. You have both been great parents and I am eternally grateful. To my Mum Lesley in particular, thank you for putting a roof over my head throughout my PhD and for providing such a great living environment for me. You have always believed so strongly in me; I may not have had the confidence to do this without that. Also, the endless stream of great coffee that you made sure flowed was absolutely crucial to the thesis writing effort. Thanks also for helping in the printing and in proof-reading my thesis for typos; that can't have been much fun and there were plenty of them!

To Kathy and Jane, thanks for being great big sisters. I have always looked up to you both. Thanks for always being more or less nice to a younger brother who was only very occasionally annoying! To Jane in particular, thanks for being interested in my science

stories and theories, and for leaving me alone (most of the time!) when I needed to study for weeks on end. Thanks most for helping to fight for my right to be a cool science nerd!

Thank you to my friends for sticking by me, for understanding why I need to work on weekends at times, for tolerating my warped sense of punctuality, and for finding science-nerdiness amusing! I really am lucky to have such a great bunch of mates.

To my supervisors, Michael Gillings and especially Andrew Holmes, I thank you for the endless guidance you given me throughout my PhD. I have learnt a huge amount from you both and it has been an honour to have each of you as a mentor. To Andy, thank you for always having your office door open to me. Your willingness to help and advice went above and beyond the call of duty. To Nick Coleman, you were a great mentor in the lab throughout my PhD. If you had not been in the lab, I would have had less fun, but more importantly I would have learned far less, generated far less data and not become as accomplished a scientist. Thank you.

Thank you to both Sasha and Claire being my fellow PhD lab buddies. The moral support, good fun and philosophical conversations were invaluable. We will forevermore share a weird bond, a mutual understanding of the pain we have endured! Seriously though, the value of having other people in the same boat should not be undervalued, especially when they are fun to work with too! It wouldn't have been the same without you guys. Good luck finishing up your respective theses, and whatever you do, don't take as long as I did!

Thank you to all the people in the Holmes lab at Sydney University and the EMMA lab at Macquarie University who have assisted me and/or shared a lab-space with me throughout my PhD. It is a pleasure to work each day with so many people who are

passionate about what they do. Thank you to Johannes Sikorski for supplying most of the *Pseudomonas* strains analysed. Thank you to Ruth Hall for helpful guidance throughout both the research and writing-up component of my PhD.

## **ABBREVIATIONS**

59-be – 59-base element

bp – Base pairs

CA – correspondence analysis

CI – chromosomal integron

CTAB – Hexadecyltrimethylammonium bromide

DIG-6-dUTP – digoxigenin-6-dUTP

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

Gv. – Genomovar

HGT – horizontal gene transfer

IGS1 – 16S-23S rDNA intergenic spacer

IntI – integron integrase

LB broth – Luria-Bertani broth

MI – mobile integron

PCA – principal components analysis

PCR – polymerase chain reaction

RFLP – Restriction fragment length polymorphism

RSCU – relative synonymous codon usage

SSC – Standard Saline Citrate

SDS – Sodium dodecyl sulfate

TBE buffer – Tris borate EDTA buffer

TE buffer – Tris EDTA buffer