Use of Non-mammalian Models to Assess the
Virulence of *Pseudomonas aeruginosa*

by

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*Pseudomonas aeruginosa* (P. aeruginosa) is the archetypical opportunistic pathogen that accounts for significant numbers of cases of hospital-acquired infections. It adapts to diverse environments in part because it may “modify” its genome through horizontal gene transfer (HGT). *Caenorhabditis elegans* (C. elegans) and *Acanthamoeba* spp. (*A. castellanii* and *A. polyphaga*) can be employed as *in vivo* surrogates to investigate the virulence associated with *P. aeruginosa* clinical isolates and with the presence of genomic islands, genetic elements acquired through HGT.

A PA14 based *P. aeruginosa* pathogenicity island (PAPI)-2 minus mutant exhibited only a minor reduction in virulence as measured by *C. elegans* survival using the slow-killing assay (SKA) model of virulence but significant attenuation of virulence was found as assessed by the expansion of *A. castellanii* but not *A. polyphaga* film on non-nutrient agar.

A *C. elegans* highthroughput assay (HTA) was also established as model of infection and validated against the SKA. The HTA was employed to screen a panel of *P. aeruginosa* mutants created by deletion of tRNA-associated genomic islands. Few mutants (5 out of 21) showed attenuated virulence toward *C. elegans* in HTA many of which were confirmed to be less virulent toward the nematode using SKA.

Screening of clinical isolates linked the strains isolated from blood culture of a patient with lower survival of *C. elegans* in HTA compared with strains isolated from sputum. Moreover, type III secretion system effector (T3SS) *exoU*-positive strains and strains developing pigments during growth were more virulent toward *C. elegans* in HTA than T3SS effector *exoS*-positive strains and pigment-less strains, respectively. Since pigment development was also linked to *exoU* and this gene has been associated with genomic islands integrated at *tRNA^{Lys10}*, it was inferred that these genomic islands may bear functions affecting *C. elegans* survival possibly enhancing iron scavenging.
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Dedication

To my grandmother, Lucia, for showing me what real fortitude is

And my nice, Emma, for giving me joy
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>3-oxo-C₁₂-HSL</td>
<td>N-3-oxo-dodecanoyl homoserine lactone</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADPRT</td>
<td>ADP ribosyl transferase</td>
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<tr>
<td>AHL</td>
<td>N-Acyl homoserine lactone</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BHI</td>
<td>brain-heart infusion broth</td>
</tr>
<tr>
<td>bp</td>
<td>base-pairs</td>
</tr>
<tr>
<td>C₄-HSL</td>
<td>N-butanoyl homoserine lactone</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>cyclic di-guanosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeat</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FKA</td>
<td><em>C. elegans</em> fast-killing assay</td>
</tr>
<tr>
<td>g</td>
<td>gravitational acceleration</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GI</td>
<td>genomic island</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HGT</td>
<td>horizontal gene transfer</td>
</tr>
<tr>
<td>HSI-I, -II, -III</td>
<td>haemolysin co-regulated protein secretion island I, II, III</td>
</tr>
<tr>
<td>HTA</td>
<td><em>C. elegans</em> highthroughput assay</td>
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<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>LA</td>
<td>Luria-Bertani agar</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>LES</td>
<td>Liverpool epidemic strain</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NNA</td>
<td><em>Acanthamoeba</em> non-nutrient agar</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAIs</td>
<td>pathogenicity islands</td>
</tr>
<tr>
<td>PAPI</td>
<td><em>Pseudomonas aeruginosa</em> Pathogenicity Island</td>
</tr>
<tr>
<td>PAR-1, -2, -3</td>
<td>protease-activated receptor-1, -2, -3</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGS</td>
<td>Peptone-glucose-sorbitol medium</td>
</tr>
<tr>
<td>PQS</td>
<td><em>Pseudomonas</em> quinolone signal</td>
</tr>
<tr>
<td>PYG</td>
<td>peptone-yeast extract-glucose medium</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>QS</td>
<td>quorum sensing</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Sec</td>
<td>general secretory pathway</td>
</tr>
<tr>
<td>SKA</td>
<td><em>C. elegans</em> slow-killing assay</td>
</tr>
<tr>
<td>st.dev.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>T1SS</td>
<td>type I secretion system</td>
</tr>
<tr>
<td>T2SS</td>
<td>type II secretion system</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
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<td>type VI secretion system</td>
</tr>
<tr>
<td>T7SS</td>
<td>type VII secretion system</td>
</tr>
<tr>
<td>Tat</td>
<td>twin-arginine translocation pathway</td>
</tr>
<tr>
<td>TD$_{50}$</td>
<td>time required to kill 50% of the nematodes</td>
</tr>
<tr>
<td>TD$_{m}$</td>
<td>average time-to-death of nematodes</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TPS</td>
<td>two-partner secretion</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptone soya agar</td>
</tr>
<tr>
<td>UKM</td>
<td>Universiti Kabangsaan Malaysia</td>
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<tr>
<td>UoL</td>
<td>University of Leicester</td>
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</tbody>
</table>
# Table of content

**List of Figures** ................................................................................................. 11

**List of Tables** ................................................................................................. 13

**Chapter 1: Introduction** ............................................................................. 15

*Pseudomonas aeruginosa* ............................................................................ 16

Clinical relevance ............................................................................................... 16

*P. aeruginosa* genomics ........................................................................... 17

Horizontal gene transfer ......................................................................................... 18

Components of the accessory genome ............................................................... 19

Integrative and conjugative elements .................................................................... 19

Replacement islands ............................................................................................. 21

Prophages and phage-like elements ..................................................................... 21

Transposons, insertion sequences and integrons .................................................. 22

**Virulence factors** .......................................................................................... 26

Secretion systems ................................................................................................. 26

Secreted enzymes associated with virulence ....................................................... 30

Proteases .............................................................................................................. 30

Lipases and phospholipases ................................................................................. 31

Enzymes leading to substrate modifications ....................................................... 32

Enzymes degrading bacterial components ......................................................... 33

*P. aeruginosa* cell surface components involved in virulence ......................... 34

Factors involved in iron recruitment ..................................................................... 35

Formation of biofilm ............................................................................................ 37

**Regulatory networks and cell-to-cell communication** ................................. 38

Quorum sensing .................................................................................................. 38

AHLs system ....................................................................................................... 39

PQS system ......................................................................................................... 39

Two-component systems ...................................................................................... 40

Nucleotide-based signals .................................................................................... 41

**Non-mammalian model organisms to study *P. aeruginosa*** .......................... 42

*Caenorhabditis elegans*, the multicellular model .................................................. 43

*C. elegans* anatomy and life cycle ...................................................................... 43

Importance of *C. elegans* as a model of infection .............................................. 46

Use of *C. elegans* to study *P. aeruginosa* infection ........................................ 49

Slow-killing assay ................................................................................................. 49

Fast-killing assay .................................................................................................. 50

Killing by paralysis assay ..................................................................................... 51
Chapter 2: Materials and Methods ................................................................. 61

Pseudomonas aeruginosa ........................................................................ 62
Collection of clinical isolates ...................................................................... 62
Maintenance of bacterial cultures ................................................................. 62
T3SS effector genes colony PCR assays ...................................................... 62

Caenorhabditis elegans assays ..................................................................... 70
Maintenance of C. elegans .......................................................................... 70
Slow-killing assay (SKA) ............................................................................ 70
Protocol used at University of Leicester (UoL) ........................................... 70
Protocol used at Universiti Kebangsaan Malaysia (UKM) ............................ 73
Fast-killing assay (FKA) ............................................................................. 76
Survival analysis ....................................................................................... 76
Highthroughput assay (HTA) .................................................................... 77

Acanthamoeba spp. assays .......................................................................... 82
Maintenance of Acanthamoeba cultures ..................................................... 82
Non-nutrient agar (NNA) assay ................................................................. 82

Chapter 3: Use of Acanthamoeba spp. and C. elegans infection models
revealed the involvement of PAPI-2 but not PAPI-1 in P. aeruginosa
virulence ...................................................................................................... 86

Introduction ................................................................................................ 87
PAPI-1 and PAPI-2 islands ......................................................................... 87
Features of PAPI-1 .................................................................................... 87
Features of PAPI-2 .................................................................................... 89
Contribution of PAPI-1 and PAPI-2 to PA14 virulence ............................... 89
Aims of this chapter .................................................................................. 91
Results .................................................................................................................. 92

Survival of *C. elegans* using the slow-killing assay ........................................... 92

Preliminary evidence of PAPI-2 involvement in virulence as demonstrated by the slow-killing assay ................................................................. 92

The PA14Δ1Δ2 mutant showed attenuated virulence in a large-scale slow-killing assay ................................. 96

Changes to slow-killing assay protocol erased the difference between PA14 wildtype and PA14Δ1Δ2 ........................................................................................................ 99

The addition of PAPI-1 did not increase PAO1 virulence ........................................ 99

Survival of *C. elegans* as measured by the fast-killing assay ................................ 103

Fast-killing assay using *C. elegans* N2 ................................................................ 103

Fast-killing assay using *C. elegans* CF512 ............................................................ 104

Grazing of *A. castellanii* and *A. polyphaga* on *P. aeruginosa* ....................... 109

*A. castellanii* growth film expanded faster on PA14 mutants .................................. 109

*A. polyphaga* growth film expansion was similar between *P. aeruginosa* strains ................................................................. 111

Conclusions and Discussion ........................................................................ 118

PAPI-2 is not consistently involved in *C. elegans* virulence ................................ 118

*A. castellanii* growth film expanded faster on PA14 when PAPI-2 was deleted .......... 120

Chapter 4: Identification of genomic islands involved in *P. aeruginosa* virulence by a newly established *C. elegans* high-throughput assay ... 124

Introduction ........................................................................................................ 125

Genomic islands associated with *tRNAs* in *P. aeruginosa* ................................ 125

Association between *tRNA*-integrated accessory elements and virulence .............. 125

Genomic islands associated with *tRNA_{Pro21}* (RGP28) ...................................... 126

Genomic islands associated with *tRNA_{Gly19}* (RGP27) ....................................... 127

Genomic islands associated with *tRNA_{Ser11}* (RGP8) ........................................ 128

Aims of this chapter ................................................................................................ 129

Results .................................................................................................................. 130

Validation of a new *C. elegans* high-throughput assay ...................................... 130

To set up a multi-well high-throughput *C. elegans* assay .................................. 130

To equate the high-throughput assay with the slow-killing assay .......................... 134

Reproducibility of the high-throughput assay .................................................... 134

Screening of *P. aeruginosa* wildtype/deletion mutants pairs ................................ 137

Deletion of *tRNA_{Pro21}*-, *tRNA_{Gly19}*- and *tRNA_{Ser11}*-inserted genomic islands and preliminary growth tests ................................................................. 137

Screening of *P. aeruginosa* mutants with deletion of *tRNA_{Pro21}* genomic island ................................................................. 138

Screening of *P. aeruginosa* mutants with deletion of the *tRNA_{Gly19}* genomic island ................................................................. 139

Screening of *P. aeruginosa* mutants with deletion of *tRNA_{Ser11}* genomic island ................................................................. 139
Chapter 5: Investigation of the links between survival of\textit{C. elegans} and\textit{P. aeruginosa} source of isolation, type III secretion system effectors and pigment production

Introduction ............................................................................................ 158

Phenotypes associated with \textit{P. aeruginosa} clinical isolates ............................... 158
Type III secretion system effectors and virulence ............................................. 159
  Contribution of T3SS effectors to \textit{P. aeruginosa} virulence ........................... 159
  Exclusiveness of \textit{exoU} and \textit{exoS} .................................................................. 161
Aims of this chapter ......................................................................................................... 163

Results .................................................................................................... 164

Association between \textit{P. aeruginosa} isolation source and virulence in \textit{C. elegans} ....... 164
  \textit{P. aeruginosa} bloodstream isolates were more virulent against \textit{C. elegans} than isolates from sputum ............................................................. 164
Prevalence of \textit{P. aeruginosa} T3SS effector genes and virulence in \textit{C. elegans} ......... 165
  \textit{exoU} and \textit{exoY} are associated with strains exhibiting lower HTA scores ............ 166
  Lack of association between isolation source and presence of T3SS effector genes ....... 169
Analysis of the \textit{exoU} locus ................................................................................. 169
  \textit{exoU}/\textit{spcU} pair is always part of genetic element associated with \textit{tRNA}^{\text{Leu}}^{\text{ex10}} ................. 171
  Comparison between \textit{exoU}/\textit{spcU} carrying genetic elements .................................... 171
Production of pigment by \textit{P. aeruginosa} and survival of \textit{C. elegans} .................. 174
  Association between production of pigmented compounds and HTA score ........... 174
  Investigation of the association between production of pigment and source of isolation or the presence of T3SS effector genes ................................................................. 175

Conclusions and discussion ............................................................................. 179
  \textit{C. elegans} as model organisms to gauge the virulence of \textit{P. aeruginosa} clinical isolates 179
  \textit{exoU} as a marker for genomic islands affecting \textit{C. elegans} survival ...................... 181
  Production of pigment by \textit{P. aeruginosa} as predictor of virulence against \textit{C. elegans} .... 183

Chapter 6: Final conclusions and future work.............................................. 187
Evidences supporting the use of *A. castellanii* as model organism to study *P. aeruginosa* virulence factors ................................................................. 188
Genomic islands involved in *P. aeruginosa* virulence toward *C. elegans* ............ 189
Are *P. aeruginosa* and *C. elegans* competing for acquisition of iron? .............. 191

Appendix A ............................................................................................................ 193
Diameter measured for growth film of *Acanthamoeba* spp. grazing on *P. aeruginosa* PA14 WT, PA14ΔPAPI-1, PA14ΔPAPI-2, PA14Δ1Δ2 and PAO1 ........................................ 193

Appendix B ............................................................................................................ 195
ORFs associated with genomic islands integrated at tRNAPro21, tRNAGly19 and tRNASer11 195

Appendix C ............................................................................................................ 203
Growth curves for selected wildtype/tRNA-associated genomic island deletion mutant pairs ........................................................................................................ 203

Appendix D ............................................................................................................ 210
*C. elegans* N2 highthroughput assay score for tRNA-associated genomic island deletion mutants and their isogenic wildtype strains ......................................................................... 210

Appendix E ............................................................................................................ 212
Contingency tables and χ² tests ........................................................................... 212

Appendix F ............................................................................................................ 218
To elucidate *A. castellanii* film grazing on *P. aeruginosa* ........................................ 218
Expansion of *A. castellanii* film is facilitated when *P. aeruginosa* PA14 was plated at higher density ................................................................................................. 218
Expansion of *A. castellanii* film is bacterial growth conditions-dependent and strain-dependent ................................................................................................. 220
Expansion of *A. castellanii* film is not affected by type three secretion system effectors ... 221

Appendix G ............................................................................................................ 222
To elucidate *A. polyphaga* film grazing on *P. aeruginosa* strains ....................... 222
Expansion of *A. polyphaga* film is not affected by plating *P. aeruginosa* strains at different densities ......................................................................................... 222
Expansion of *A. polyphaga* film is independent from bacterial growth conditions ... 224

References ............................................................................................................. 225
List of Figures

Figure 1-1: *C. elegans* anatomy ................................................................. 44
Figure 1-2: *C. elegans* life cycle ............................................................. 45
Figure 2-1: SKA at UoL ........................................................................... 72
Figure 2-2: SKA at UKM .......................................................................... 74
Figure 2-3: HIghthroughput assay .......................................................... 79
Figure 2-4: NNA assay ........................................................................... 84
Figure 2-5: Measurement of amoeba film on NNA plates ................. 85
Figure 3-1: *C. elegans* survival in the small-scale slow-killing assay 94
Figure 3-2: *C. elegans* survival in the large-scale slow-killing assay 97
Figure 3-3: *C. elegans* survival in slow-killing assay done at Universiti Kabangsaan Malaysia ................................................................. 100
Figure 3-4: *C. elegans* survival on PAO1::PAPI-1 and PAO1 .......... 102
Figure 3-5: *C. elegans* N2 survival in fast-killing assay ..................... 105
Figure 3-6: *C. elegans* CF512 survival in fast-killing assay ............... 107
Figure 3-7: Grazing of *A. castellanii* on *P. aeruginosa* ................. 112
Figure 3-8: Amount of bacteria plated for *A. castellanii* NNA assay 114
Figure 3-9: Grazing of *A. polyphaga* film on *P. aeruginosa* .......... 115
Figure 3-10: Amount of bacteria plated for *A. polyphaga* NNA assay 117
Figure 4-1: Slow-killing assay for *P. aeruginosa* clinical isolates ......... 132
Figure 4-2: *C. elegans* brood size when seeded on PA14 or PAO1 .... 133
Figure 4-3: Relationship between highthroughput assay (HTA) score and slow-killing assay (SKA) TD\textsubscript{50} ................................. 135
Figure 4-4: HTA scores for wildtype and mutants strains .................... 140
Figure 4-5: PA14-related strains assessed with slow-killing assay ....... 143
Figure 4-6: LES431-related strains assessed with slow-killing assay 145
Figure 4-7: KR877-related strains assessed with slow-killing assay......................... 148

Figure 5-1: HTA scores for *P. aeruginosa* clinical isolates grouped by their source of isolation........................................................................................................................................ 167

Figure 5-2: HTA scores for *P. aeruginosa* clinical isolates characterized by the presence of T3SS effector genes.................................................................................................................. 168

Figure 5-3: Sequence comparison between genetic elements containing *exoU/spcU*. 173

Figure 5-4: HTA scores for *P. aeruginosa* clinical isolates characterized by the production of pigmented compounds .................................................................................................................. 177

Figure C-1: Growth curves for KR877-related strains ............................................. 203

Figure C-2: Growth curves for KR373-related strains ............................................. 204

Figure C-3: Growth curves for MID9245-related strains ........................................ 205

Figure C-4: Growth curves for PA103-related strains............................................. 206

Figure C-5: Growth curves for KR873-related strains .......................................... 207

Figure C-6: Growth curves for KR352-related strains .......................................... 208

Figure C-7: Growth curves for KR822-related strains .......................................... 209

Figure F-1: *A. castellanii* film expansion on *P. aeruginosa* plated at different densities ........................................................................................................................................ 219

Figure F-2: *A. castellanii* film expansion on *P. aeruginosa* grown on different media........................................................................................................................................ 220

Figure F-3: *A. castellanii* expansion on PA14 mutants lacking T3SS effector genes ........................................................................................................................................ 221

Figure G-1: *A. polyphaga* film expansion on *P. aeruginosa* plated at different densities ........................................................................................................................................ 223

Figure G-2: *A. polyphaga* film expansion on *P. aeruginosa* grown on different media........................................................................................................................................ 224
List of Tables

Table 1-1: pKLC102-related ICEs ................................................................. 23
Table 1-2: pKLC102-related ICEs carrying exoU .............................................. 24
Table 1-3: PAGI-2-related ICEs ....................................................................... 25
Table 1-4: Secretion systems characterized in P. aeruginosa .............................. 29
Table 1-5: Nematodes vs. mice as models to study human bacterial pathogen infectivity ........................................................................................................... 48
Table 1-6: P. aeruginosa virulence-related genes involved in SKA ...................... 53
Table 2-1: Bacterial strains used in this project .................................................. 64
Table 2-2: Primers used for T3SS effector genes PCR assays .............................. 69
Table 2-3: Differences between UoL and UKM SKA protocols ........................... 75
Table 2-4: Scoring system for HTA .................................................................... 80
Table 3-1: Survival analysis of C. elegans CF512 in small scale slow-killing assay 95
Table 3-2: Survival analysis of C. elegans CF512 in large scale slow-killing assay 98
Table 3-3: Survival analysis of C. elegans in slow-killing assay done at Universiti Kabangsaan Malaysia ................................................................. 101
Table 3-4: Survival analysis of C. elegans N2 in fast-killing assay ....................... 106
Table 3-5: Survival analysis of C. elegans CF512 in fast-killing assay .................. 108
Table 3-6: Statistical analysis for A. castellanii grazing on P. aeruginosa .......... 113
Table 3-7: Statistical analysis for A. polyphaga grazing on P. aeruginosa .......... 116
Table 4-1: Virulence phenotype assigned to P. aeruginosa strains .................... 136
Table 4-2: Reproducibility of HTA scores ........................................................ 136
Table 4-3: Survival analysis of C. elegans seeded on PA14-related strains .......... 144
Table 4-4: Survival analysis of C. elegans seeded on LES431-related strains ....... 146
Table 4-5: Survival analysis of C. elegans seeded on KR877-related strains ......... 149
Table 5-1: Prevalence of T3SS effector genes in *P. aeruginosa* clinical isolates derived from blood or sputum

Table 5-2: Distribution of *P. aeruginosa* strains based on their source of isolation or presence of T3SS effector gene among pigment-producing isolates

Table A-1: Diameter of *A. castellanii* film grazing on *P. aeruginosa* strains

Table A-2: Diameter of *A. polyphaga* film grazing on *P. aeruginosa* strains

Table B-1: ORFs annotation for representative tRNA^{Pro21}-associated genomic islands

Table B-1 (continued): ORFs annotation for representative tRNA^{Pro21}-associated genomic islands

Table B-2: ORFs annotation for representative tRNA^{Gly19}-associated genomic islands

Table B-3: ORFs annotation for representative tRNA^{Ser11}-associated islets and genomic island

Table D-1: tRNA^{Pro21}-GI deletion mutants vs. wildtype

Table D-2: tRNA^{Gly19}-GI deletion mutants vs. wildtype

Table D-3: tRNA^{Ser11}-GI deletion mutants vs. wildtype

Table E-1: Production of pigment during HTA vs. source of isolation of *P. aeruginosa* clinical isolates

Table E-2: Production of pigment vs. presence of *exoU* in *P. aeruginosa* clinical isolates

Table E-3: Source of isolation vs. presence of *exoU* and/or *exoS* in *P. aeruginosa* clinical isolates

Table E-4: Source of isolation vs. presence of *exoY* in *P. aeruginosa* clinical isolates

Table E-5: Production of pigment in King’s broth vs. production of pigment on NGM for *P. aeruginosa* clinical isolates

Table E-6: Production of pigment vs. presence of *exoY* in *P. aeruginosa* clinical isolates
Chapter 1

Introduction
**Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative, rod-shaped bacterium belonging to the γ-proteobacteria class. This bacterium can be found in both natural and man-made environments showing high-level of adaptability to different habitats (Campa, Bendinelli & Friedman 1993). This flexibility is also revealed by the aptitude to infect a wide range of hosts from mammals to insects and nematodes to plants (Rahme *et al.* 1995).

**Clinical relevance**

*P. aeruginosa* is the archetypal opportunist pathogen causing illness in humans when underlying disease or treatment renders the patient immunocompromised, unable to cough properly (Williams, Dehnbostel & Blackwell 2010), thermally or otherwise injured (Shankowsky, Callioux & Tredget 1994). Because it can persist on hospital surfaces (Trautmann, Lepper & Haller 2005, Chitkara, Feierabend 1981) and can be carried asymptotically by health-care personnel (Beer *et al.* 2006), *P. aeruginosa* is a leading cause of nosocomial pneumonia (Bergone-Berezin, Richards *et al.* 2000) with the highest mortality rate (up to 60% in some settings) for ventilator-acquired pneumonia (Heyland *et al.* 1999). In contrast, community-acquired pneumonia is only occasionally caused by *P. aeruginosa* (Almirall *et al.* 2000).

*P. aeruginosa* also represents an important problem for patients with damaged mucociliary function as it occurs for example during cystic fibrosis or bronchiectasis because it may become a permanent resident of the lungs (Williams, Dehnbostel &
Blackwell 2010) whereas is associated with worst prognosis (Jacques et al. 1998). In order to endure in the lung of cystic fibrosis patients, \textit{P. aeruginosa} undergoes significant adaptations including adopting a mucoid phenotype (Hassett et al. 2009). Due to its ubiquitous presence, \textit{P. aeruginosa} can infect deep wounds and burns causing life-threatening infections (Pier 2012) and it is also a relevant pathogen for soft contact lenses wearers (Pier 2012).

Notwithstanding the initial site of the infection, \textit{P. aeruginosa} becomes a major clinical concern if it reaches the bloodstream (Wisplinghoff et al. 2004). In England, Wales and Northern Ireland, \textit{Pseudomonas} spp. were reported as the eighth most common cause of monomicrobial bacteraemia in 2012 with \textit{P. aeruginosa} isolated from the blood of 91\% of the cases (HPA 2013). The recovery of this bacterium in the bloodstream is a clear sign of deteriorating patient health. In fact, the mortality rate for bloodstream infections has been reported to be between 80 to 100\% compared to infections restricted to the lung, where mortality is reported to be less, between 30 to 60\% (Aksamit 1993, Fick 1993).

\textbf{\textit{P. aeruginosa} genomics}

The ecological properties of \textit{P. aeruginosa} and in particular its ability to thrive on both inanimate surfaces and living organisms reside in its unusually large (Klockgether et al. 2011) and non-redundant genome (Kung, Ozer & Hauser 2010) specifying for its metabolic adaptability, persistence and virulence factors (Stover et al. 2000). In this bacterium, the genome has a mosaic structure formed by a core component and an accessory component (Klockgether et al. 2011). The core genome represents 90\% of the genetic material and it is highly conserved. It encodes for factors that are shared by many strains (Wolfgang et al. 2003). In contrast, the accessory genome is formed by
genetic elements found in one or few strains (Mathee et al. 2008) and represents a relevant source of genome plasticity in *P. aeruginosa* (Klockgether et al. 2011).

**Horizontal gene transfer**

Bacteria may evolve thorough genome reduction (small-scale or large-scale deletions), mutations, rearrangements and gene acquisition by horizontal gene transfer (HGT) (Dobrindt et al. 2004). The latter mechanism may allow the concurrent acquisition of organized genes resulting in the gain of complex phenotypes in one step (Ochman, Lawrence & Groisman 2000), providing an advantage in niches where there is competition among different species of bacteria or with predators. In *P. aeruginosa*, HGT occurs mainly through phage-related transduction and type IV pili-dependent conjugation and represents a major force in creating distinctive genomic regions (Kung, Ozer & Hauser 2010).

The efficiency of the transfer/acquisition is dependent on many factors. High degree of similarities between donor and recipient cells usually increases the probability of success in the transfer (Kung, Ozer & Hauser 2010). However, LPS replacement island codifying for O antigen was likely acquired through transduction of Gram-positive bacteria capsule biosynthesis-related genes (Dean et al. 1999) and a PAO1 phospholipase was of eukaryotic origin (Wilderman et al. 2001). The acquisition of new accessory elements may be hindered by the presence of same/similar elements as it is in the case of PAPI-1 (Qiu, Gurkar & Lory 2006) and presence of mechanisms such as clustered regularly interspaced short palindromic repeats (Barrangou et al. 2007) and restriction modification systems (Smith 1979).
Components of the accessory genome

The *P. aeruginosa* accessory genome comprises both extra-chromosomal (e.g. plasmids) and chromosomal elements (Klockgether *et al.* 2011). Each completely sequenced *P. aeruginosa* genome contains 30-50 elements defined as accessory (Klockgether *et al.* 2011) identifiable because of their difference in G+C content, codon and tetranucleotide usage, their association with mobility genes/functions and their insertion at specific loci (Kung, Ozer & Hauser 2010). The availability of sequenced genomes allowed the definition of 89 loci, termed ‘regions of genome plasticity’ (RGPs; Mathee *et al.* 2008, Klockgether *et al.* 2011) flanked by DNA sequences that are conserved between strains but contain four or more ORFs that are unique and not present in all strains (Mathee *et al.* 2008). The genetic elements found in RGPs are often denoted as genomic island, if above 10kb, or islet, if below 10kb (Kung, Ozer & Hauser 2010). The large majority of RGPs are located in intergenic regions, 20 are located at the 3’ end of tRNAs and only 3 are located inside coding ORFs (Klockgether *et al.* 2011).

The accessory elements may be grouped in different categories depending on their features for example presence of direct or inverse repeats or the type of integrase they are associated with. However, some cannot be assigned to a specific type because they are degenerate or they have not been characterized sufficiently (Kung, Ozer & Hauser 2010).

Integrative and conjugative elements

Integrative and conjugative elements (ICEs) present both plasmid and phage-like features. Similarly to plasmids, ICEs may be maintained episomically by the partitioning protein Soj (Qiu, Gurkar & Lory 2006) and mediate transfer by conjugation through the island-specific type IV pili (Juhas *et al.* 2007). They also carry a phage-like
integrate to mediate the site-specific recombination between \textit{attP} on the ICE and the \textit{attB} on the chromosome (Kung, Ozer & Hauser 2010) usually at the 3’ end of \textit{tRNAs} (Williams 2002). These islands are often unique, characterized by the presence of strain-specific cargo genes (Wurdemann, Tummler 2007). In \textit{P. aeruginosa}, ICEs may be divided in two large families called pKLC102-related elements (Kung, Ozer & Hauser 2010) or PAGI-2-related elements (Klockgether \textit{et al.} 2011) depending of the integrase they carry.

The pKLC102-related islands (Table 1-1 and Table 1-2) contain the XerC/XerD-like integrase able to drive recombination with the 3’end of \textit{tRNA}^{Lys} (Klockgether \textit{et al.} 2011). Some of the elements (e.g. pKLC102 and pKLK106) can excise frequently and are found extra-chromosomally in high numbers while others (e.g. PAPI-1) excise at much lower but detectable level (Klockgether \textit{et al.} 2007). However, some elements (e.g. PAGI-4) have become fixed in the chromosome (Kung, Ozer & Hauser 2010). The functions encoded by the cargo genes carried in these islands (Table 1-1 and Table 1-2) are disparate, including metabolic pathways and virulence-related factors to regulatory elements but many ORFs remain putative or hypothetical. Nonetheless, contribution to virulence was demonstrated for some of the ORFs-associated with PAPI-1 (He \textit{et al.} 2004), the NR-I and NR-II regions of PAGI-5 (Battle \textit{et al.} 2008) and PAPI-2 (Harrison \textit{et al.} 2010).

The PAGI-2-related islands (Table 1-3) carry the phage P4-like integrase able to catalyse integration at the 3’end of \textit{tRNA}^{Gly} (Klockgether \textit{et al.} 2011). Elements such as \textit{clc} island are known to be transferred readily between \textit{\gamma}-proteobacteria and \textit{\beta}-proteobacteria (Gaillard \textit{et al.} 2008) while others, such as PAGI-2 and PAGI-3, appear to have undetectable mobility (Klockgether \textit{et al.} 2007). The cargo genes of these elements (Table 1-3) are involved in degradation of aromatic compounds (Gaillard \textit{et al.}
and the detoxification and resistance to heavy metal (Larbig et al. 2002). These elements are not likely to contribute directly in virulence but may provide increased fitness in certain environments (Dobrindt et al. 2004).

Replacement islands

Replacements islands are similar to genomic islands because their ORFs are clustered together, they have horizontal gene transfer features and they are highly divergent between strains (Kung, Ozer & Hauser 2010). In contrast with genomic islands, replacement islands are present in all the strains (Kung, Ozer & Hauser 2010). Siderophores, enzymes involved in LPS synthesis and motility-related components are among the functions encoded by replacement islands-specific genes (Klockgether et al. 2011).

Prophages and phage-like elements

Bacteriophages or phages may be responsible for horizontal gene transfer and represent a reservoir of genetic diversity for bacteria (Kung, Ozer & Hauser 2010). As for ICEs, integration of phage genomes in bacterial chromosome during lysogenic cycle is both recombinase-driven (Kung, Ozer & Hauser 2010) and site-specific, usually occurring at or next to a tRNA (Dobrindt et al. 2004). Once integrated in the bacterial chromosome, phages are labelled prophages (Kung, Ozer & Hauser 2010) and P. aeruginosa strains may contain several. For example, the Liverpool epidemic strain (LES) B58 has six prophages, which have been demonstrated to contribute to virulence (Winstanley et al. 2009). Other prophages are also known for conferring phenotypes promoting virulence (Nakayama et al. 1999, Nakayama et al. 2000, Webb, Lau & Kjelleberg 2004).
Transposons, insertion sequences and integrons

Transposition allows the excision of elements by the action of a transposase on short inverted repeats at the edge of the element followed by integration at another site (Kung, Ozer & Hauser 2010). Genetic elements that carry a transposase are classified as insertion sequences (IS) or transposons depending on the size and complexity (Kung, Ozer & Hauser 2010). Integrons can mobilize only by associating with other mobile elements (Mazel 2006). Their main features are the presence of an integrase belonging to the tyrosine-recombinase family, a promoter that drives the expression of the genes associated with the integron and a recombination site downstream of the promoter (Mazel 2006). The relevance of transposons and integrons in the clinical setting is due to their association with development of antibiotic resistance (Kung, Ozer & Hauser 2010).
<table>
<thead>
<tr>
<th>Island (Strain)</th>
<th>Size (kb)</th>
<th>Integration site</th>
<th>Status</th>
<th>Cargo genes</th>
<th>Contribution to virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKLC102</td>
<td>103.5</td>
<td>tRNA&lt;sub&gt;lys10&lt;/sub&gt; (RGP7)</td>
<td>Episomal and/or Integrated</td>
<td>Fatty acid synthase; Chemotaxis (p); Cold adaptation protein; Polyketide synthase; Phage antirepressor; Transcriptional regulators (p); Synthase for cyclic-β-(1,2)-glucan</td>
<td>Unknown</td>
</tr>
<tr>
<td>pKLK106</td>
<td>106.0</td>
<td>tRNA&lt;sub&gt;lys10&lt;/sub&gt; (RGP7)</td>
<td>Episomal and/or Integrated</td>
<td>Similar to pKLC102</td>
<td>Unknown</td>
</tr>
<tr>
<td>PAGI-4</td>
<td>23.4</td>
<td>tRNA&lt;sub&gt;lys10&lt;/sub&gt; (RGP7)</td>
<td>Integrated only</td>
<td>Hypothetical ORFs</td>
<td>Unknown</td>
</tr>
<tr>
<td>PAGI-5</td>
<td>99.3</td>
<td>tRNA&lt;sub&gt;lys10&lt;/sub&gt; (RGP7)</td>
<td>Integrated</td>
<td>NR-I; NR-II; Mercury metabolism</td>
<td>NR-I and NR-II associated with higher LD&lt;sub&gt;50&lt;/sub&gt;; attenuation upon deletion</td>
</tr>
<tr>
<td>PAPI-1</td>
<td>108.0</td>
<td>tRNA&lt;sub&gt;lys47&lt;/sub&gt; (RGP41)</td>
<td>Integrated but excisable</td>
<td>Type IV pili; Two-component systems; Pyocin S5; virulence factors present in other bacteria (p)</td>
<td>Two-component system involved in biofilm formation; 19 ORFs showed attenuation when interrupted</td>
</tr>
</tbody>
</table>

**Table 1-1: pKLC102-related ICEs**

Integrative and conjugative elements related to pKLC102. The bacterial strain in which each island was originally identified is shown in parenthesis. Also, genomic island preferred integration site, integrated or episomal status, cargo genes and known contributions to virulence are shown; “integrated” refers to integration in the bacterial chromosome. PAPI = *Pseudomonas aeruginosa* pathogenicity island; PAGI = *Pseudomonas aeruginosa* genomic island; RGP = region of genome plasticity; (p) = putative function was assigned to ORF. Table information was assembled from: Klockgether et al. (2011); Kung, Ozer & Hauser (2010); Kulasekara et al. (2006); He et al. (2004).
<table>
<thead>
<tr>
<th>Island (Strain)</th>
<th>Size (kb)</th>
<th>Integration site</th>
<th>Status</th>
<th>Cargo genes</th>
<th>Contribution to virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAPI</td>
<td>10.8</td>
<td>tRNA&lt;sup&gt;Lys10&lt;/sup&gt; (RGP7)</td>
<td>Integrated</td>
<td>exoU/spsU</td>
<td>Deletion of island decreases virulence</td>
</tr>
<tr>
<td>ExoU-A (6077)</td>
<td>81.2</td>
<td>tRNA&lt;sup&gt;Lys10&lt;/sup&gt; (RGP7)</td>
<td>Integrated</td>
<td>exoU/spsU; Synthesis of antifungal pyoluteorin (p); Catalase isozyme A; Glutathione S-transferase (p); Pirin-related protein; α/β hydrolase family protein; Amidohydrolase family protein; LysR binding protein (p); HlyD/EmrA family membrane protein; O-methyl transferase family protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ExoU-B (19660)</td>
<td>29.8</td>
<td>tRNA&lt;sup&gt;Lys10&lt;/sup&gt; (RGP7)</td>
<td>Integrated</td>
<td>exoU/spsU; GNAT family acetyltransferase; Colicin immunity protein; Pyocin S5 (p); RNA polymerase σ-70 factor, extracytoplasmic function family; Oxygen-independent coproporphyrinogen III oxidase (p); nitric oxide reductase transcriptional regulator (p); quinol-dependent nitric oxide reductase (p); Regulator of mercury resistance proteins (p); NADH:flavin oxidoreductase family protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>ExoU-C (X13273)</td>
<td>3.9</td>
<td>tRNA&lt;sup&gt;Lys10&lt;/sup&gt; (RGP7)</td>
<td>Integrated</td>
<td>exoU/spsU</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 1-2: pKLC102-related ICEs carrying exoU**

Integrative and conjugative elements related to pKLC102 carrying the type III secretion system effector gene exoU. The bacterial strain in which each island was originally identified is shown in parenthesis. Also, genomic island preferred integration site, integrated or episomal status, cargo genes and known contributions to virulence are shown; “integrated” refers to integration in the bacterial chromosome. PAPI= *Pseudomonas aeruginosa* pathogenicity island; RGP= region of genome plasticity; (p)= putative function was assigned to ORF. Table information was assembled from: Klockgether et al. (2011); Kung, Ozer & Hauser (2010); Kulasekara et al. (2006); He et al. (2004); Harrison et al. (2010).
<table>
<thead>
<tr>
<th>Island (Strain)</th>
<th>Size (kb)</th>
<th>Integration site</th>
<th>Status</th>
<th>Cargo genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>clc (B13*)</td>
<td>105</td>
<td>tRNA\textsubscript{Gly} (RGP27 or 29)</td>
<td>Integrated but excisable</td>
<td>clcRABCD (degradation of 3- and 4- chlorocatehol); 2-aminophenol degradation; aromatic compounds transporter (p); aromatic ring dioxygenase</td>
</tr>
<tr>
<td>PAGI-2 (C)</td>
<td>105</td>
<td>tRNA\textsubscript{Gly} (RGP29)</td>
<td>Integrated only</td>
<td>Homologous to Cupriavidus metallidurans</td>
</tr>
<tr>
<td>PAGI-3 (SG17M)</td>
<td>103</td>
<td>tRNA\textsubscript{Gly} (RGP29)</td>
<td>Integrated only</td>
<td>Metabolic genes (p); transport genes (p); resistance genes (p)</td>
</tr>
<tr>
<td>LESGI-3 (LESB58)</td>
<td>111</td>
<td>tRNA\textsubscript{Gly} (RGP27)</td>
<td>Integrated</td>
<td>Complex of heavy metals; transport of heavy metals</td>
</tr>
</tbody>
</table>

**Table 1-3: PAGI-2-related ICEs**

Integrative and conjugative elements related to PAGI-2. The bacterial strain in which each island was originally identified is shown in parenthesis. Also, genomic island preferred integration site, integrated or episomal status and cargo genes are shown; “integrated” refers to integration in the bacterial chromosome. PAGI= Pseudomonas aeruginosa genomic island; LESGI= LES (Liverpool epidemic strain) genomic island. RGP= region of genome plasticity; (p)= putative function was assigned to ORF. Table information was assembled from: Klockgether et al. (2011); Kung, Ozer & Hauser (2010).

* = this element was originally identified in Pseudomonas knackmussii B13 strain
**Virulence factors**

The degree of disease severity after *P. aeruginosa* infection depends on the efficacy of the host defences (Safdar, Crnich & Maki 2005), the effectiveness of antibiotic treatment (Lodise et al. 2007) and the virulence associated with the bacterial strain (Hauser et al. 2002). In its large genome, *P. aeruginosa* harbours the capacity to produce many elements known to contribute to its virulence and also many ways to deliver them outside the bacterial cell.

**Secretion systems**

Gram-negative bacteria possess secretion machineries that allow crossing of molecules through the inner membrane, the periplasm containing a thin layer of peptidoglycan and the outer membrane in contact with the extracellular milieu (Silhavy, Kahne & Walker 2010). Seven distinct secretion systems have been identified and they are labelled type I (T1SS) to type VII secretion system (T7SS). They are all widespread among Gram-negative bacteria (Economou et al. 2006) with the exception of T7SS that has been characterized only in *Mycobacterium* spp. (Abdallah et al. 2009).

In *P. aeruginosa*, secretion systems belonging to most of the classes have been described (Table 1-4). They can be classified in different groups depending on the necessity to use either the general secretory (Sec) or the twin-arginine translocation pathway (Tat) and on requiring direct contact with the target cell. When a “two-steps” mechanisms is involved, the secretion system *per se* is located in the outer membrane and translocate compounds outside the cell from the periplasm. This is preceded by the intervention of general secretion pathways (Sec or Tat) to allow crossing of the inner membrane. T2SS and T5SS are included in this category. Other secretion systems do not require the intervention of Sec or Tat and they can secrete molecules directly from
the cytoplasm to the extracellular milieu (T1SS) or the cytoplasm of a target cell (T3SS or T6SS (Bleves *et al.* 2010).

Type III and type VI secretion systems (T3SS and T6SS) are Sec- and Tet-independent and translocate proteins directly into the cytosol of target cells for which they require direct contact (Bleves *et al.* 2010). Both T3SS and T6SS have a complex organization but interestingly, T3SS is akin to flagella (Soscia *et al.* 2007) and targets only eukaryotic cells while the T6SS structure is similar to T4 bacteriophage tail and targets both eukaryotic and prokaryotic cells (Basler, Mekalanos 2012, Leiman *et al.* 2009).

The proteins translocated by T3SS are called effectors. They are recruited by the complex machinery in their unfolded state (Cornelis 2006) and require the presence of chaperones when they are in the cytoplasm (Letzelter *et al.* 2006). The effectors represent the major contribution to virulence exerted by T3SS (Roy-Burman *et al.* 2001) even though the apparatus itself may be cytotoxic (Lee *et al.* 2005). The expression of T3SS is affected by general pathways such as stress signals (Yahr, Wolfgang 2006), cAMP concentration (Wolfgang *et al.* 2003), two-component regulators (Goodman *et al.* 2004, Mulcahy *et al.* 2006), metabolism (Rietsch, Mekalanos 2006) and is repressed by quorum sensing signals (Bleves *et al.* 2005). The T3SS is also regulated directly by a dedicated AraC-like transcriptional factor called ExsA (Frank 1997). It has been noted that T3SS expression occurs only at low bacterial cell density, as is found at the beginning of an infection (Hogardt *et al.* 2004, Bleves *et al.* 2005), and it is not produced during chronic infection (Bleves *et al.* 2010). These observations led to the hypothesis that repression of T3SS may be a way to prolong the residence of *P. aeruginosa* in the host (Bleves *et al.* 2010).

Initially, only few effectors able to be translocated by T3SS were identified and extensively studied, called ExoU, ExoS, ExoY and ExoT. They represented a relatively
small number of effectors when compared to the number present in other bacteria using T3SS (Gellatly, Hancock 2013). However, more recent work has identified new protein able to be targeted by T3SS for translocation into an eukaryotic cells named NDK (Neeld et al. 2014), PemA and PemB (Burstein et al. 2015) and highlighted the possibility that new effectors may be yet to be discovered (Burstein et al. 2015).

Alternatively to membrane proteins-based system, secretion may also be achieved through production of outer membrane vesicles or OMVs (Bleves et al. 2010). OMVs are round bodies formed from the bacterial outer membrane and enclosing soluble and insoluble proteins from the periplasm (Kulp, Kuehn 2010). They have been observed in biopsies of tissues infected by different pathogens including P. aeruginosa (Irazoqui et al. 2010) in which they could release inflammatory and/or virulence factors (Ellis, Kuehn 2010). Production of outer membrane vesicles is a regulated process and has been identified as a mechanism of stress response in P. aeruginosa in particular regarding oxidative stress or stress due to treatment with antimicrobials (Macdonald, Kuehn 2013).
<table>
<thead>
<tr>
<th>Secretion System</th>
<th>Alternative name</th>
<th>Mechanism of secretion</th>
<th>Known systems</th>
<th>Secreted compounds/effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1SS</td>
<td></td>
<td>Direct transfer from cytoplasm to extracellular milieu</td>
<td>Apr</td>
<td>AprA, AprX</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Has</td>
<td>HasAp (binds haem from haemoglobin)</td>
</tr>
<tr>
<td>T2SS</td>
<td>Secreton</td>
<td>Present in outer membrane; requires Sec or Tat</td>
<td>Xpc</td>
<td>LasA, LasB, PrpL, LipA, LipC, PlcH, PlcN, PlcB, ToxA, PhoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hxc</td>
<td>LapA (alkaline phosphatase release under phosphate-limiting conditions)</td>
</tr>
<tr>
<td>T3SS</td>
<td>Injectisome</td>
<td>Direct contact with eukaryotic target cell</td>
<td>/</td>
<td>ExoU, ExoS, ExoT, ExoY</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NDK, PemA, PemB</td>
</tr>
<tr>
<td>T5SS</td>
<td>Autotransporters (AT or T5aSS T5cSS)</td>
<td>Present in outer membrane; requires Sec</td>
<td>TSP5</td>
<td>LepA</td>
</tr>
<tr>
<td></td>
<td>2-partner secretion systems (TPS or T5bSS)</td>
<td></td>
<td>TSP6</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td></td>
<td>T5dSS</td>
<td></td>
<td>PlpD</td>
<td>Lipase</td>
</tr>
<tr>
<td>T6SS</td>
<td></td>
<td>Direct contact with eukaryotic or bacterial target cell</td>
<td>HIS-I</td>
<td>Tse1, Tse2, Tse3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HIS-II</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HIS-III</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1-4: Secretion systems characterized in P. aeruginosa**

A summary of the major features of secretion systems characterized in *P. aeruginosa* is shown. Table information was assembled from: Bleves et al. (2010) for T1SS, T2SS, T5SS; Houser (2009), Bleves et al. (2010), Burstein et al. (2015) and Neeld et al. (2014) for T3SS; Lesic et al. (2009) and Bleves et al. (2010) for T6SS.
Secreted enzymes associated with virulence

Proteases

The importance of proteases in *P. aeruginosa* virulence has been long established (Liu, Abe & Bates 1961) and recently some of the mechanisms underlying their association with virulence have been elucidated.

AprA is an alkaline protease secreted by the Apr system (T1SS) relevant in various diseases (Matsumoto 2004) and in particular corneal infections (Guzzo *et al*. 1991). AprA has been implicated in the degradation of complement and fibronectin (Laarman *et al*. 2012) and in immune avoidance by interfering with the binding of flagellin to Toll-like receptors (TLR)-5 (Bardoel *et al*. 2011).

LepA is a protease released through a type V secretion system (Bleves *et al*. 2010) and is capable of digesting the PAR-1, PAR-2 and PAR-4 receptors on human cells inducing activation of eukaryotic transcriptional factors NF-κB (Kida *et al*. 2008). It is also detectable in supernatant of clinical isolates cultures but not from the lab strain PAO1 (Kida *et al*. 2008).

The Xpc type 2 secretion system is responsible for the release of several enzymes with proteolytic activity (Bleves *et al*. 2010). One of the most studied is the elastase LasB which is capable of breaking down elastin, a component of the lung tissue (Alcorn, Wright 2004), and also plays a pivotal role in keratosis (Matsumoto 2004). Since the LasB product may degrade the surfactant protein D (Alcorn, Wright 2004) altering alveolar macrophage function, when compared with wildtype strains, ΔlasB mutants were more susceptible to phagocytosis and showed an overall virulence attenuation (Kuang *et al*. 2011). LasB activity is amplified by a second elastase, LasA (Matsumoto 2004), known as staphylolysin because of the lytic effect on the *Staphylococcus aureus*
cell wall (Toder et al. 1994). Also secreted by the Xpc T2SS, protease IV or PrpL has been implicated in corneal erosion (Engel et al. 1998) and is involved in degradation of complement, immunoglobulin, fibrinogen (Gellatly, Hancock 2013) and surfactant proteins A and D, collectively affecting the ability of alveolar macrophages to bind *P. aeruginosa* (Malloy et al. 2005).

Lipases and phospholipases

The Xpc T2SS is also capable of secreting several enzymes which can degrade esters of glycerol (lipases) such as LipA and LipC (Jaeger et al. 1994, Martinez, Ostrovsky & Nunn 1999), and so are able to break down the lipid component of pulmonary surfactant during lung infection (Kipnis, Sawa & Wiener-Kronish 2006).

Phospholipases are lipases acting on phospholipids, the major component of all cell membranes, released either by type II or by type III secretion systems. PlcN, PlcH and PlcB are secreted in the extracellular milieu by Xpc T2SS and specifically target the eukaryotic membrane (Barker, Humphrey & Brown 1999).

One of the most powerful phospholipases at *P. aeruginosa* disposal is the T3SS effector ExoU. Interestingly, only ~ 30% (Garey et al. 2008) of *P. aeruginosa* isolates carry *exoU* and *spcU*, which codes for the ExoU chaperon, possibly because they are usually found in genomic islands associated with 3’ end of *tRNA^{Lys}^A* (Kulasekara et al. 2006, He et al. 2004). Once translocated inside the target cell, ExoU associates with eukaryotic cell membrane (Rabin et al. 2006) and causes its disarray followed by rapid death through cell necrosis (Finck-Barbancon et al. 1997). In a rabbit model where the bacterium was instilled in the lungs and followed by bacteraemia and septic shock, ExoU cause decompartmentalization of the inflammatory response (Kurahashi et al. 1999) while in humans, its secretion was more frequently found in strains recovered
from patients with ventilator associated pneumonia that were more severely ill (Roy-Burman et al. 2001).

Enzymes leading to substrate modifications

In order to hinder the normal functioning of a target eukaryotic cell, *P. aeruginosa* can produce enzymes that directly modify proteins inside the cell or indirectly influence their regulation. In this category reside the other type III secretion system effectors: ExoY, ExoT and ExoS.

ExoY is an adenylate cyclase catalysing the conversion of ATP to cAMP (Yahr et al. 1998). Elevated concentrations of cAMP in eukaryotic cells has a direct effect on actin stability with consequent cytoskeleton disorganization (Cowell, Evans & Fleiszig 2005), reduced ability to uptake bacteria and higher cell permeability followed by increased lung permeability (Sayner et al. 2004).

ExoS and ExoT are very similar proteins comprising two catalytic domains: a GTPase-activating protein (GAP) and ADP ribosyl transferase (ADPRT). The former hydrolyses GTP while the latter transfers ADP-ribose to a suitable substrate (Hauser 2009). The GAP activity targets several proteins affecting the cytoskeleton functionality (Pederson et al. 1999, Goehring et al. 1999) and reducing phagocytosis (Garrity-Ryan et al. 2000, Frithz-Lindsten et al. 1997). The ADPRT domain is a common feature shared by many bacterial toxins including cholera and diphtheria toxins (De Haan, Hirst 2004) but showing different specificity in ExoS and ExoT (Hauser 2009). The ExoS ADPRT compromises the eukaryotic cell cytoskeleton and reduces cell adherence therefore allowing the bacteria access to the target cell (Rocha et al. 2003). Furthermore, ExoS has a role in modulation of the host response because its C-terminal portion may bind TLR-2 while its N-terminal portion may bind TLR-4 (Epelman et al. 2004). The ExoT ADPRT may block the cell division cycle (Shafikhani, Engel 2006) resulting in
phagocytosis stalling, epithelial barrier compromising (Lee et al. 2005) and wound healing delay (Geiser et al. 2001).

ToxA or exotoxin A also includes an ADPRT domain but is secreted by Xpc T2SS in to the extracellular milieu (Lu, Mizushima & Lory 1993). Here, it is able to find and enter a target cell (Bleves et al. 2010), hinder protein synthesis and cause cell death (Pavlovskis, Iglewski & Pollack 1978). Alongside this, it acts to dampen the host immune response (Schultz et al. 2001). Although exotoxin A appears to have a less relevant role in virulence than T3SS effectors (Kudoh et al. 1994), toxA-negative strains were found to be less virulent than corresponding toxA-positive strains (Miyazaki et al. 1995).

The Xpc T2SS is also responsible for the secretion of PhoA, an alkaline phosphatase responsible for substrate desphosphorylation (Filloux et al. 1988). This enzyme is likely to boost phosphate absorption under deficient condition (Horiuchi, Horiuchi & Mizuno 1959) as it may occur for example after major surgery and it is a predictor of subsequent sepsis (Shor et al. 2006).

**Enzymes degrading bacterial components**

In order to establish an infection, a pathogen must compete with both the host immune system and the other microbes usually residing in the host. Accordingly, *P. aeruginosa* is able to translocate Tse1, Tse2 and Tse3 effectors to other Gram-negative bacteria through the HIS-I T6SS (Russell et al. 2011). Tse2 exerts growth arrest in other prokaryotes as well as in eukaryotes (Hood et al. 2010) while Tse1 and Tse3 are delivered to the periplasm of other bacterial cells where they hydrolyse the peptidoglycan with consequent cell lysis (Russell et al. 2011).
**P. aeruginosa cell surface components involved in virulence**

Beside proteins, there are other components of the bacterial cell surface that are directly or indirectly involved in virulence such as pili, flagella, LPS and alginites.

*P. aeruginosa* cell may possess one flagellum, a long whip-like structure, and several shorter appendages, called pili (type IV). Although structurally different, they are required to establish an infection (Gellatly, Hancock 2013) and for motility. The involvement in virulence has been established in several models of infection where the mutants without flagellum or pili were less virulent than the original strain with the appendages (Feldman *et al.* 1998, Brimer, Montie 1998, Gellatly, Hancock 2013). It has been hypothesized that the presence of type IV pili on the cell surface may lead to bacterial aggregation and formation of microcolonies, necessary to create biofilms and shelter bacteria from immune system (Craig *et al.* 2003, Sriramulu *et al.* 2005). Interestingly, the flagellum is down-regulated during chronic infection (Wolfgang *et al.* 2003) similarly to T3SS. Regarding motility, the flagellum confers the ability to move in aqueous environs, called swimming (Gellatly, Hancock 2013) while pili enable movement on solid surfaces, named twitching (Kipnis, Sawa & Wiener-Kronish 2006). When present together, they allow for movement on semi-solid surfaces, a form of motility known as swarming (Kohler *et al.* 2000).

As other Gram-negative bacteria, the surface layer of *P. aeruginosa* outer membrane is composed principally by a complex glycolipid known as lipopolysaccharide (LPS) or endotoxin (Kamio, Nikaido 1976) that comprises three parts: the lipid A, the polysaccharide core domain and the O-antigen (Lam *et al.* 2011). Lipid A is the portion of LPS anchored to the outer membrane (Gellatly, Hancock 2013) and elicits the production of pro-inflammatory cytokines through TLR-4 recognition (Akira, Uematsu & Takeuchi 2006). The extent of the inflammatory response depends on the specific
composition of the lipid A which is in turn dependent on strain-specific characteristics and on environmental factors (Gellatly, Hancock 2013). The O-antigen represents the most external portion of LPS, it forms the basis of strain serotyping (King et al. 2009) and may elicit a strong humoral response depending on its specific composition (Hancock et al. 1983).

Alginates are exopolysaccharides created by the combination of several manuronic and glucoronic acid molecules (Kipnis, Sawa & Wiener-Kronish 2006). P. aeruginosa strains that colonize cystic fibrosis patients often undergo mutations resulting in overexpression of alginates with consequent conversion to the mucoid phenotype (Davies 2002). In fact, this phenotype confers protection against the host immune response, including phagocytosis, and enhances antibiotics resistance (Cobb et al. 2004).

**Factors involved in iron recruitment**

Iron (Fe$^{3+}$ or Fe$^{2+}$) is an abundant but essential element required for bacterial metabolism and virulence (Andrews, Robinson & Rodriguez-Quinones 2003, Cornelis, Dingemans 2013). Bacteria compete with each other to acquire iron (Traxler et al. 2012) but during an infection, they also compete with the host where this element is sequestered by heme or strongly chelated to transfer proteins such as lactoferrin and transferrin (Cornelissen, Sparling 1994). P. aeruginosa may employ several mechanisms to acquire iron including production of siderophores and phenazines.

Siderophores are low-molecular weight proteins that are able to bind Fe$^{3+}$ ions to high affinity (Hider, Kong 2010) and to internalize them through a TonB-dependent mechanism (Cornelis 2010). In P. aeruginosa, two siderophores have been shown to have relevance in virulence: pyoverdine and pyochelin.
Pyoverdine is a green-yellow fluorescent compound and represent the primary siderophore produced when iron concentration is low (Visca, Imperi & Lamont 2007). *P. aeruginosa* strains may possess one of three possible pyoverdines (PVDI, PVDII or PVDIII) characterized by different peptide chain (Meyer *et al.* 1997). The synthesis of pyoverdine is complex involving several cell compartments (Schalk, Guillon 2013), many genes organized in clusters (Spencer *et al.* 2003) that bear the signs of accessory elements, and requires non-ribosomal peptide synthetases (Schalk, Guillon 2013). Pyoverdine is required by *P. aeruginosa* in order to exert full virulence in burn or acute pneumonia mouse models and for biofilm formation (Meyer *et al.* 1996, Takase *et al.* 2000a). Pyoverdine controls its own expression and the expression of other virulence factors such as the protease PrpL and exotoxin A (Cornelis 2010).

Pyochelin is a peptide-based siderophore produced by all *P. aeruginosa* strains. It has lower affinity for Fe$^{3+}$ ions than pyoverdine (Brandel *et al.* 2012) but requires a smaller number of genes for its synthesis (Britigan, Rasmussen & Cox 1997). The pyochelin-iron complex may cause oxidative damage and be responsible for inflammation in particular if pyocyanin is also present (Britigan, Rasmussen & Cox 1997). In contrast to pyoverdine, pyochelin seems to have a more prominent role in chronic infection (Hare *et al.* 2012).

Pyocyanin, a green-blue pigment, and its precursor, phenazine-I-carboxylic acid, are phenazines produced by *P. aeruginosa* and involved in iron uptake (Wang, Newman 2008). They are able to reduce Fe$^{3+}$ to Fe$^{2+}$ ions that can freely diffuse across the outer membrane (Wang *et al.* 2011). Once in the periplasm, ferrous ions are transported inside the cell through the FeOABC system (Cartron *et al.* 2006). Both phenazines and FeOABC system are important in the long-term colonization of cystic fibrosis patients
where phenazines, and in particular pyocyanin, have been shown to accumulate and be detectable in the sputum (Yoon et al. 2002).

Because of its importance and reactivity, iron uptake in *P. aeruginosa* is tightly regulated by Fur, the major-iron-responsive general regulator (Cornelis 2010). *fur* appears to be essential in *P. aeruginosa* because it cannot be deleted (Barton et al. 1996) as it may act both directly and indirectly to regulate many genes involved in iron uptake (Cornelis, Matthijs & Van Oeffelen 2009).

**Formation of biofilm**

Biofilms are multicellular bacterial communities that can form on both biotic settings, such as human teeth, skin or urinary tract (Hatt, Rather 2008), and non-biotic settings, such as catheters, medical implants (Hall-Stoodley, Costerton & Stoodley 2004, Donlan 2008) or pipelines (de Carvalho 2007). The ability to form biofilm contributes to *P. aeruginosa* pathogenesis (Lopez, Vlamakis & Kolter 2010). Biofilms increase the antibiotic tolerance of bacteria (Mah, O'Toole 2001), protect them from protozoan predation (Matz, Kjelleberg 2005) or host immune system (Anderson, O'Toole 2008) and increase the portion of persister cells, non-dividing, genetically identical cells with higher resistance to environmental stresses (Lewis 2005).

The initial stage of biofilm formation usually requires the change from transient to permanent attachment to a surface (Karatan, Watnick 2009) followed by encasing in an extracellular matrix produced by the bacteria themselves (Karatan, Watnick 2009). During these stages, bacterial cells undergo enormous physiological alterations reflected by huge transcriptional changes (Sauer et al. 2002). The extracellular matrix is formed by exopolysaccharides, proteins and extracellular DNA (Karatan, Watnick 2009). In mucoid *P. aeruginosa* strains, the matrix polysaccharide is constituted mainly by alginates (Evans, Linker 1973). In non-mucoid strains, the matrix may be formed by
glucose-rich polysaccharides specified by the *pel* locus (Karatan, Watnick 2009) and/or mannose- and galactose-rich polysaccharides specified by the *psl* locus (Friedman, Kolter 2004). The proteins usually found in the matrix belong either to the pili/fimbrie family or to the lectins family (Karatan, Watnick 2009). The function of extracellular DNA in biofilm is uncertain but its presence is essential for the integrity of the biofilm (Whitchurch *et al.* 2002). Many systems intervene in the regulation of biofilm formation, including quorum sensing, secondary messengers and two-component systems (Karatan, Watnick 2009). Remarkably, some systems seems to regulate biofilm and T3SS in opposite manner: the sensor kinase RetS represses biofilm formation but increases T3SS expression while the two-component system GacSA increases biofilm formation but represses T3SS expression (Karatan, Watnick 2009).

**Regulatory networks and cell-to-cell communication**

In order to achieve high adaptability to different environments, a large proportion of the *P. aeruginosa* genome is devoted to regulation of the expression of genes involved in response to changing environmental conditions (Stover *et al.* 2000) and to communicate the changes required for adaptation to other surrounding cells. For these purposes, *P. aeruginosa* has complex signalling systems based on diffusible molecules.

**Quorum sensing**

Quorum sensing allows bacteria to sense their population density (Jimenez *et al.* 2012) and regulate the expression of many genes by employing low-molecule weight compounds called autoinducers (Williams, Camara 2009).
**AHLs system**

*N*-Acyl homoserine lactones (AHLs)-based quorum sensing systems were the first systems to be discovered and are most frequently found in Gram-negative bacteria (Whitehead et al. 2001). AHLs are synthesized by LuxI-like proteins and sensed by LuxR-like transcriptional regulators (Fuqua, Winans & Greenberg 1996). In *P. aeruginosa*, there are two known AHLs systems: LasI/LasR (Pearson et al. 1994) and RhlII/RhlR (Pearson et al. 1995). They produce and sense two different autoinducers, 3-oxo-C12-HSL for Las and C4-HSL for Rhl (Jimenez et al. 2012). They both control the expression of *lasA* and *lasB* (Toder, Gambello & Iglewski 1991, Jones et al. 1993), *aprA* (Gambello, Kaye & Iglewski 1993) and *xpcP* and *xpcR* codifying for T2SS components (Jimenez et al. 2012). Furthermore, the RhlR/C4-HSL complex represses the expression of T3SS genes while activates *rhlAB* inducing production of rhamnolipids (Bleves et al. 2005). It also controls production of alternative σ factor *rpoS* (Latifi et al. 1996), pyocyanin and hydrogen cyanide (Jimenez et al. 2012). On the other hand, LasR/3-oxo-C12-HSL controls expression of *toxA* (Gambello, Kaye & Iglewski 1993) and induces *rhlI*, evidence of the hierarchical structure of the two systems (Latifi et al. 1996) at least in some growth conditions (Duan, Surette 2007). Considering the large numbers of virulence factors controlled by AHL systems in *P. aeruginosa*, it is not surprising that deletion of genes encoding either LasI/LasR or RhlII/RhlR leads to reduced virulence in many models of infection (Rumbaugh et al. 1999, Smith et al. 2002, Pukatzki, Kessin & Mekalanos 2002, Tan et al. 1999).

**PQS system**

The PQS system is exclusively found in *Pseudomonas* and *Burkholderia* spp. (Pesci et al. 1999) and it employs 4-quinolones as signal molecules. In *P. aeruginosa*, the precursor HHQ [2-heptyl-4(1H)-quinolone] is synthesized by the products of the first...
four genes of the *pqsABCDE* operon and transformed in the final *Pseudomonas* quinolone signal or PQS [2-heptyl-3-hydroxy-4-quinolone] by PqsH (Jimenez et al. 2012, Bredenbruch et al. 2005). Once PQS has reached a certain threshold, it binds with its receptor PqsR (or MvfR) and activates expression of *pqsABCDE* and of the adjacent *phnAB* operon with consequent increased production of PQS and pyocyanin, respectively (Deziel et al. 2005). PqsE is not involved in HHQ synthesis but it affects expression of pyocyanin, lectin, rhamnolipids and hydrogen cyanide (Gallagher et al. 2002, Jimenez et al. 2012). As expected, mutations in either *pqsA* or *pqsE* reduced the virulence of the bacteria in several models of infection (Rampioni et al. 2010).

**Two-component systems**

Two-component systems (TCSs) are a widespread signalling system among bacteria where they provide the vital ability to sense the extracellular milieu and to tune bacterial output accordingly (Mikkelsen et al. 2013). Many TCSs consist of a transmembrane sensor kinase capable of autophosphorylation upon detection of a specific signal and of a response regulator usually working as transcription factor (Galperin 2010) and leading to changes in gene expression (Jimenez et al. 2012).

One of the best characterized TCS in *P. aeruginosa* is the global activator of antibiotic and cyanide synthesis (GAC) system that has been implicated in bacterium-host interactions (Mulcahy et al. 2008). Once activated by as yet unknown signals, the transmembrane sensor kinase GacS phosphorylates the GacC regulator which in turn upregulates the expression of two small RNA, RsmZ and RsmY (Jimenez et al. 2012). The overall effect of activating these small RNAs is to turn down phenotypes associated with acute infection and turn up phenotypes associated with chronic infection (Mulcahy et al. 2008).
**Nucleotide-based signals**

In bacteria, some of the intracellular signals are relayed by nucleotide-based molecules. Cyclic AMP, or cAMP, is synthesised in *P. aeruginosa* by the action of two adenylate cyclases, CyaB and CyaA (Wolfgang *et al.* 2003). cAMP may increase the expression of *toxA* (exotoxin A), type IV pili, T3SS and LasI/LasR quorum sensing genes by binding to the virulence factor regulator VfR (Beatson *et al.* 2002, Albus *et al.* 1997) whose deletion lead to loss of virulence (Smith, Wolfgang & Lory 2004).

The level of cyclic di-GMP (c-di-GMP) is controlled in bacterial cells by two types of enzyme: diguanylate cyclases that synthesise it and phosphodiesterases that degrade it (Jimenez *et al.* 2012). c-di-GMP controls a large number of phenotypes including biofilm formation, long-term survival, small-colony variant adaptation and virulence in plants and animals (Jimenez *et al.* 2012).
Non-mammalian model organisms to study \textit{P. aeruginosa}

The use of mammalian models has been invaluable to establish the method of action of many virulence factors and to study the host immune response. In the field of \textit{P. aeruginosa}, murine models (\textit{Mus musculus}) have been employed to mimic infection occurring after burns (Calum, Hoiby & Moser 2014), from wounds (Trostrup \textit{et al.} 2013), in lungs or in bloodstream depending on how the bacterium is delivered to the mouse (Takase \textit{et al.} 2000b). Due to the ethical issues and high costs attached to the use of mammals in research setting, non-mammalian models have been introduced by exploiting the ability of many human pathogens to produce universal virulence factors affecting vertebrate, invertebrates and unicellular bacterial predators (Kurz, Ewbank 2007). \textit{Dictyostelium discoideum}, an amoeba, \textit{Caenorhabditis elegans}, a nematode, \textit{Drosophila melanogaster}, an insect, and \textit{Danio rerio}, a fish also known as zebrafish, have been instrumental in expansion of our knowledge of host-pathogen interactions because they are easy to culture or rear (Steinert, Heuner 2005), such organisms can be modified genetically (Pradel, Ewbank 2004) and they can be used in relatively high-throughput screenings (Styer \textit{et al.} 2005, Benghezal \textit{et al.} 2006). Moreover, the evolution of bacterial traits to avoid predation from organisms such as amoeba and nematodes is likely to predate the use of these factors in human infection (Kurz, Ewbank 2007, Irazoqui, Urbach & Ausubel 2010) and simpler organisms may provide the advantage to focus on few aspects of the host-pathogen interaction (Irazoqui, Urbach & Ausubel 2010).
**Caenorhabditis elegans, the multicellular model**

*Caenorhabditis elegans* (*C. elegans*) is a free-living organism first described in the 1900 and classified in the *Nematoda* phylum (Riddle *et al.* 1997) [also referred here as nematode or worm]. It dwells in the soil or on vegetation of temperate zones where it feeds on bacteria (Altun, Hall 2009).

**C. elegans anatomy and life cycle**

In its adult stage, *C. elegans* reaches ~1 mm in length (Altun, Hall 2009) and its body can be divided in three parts: the head, the mid-body and the tail (Figure 1-1). The head contains the grinder, to crush food, the pharynx, to push food into the grinder, and the pharyngeal-intestinal valve, to allow the passage of crushed food into the intestine (McGhee 2007). The mid-body section is surrounded by the hypodermis that secretes cuticles functioning as exoskeleton (Altun, Hall 2009). The intestine, where degradation of bacteria, absorption and storage of nutrients occurs (McGhee 2007), the gonads, the pseudocoelom (or body cavity) and the muscles reside beneath the hypodermis (Altun, Hall 2009). Finally the tail contains the anus where defecation occur (Altun, Hall 2009).

The *C. elegans* population is predominately formed by hermaphrodites that produce both sperm and oocytes and lay fertilized eggs in which early embryogenesis takes place (Figure 1-2). After hatching, the nematode undergoes four mouls (larval stages named L1 to L4) before becoming an adult able to lay eggs (Riddle *et al.* 1997, Altun, Hall 2009). Interestingly, the time spent at each stage is temperature-dependent taking ~ 2.5 days at 25°C or ~ 6 days at 15°C to complete the cycle (Byerly, Cassada & Russell 1976).
Figure 1-1: *C. elegans* anatomy

A. Schematic drawing of *C. elegans* anatomical structures, left lateral side [Source: Altum, Hall (2009)]  

B. Image of *C. elegans* obtained with 40× objective in bright field at Universiti Kabangsaan Malaysia; the lack of eggs is due to RNAi treatment and allows for clearer imaging of other organs in the mid-body section of nematodes in particular the intestine. Images of six sections of the nematode were taken and assembled using GIMP 2 software.
Figure 1-2: *C. elegans* life cycle

Schematic representation of the *C. elegans* development [Source: Altum, Hall (2009)]. Blue numbers along the arrows indicated the time required for each stage at 22°C; L1, L2, L3 and L4 represent the nematode larval stages; Dauer is a facultative stage that may appear in hostile environments. Inside the adult nematode, sperm and oocytes combine to produce eggs that are laid at the gastrula stage. After development outside the utero, the eggs may hatch producing nematode at L1 stage. If food and favourable growth conditions are present, the normal development of the nematode continues until it become adult and may start to produce eggs.
Importance of *C. elegans* as a model of infection

There are several characteristics that set apart *C. elegans* to be a model organism to study infection. This nematode can be stored unaltered for years and maintained in lab by feeding on an *Escherichia coli* (*E. coli*) strain called OP50 (Stiernagle 2006) generating ~ 300 offspring that are identical to the progenitor (Altun, Hall 2009). Moreover, it has a transparent body (Altun, Hall 2009) allowing for direct microscope examination in particular when using Green Fluorescent Protein (GFP) or other fluorescent compounds (Irazoqui, Ausubel 2010). *C. elegans* genome was completely sequenced in 1998 (C. elegans Sequencing Consortium 1998) allowing its genetic manipulation (Fay 2013). More recently, the discovery of RNA interference (RNAi) has permitted the control of expression of genes without the need for genetic modifications (Ahringer 2006, Billi, Fischer & Kim 2014).

*C. elegans* was initially employed as model organism by Sydney Brenner who used N2 (Bristol) strain to study its development and nervous system (Brenner 1973, Brenner 1974, Brenner 1988). Subsequently, *C. elegans* has been employed as a model to study programmed-cell death (Metzstein, Stanfield & Horvitz 1998), signal transduction (Kornfeld 1997), sex determination (Marin, Baker 1998), embryogenesis, nervous and muscle systems development, ageing (Hekimi *et al.* 1998) and RNA interference (Fire *et al.* 1998). Moreover, it has more recently been used to study microorganism infections (Kurz, Ewbank 2007) because different bacterial strains can be fed to the nematodes by growing them on media prepared in lab (Sifri, Begun & Ausubel 2005).

Since the second half of 1990s, researchers have employed *C. elegans* to study host-pathogen interactions (Darby 2005). The pathology exerted on *C. elegans* may be monitored by following nematode survival (Tan, Mahajan-Miklos & Ausubel 1999), changes in behaviour (Zhang, Lu & Bargmann 2005) or in appearance (Hodgkin,
Kuwabara & Cornelissen 2000), accumulation of bacteria in the intestine (Aballay, Yorgey & Ausubel 2000) and by monitoring gene expression via qRT-PCR (Troemel et al. 2006) or reporter transgenic systems (Irazoqui et al. 2008).

In order to validate it, several publications have compared *C. elegans* with murine models of infection. *C. elegans* and *M. musculus* share some similarities (they both have innate immunity) but *C. elegans* does not have circulatory system, professional phagocytes, an adaptive immune system (Sifri, Begun & Ausubel 2005) and cannot be reared at 37°C, temperature at which some virulence factors are expressed (Wurtzel et al. 2012). Notwithstanding these dissimilarities, many mutants that appear attenuated in *C. elegans* infection also displayed attenuation in the mouse models (Table 1-5).

*C. elegans* actively employ innate immunity as defence against infection (Ewbank 2006). Some innate immune pathways are conserved when compared to mammalian cells such as the p38 MAPK and the insulin-like pathways. p38 mitogen-activated protein kinase (MAPK) pathway leads to activation of antimicrobial peptides and programmed cell death important to confer resistance to *P. aeruginosa* infection (Kim et al. 2002). With the DAF-2/DAF-16 insulin-like signalling pathway, the activation of DAF-2 has a pleiotropic outcome affecting metabolism, lifespan, reproduction, development, stress resistance and innate immunity (Wolkow et al. 2000, Garsin et al. 2003). In contrast with mammalian cells, *C. elegans* does not possess a NF-κB-like transcriptional regulator (Pujol et al. 2001) and has only one Toll-like receptor (TOL-1). TOL-1 is responsible only for part of the nematode immune response to pathogens because its expression is activated by some bacteria, for example *Salmonella enterica*, but not by others, for example *P. aeruginosa* or *Staphylococcus aureus* (Tenor, Aballay 2008, Irazoqui, Urbach & Ausubel 2010).
<table>
<thead>
<tr>
<th>Bacterium tested</th>
<th>C. elegans strain</th>
<th>Purpose of using C. elegans</th>
<th>Type of assay employed</th>
<th>Comparison with Mus musculus</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| *P. aeruginosa*  | N2                | Screen a transposon library to identify mutants with attenuated virulence | ● Slow-killing assay  
● Fast-killing assay | 21 interrupted genes showed attenuation in *C. elegans*, 19 of which were also attenuated in mouse full-thickness skin burn model | (1) |
| *Salmonella enterica* serovar Typhimurium | N2 and JK509 | Relevance of Dam activity for *S. enterica* virulence | ● Plate killing assay  
● Shifting assay  
● Liquid killing assay | *dam*-negative mutants were attenuated in *C. elegans* assay and non-virulent in mouse | (2) |
| *Staphylococcus aureus* | N2 | Effect on virulence of knocking out *dsbA* | ● Plate killing assay | No difference between WT and mutant was recorded in both *C. elegans* and mouse | (3) |
| *Yersinia pestis* | N2 and glp-4 | Establishment of *C. elegans* - *Y. pestis* model to identify non-biofilm related virulence factors | ● Plate killing assay  
● Shifting assay  
● 24-well plate assay | 50% of the strains attenuated in *C. elegans* were also attenuated in mouse | (4) |
| Extra-intestinal Pathogenic *Escherichia coli* | fer-15 | Establishment of a *C. elegans* - pathogenic *E. coli* model | ● Plate killing assay | There was a significant correlation between virulence in *C. elegans* and murine septicaemia model | (5) |
| *Salmonella enterica* serovar Typhimurium | SS104 | Assess fitness of antibiotic-resistant strains | ● Plate killing assay  
● CFU/worm count  
● Competition assay | Relative fitness measured with *C. elegans* was similar to the relative fitness measured with mice for most strains | (6) |

**Table 1-5: Nematodes vs. mice as models to study human bacterial pathogen infectivity**

Comparison between the use of *C. elegans* and *Mus musculus* to test human pathogens mutants for attenuation in virulence. *C. elegans* strain used: N2 is the original wild-type strain; JK209, glp-4, fer-15 and SS104 are temperature sterile mutants unable to produce eggs at 25°C derived from N2; Reference: (1)= Tan *et al.* (1999); (2)= Oza, Yeh & Reich (2005); (3)= Dumoulin *et al.* (2005); (4)= Styer *et al.* (2005); (5)= Diard *et al.* (2007); (6)= Paulander *et al.* (2007).
Use of *C. elegans* to study *P. aeruginosa* infection

The *C. elegans*/*P. aeruginosa* system was the first established to study host-pathogen interactions (Tan, Mahajan-Miklos & Ausubel 1999, Mahajan-Miklos *et al.* 1999, Tan *et al.* 1999). Since then, several assays have been established in which the nematode killing mechanisms depend on specific growth conditions of the bacterium. Remarkably, *P. aeruginosa* factors involved in killing showed very little overlapping between different conditions used in assays to evaluate the nematode survival (Tan *et al.* 1999, Garvis *et al.* 2009, Feinbaum *et al.* 2012, Kirienko *et al.* 2013).

**Slow-killing assay**

The slow-killing assay (SKA) is done by plating *P. aeruginosa* on modified nematode growth medium or NGM (Tan, Mahajan-Miklos & Ausubel 1999). In these conditions, worms seeded on different strains will die at different rates through an infection-like mechanism. Some bacterial strains, such as PA14, will in as little as 2.5-3 days kill all the nematodes, while others strains, such as PAO1, will take almost 2 weeks (Tan, Mahajan-Miklos & Ausubel 1999, Lee *et al.* 2006).

In the slow-killing assay conditions, *P. aeruginosa* PA14 killing has been associated with abnormal accumulation of this microbe in the intestine when compared to the non-pathogenic *E. coli* OP50 and to PA14 attenuated mutants (Tan, Mahajan-Miklos & Ausubel 1999). Infection by PA14 altered the morphology of nematode intestinal cells and ultimately resulted in bacterial invasion (Irazoqui *et al.* 2010). Moreover, PA14 cells produced outer membrane vesicles and an unknown extracellular matrix to envelop them (Irazoqui *et al.* 2010).

Extensive work has been undertaken to identify the *P. aeruginosa* genes involved in virulence against *C. elegans* in the slow-killing assay through creation of *P. aeruginosa*
mutant libraries followed by screening assays (Tan et al. 1999, Garvis et al. 2009, Feinbaum et al. 2012, Kirienko et al. 2013) or targeted gene mutations (Yorgey et al. 2001, Hendrickson et al. 2001). Interestingly, the bulk of the work has been done using the PA14 strain (Tan et al. 1999, Garvis et al. 2009, Feinbaum et al. 2012, Kirienko et al. 2013) and only recently has work been done using a strain, TBCF10839, derived from a cystic fibrosis patient (Garvis et al. 2009). Of the identified PA14 or TBCF10839 genes, several have known or putative functions (Table 1-6) but many remain uncharacterized. P. aeruginosa seems to affect C. elegans survival mainly through regulatory gene products such as transcriptional and quorum sensing regulators, signal transduction systems and σ factors. Other contributors to virulence are gene products involved in response to adverse conditions (siderophores, stress-related proteins and catabolism), motility, chemotaxis and membrane or periplasmic proteins (see Table 1-6 for references). Surprisingly, secretion systems did not appear to have a role in P. aeruginosa virulence toward C. elegans under the conditions tested, while they are known to be relevant in other models (Miyata et al. 2003). Regarding accessory genome traits, the PA14 clone C-specific region and PAPI-1 have shown strong and intermediate correlation, respectively, with C. elegans killing (Lee et al. 2006). Moreover, a replacement island, related to the LPS O-antigen synthesis, was also involved in C. elegans killing (Lee et al. 2006).

**Fast-killing assay**

In the fast-killing assay (FKA), bacteria are grown on a rich media with a high salt concentration compared to NGM named PGS (Tan, Mahajan-Miklos & Ausubel 1999). In these conditions, death occurs within few hours from the start of the experiment, quicker compared to slow-killing assay, due to the production of low-molecular-weight toxins by P. aeruginosa (Mahajan-Miklos, Rahme & Ausubel 2000). Several PA14
gene products have been implicated in killing worms in the fast-killing assay: (i) \textit{hrpM}, homologues to a \textit{P. syringae} plant virulence factor (Aballay, Ausubel 2002); (ii) two phenazines, phenazine-1-carboxilic acid and pyocyanin, has been shown to mediate PA14 death in a pH-dependent manner (Cezairliyan \textit{et al.} 2013); (iii) \textit{mexA}, a multidrug transporter (Mahajan-Miklos, Rahme & Ausubel 2000).

**Killing by paralysis assay**

The \textit{C. elegans} killing by paralysis assay is performed by plating \textit{P. aeruginosa} on brain-heart infusion (BHI) agar (Gallagher, Manoil 2001). By using this assay and screening a PAO1 transposition library, it was possible to identify several genes involved in the regulation of virulence factors such as \textit{lasR} or \textit{gacS}. In the conditions used in this assay, the regulatory genes were involved in production of cyanide because most mutants showed very little production of it compared to the wildtype. Therefore, it was concluded that paralysis induced by cyanide inhibition of mitochondrial function was the cause of nematodes demise in these conditions, a phenomenon that would contribute to the opportunist nature of \textit{P. aeruginosa} infections (Gallagher, Manoil 2001).

**Phosphate depletion assay**

Since phosphate depletion is observed after major surgery and is a predictor of subsequent sepsis (Shor \textit{et al.} 2006), it was hypothesized that lack of phosphate in the media could induce a lethal phenotype in \textit{P. aeruginosa} (Zaborin \textit{et al.} 2009). Without phosphate added to NGM, an attenuated PAO1 strain became highly virulent and death occurred after the appearance of red coloured material in the nematodes (named “red death”). As expected, subsequent analysis of \textit{P. aeruginosa} showed that phosphate signalling or uptake systems were essential to exert virulence in absence of phosphate.
but that pyoverdine (but not pyochelin) and the quinolone quorum sensing system were also required (Zaborin et al. 2009).

**Liquid medium assay**

In order to use *C. elegans* for high throughput screening of *P. aeruginosa* mutants, two assays carried out in liquid media have been established. They both allow the automated distribution of age synchronised *C. elegans* in 96-wells (Garvis et al. 2009) or 386-wells plates (Kirienko et al. 2013) but while the former is scored manually the latter is scored automatically using image analysis software. Moreover, the use of distinct liquid media, *C. elegans* strains and *P. aeruginosa* strains highlighted the involvement of different virulence factors. Garvis et al. showed that in a cystic fibrosis-derived *P. aeruginosa* strain (TBCF10839), the products of chemotaxis-related gene cheB2, thiol:disulphide interchange protein gene dbsD2, endonuclease subunit gene uvrC, histidine kinase sensor gene colS, type IV fimbrial biogenesis gene pilY1, flageller motor protein gene motB and proton-glutamate symporter gene gltP were involved in virulence. However, only some mutants (Table 1-6) were confirmed to have attenuated virulence also in the slow-killing assay (Garvis et al. 2009). On the other hand, Kirienko et al. showed that only the slow-killing assay attenuated PA14 kinB mutant was confirmed to be less virulent in these experimental conditions (Kirienko et al. 2013).
<table>
<thead>
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<th>Gene/Cluster</th>
<th>Reference</th>
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<td><em>toxA</em></td>
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</table>

**Table 1-6: P. aeruginosa virulence-related genes involved in SKA**

List of genes or cluster of genes with known or putative function involved in *P. aeruginosa* virulence against *C. elegans* in slow-killing assay. *McEwan, Kirienko, Ausubel (2012) showed no association between deletion of *toxA* and virulence using *C. elegans* N2 strain.*
Acanthamoeba spp., the unicellular infection model

Acanthamoeba spp. are unicellular, eukaryotic organisms part of the Amoebida order. They are protozoa, found either as free-living or as parasitic organisms (Marciano-Cabral, Cabral 2003). They are distributed ubiquitously, both in natural and man-made environments (Kingston, Warhurst 1969, Paszko-Kolva et al. 1991, Mergeryan 1991). Two species of Acanthamoeba are used in bacterial virulence assays: Acanthamoeba polyphaga (A. polyphaga) and Acanthamoeba castellanii (A. castellanii). A. polyphaga was isolated from dust (Puschkarew 1913) while A. castellanii was isolated from a yeast culture (Castellani 1930) but both were initially assigned different names. Subsequently the genus Acanthamoeba was created and they were both re-classified as species belonging to this genus (Volkonsky 1931, Page 1967).

Acanthamoeba life cycle and morphology

Acanthamoeba spp. may be found either as trophozoite or as cyst (Marciano-Cabral, Cabral 2003). When optimal growth conditions are present, the trophozoites predominate. In this form, the amoebas feed on other microorganisms and reproduce by binary scission (Byers 1979). In harsher environmental conditions (Chagla, Griffiths 1974), the amoebae enter in a dormant state, the cyst, which may last for very long time and its able to survive high adverse environs (Mazur, Hadas & Iwanicka 1995).

As trophozoites, Acanthamoeba possess many of the organelles that characterize eukaryotic cells (Bowers, Korn 1968): Golgi complex, smooth and rough endoplasmic reticula, ribosomes, vacuoles and mitochondria. Members of this genus are also characterized by the formation of surface projection called acanthopodia (Page 1967), a nucleus containing a distinct nucleolus and a contractile vacuole that controls the water content of the cell (Marciano-Cabral, Cabral 2003).
**Acanthamoeba feeding and culture methods**

*Acanthamoeba* spp. are heterotrophs, that is they are unable to synthesize carbon-based organic compounds from inorganic sources (Khan 2009) therefore requiring the acquisition of organic material. The uptake of food may occur through two mechanisms (Bowers 1977): pinocytosis allows absorption of nutrient from liquid medium while phagocytosis allows the uptake of particulate matter such as microorganisms. *Acanthamoeba* spp. can specifically recognize certain bacteria and internalize them while they fail to internalize others (Marciano-Cabral, Cabral 2003). Moreover, once internalized some bacteria escape or survive digestion usually because of production of pigments, toxins or outer membrane structures (Singh 1942).

In laboratory, *Acanthamoeba* cultures can be isolated or maintained on non-nutrient agar covered by a lawn of *E. coli* or *Enterobacter aerogenes* (Visvesvara 1987). They can also be maintained axenically (without bacteria as food) in undefined liquid media such as PYG (Visvesvara, Balamuth 1975), Cline medium (Marciano-Cabral 1988) or Ac^6* (Kilvington, Price 1990) or by using mammalian cells (De Jonckheere 1980).
Free-living amoeba and their interaction with human bacterial pathogens

Free-living amoebae are of paramount importance from both ecological and evolutionary point-of-view. These protozoa represent a major predator for bacterial biofilms in nature forming a prey-predator relationship with them (Pickup, Pickup & Parry 2007a). The constant grazing by protozoa may create amoeba-resistant bacteria e.g. bacteria that have evolved mechanisms to prevent internalization or digestion (Greub, Raoult 2004).

*Acanthamoeba* spp. have been used as surrogate to study eukaryotic cells functions (Marciano-Cabral, Cabral 2003). However, after the first establishment of *Acanthamoeba* spp. involvement in *Legionella pneumophila* (*L. pneumophila*) virulence traits selection and transmission (Rowbotham 1980), evidence of free-living amoeba promoting the adaptation of opportunist pathogens to survival in macrophages has been mounting (Birtles *et al.* 1997).

*L. pneumophila-Acanthamoeba* spp. interaction is probably the best known and most studied (Kilvington, Price 1990, Rowbotham 1986, Murga *et al.* 2001). Yet, a large number of clinically relevant bacteria have been associated with *Acanthamoeba* including *Mycobacterium* spp. (Salah, Ghigo & Drancourt 2009), *Burkholderia* spp. (Landers *et al.* 2000), *E. coli* O157:H7 (Barker, Humphrey & Brown 1999), *Helicobacter pylori* (Winiecka-Krusnell *et al.* 2002) and *P. aeruginosa*.

**Acanthamoeba spp. and P. aeruginosa**

The *P. aeruginosa-Acanthamoeba* system has been studied from the prey-predator prospective because *P. aeruginosa* has been isolated from *Acanthamoeba* obtained from natural environments or from contact lenses solutions (Michel, Burghardt & Bergmann 1995). In these ecological studies, *P. aeruginosa* was able to inhibit *A. castellanii*
growth when a rich medium was used compared to buffer saline solution and prevent amoebae growth when present at concentration of 100:1 bacterium:amoeba (Wang, Ahearn 1997). Moreover, *P. aeruginosa* seemed to adhere to *Acanthamoeba* cells better than *E. coli* (Bottone et al. 1994). More recently, it was established that *A. castellanii* growth was more efficient on live *P. aeruginosa* instead of heat-killed bacteria (Pickup, Pickup & Parry 2007b). *P. aeruginosa* showed anti-amoeba defence mechanisms such as formation of microcolonies (Weitere et al. 2005) and different *P. aeruginosa* strains showed a differential ability to support *Acanthamoeba* growth (de Moraes, Alfieri 2008, Qureshi et al. 1993).

Attempts to underpin the specific *P. aeruginosa* factors involved in *A. castellanii* survival have been made. Impairment of Rhl quorum sensing did not interfere with ability of PAO1 to kill *A. castellanii* while deletion in *mucA* (alginate production regulator) or *rpoN* (alternative σ factor) had a measurable impact in reducing virulence (Matz et al. 2008). The killing of *A. castellanii* was diminished by T3SS deficiency or deletion of effectors genes (Matz et al. 2008, Abd et al. 2008) but deletion of *toxA* did not affect *A. castellanii* survival (Abd et al. 2008).

**Non-nutrient agar assay**

The non-nutrient agar (NNA) has been employed in the laboratory to isolate free-living amoeba from various samples by using easy-to-digest bacteria as food. NNA medium is prepared by mixing a diluted saline solution (Page’s amoebal salt solution) with agar (Visvesvara 1987) and the bacterial suspension is spread on the surface of the agar. In these conditions the bacteria do not have any food source and cannot multiply (Neff 1958). If amoebae are present in the sample tested, they will form visible clear plaques while feeding on the bacteria.
In the non-nutrient agar assay used to explore *P. aeruginosa* virulence, *Acanthamoeba* spp. trophozoites are spotted on the centre of the plate after it has been pre-layered with *P. aeruginosa* (Fenner *et al.* 2006). In these conditions, it was shown that blood culture-derived *P. aeruginosa* strains were more likely to be resistant to *A. polyphaga* culture grazing than environmental strains (Fenner *et al.* 2006).

**PYG agar/broth assay**

PYG or peptone-yeast extract-glucose is a rich medium able to support both bacterial and amoeba growth independently. PYG agar and *A. polyphaga* were used as a model for phagocytosis in a highthroughput assay to screen *Photorhabdus asymbiotica* cosmid library (Waterfield *et al.* 2008). Among the *P. asymbiotica* interrupted genes involved in virulence toward *A. polyphaga* the following were identified: hemaglutinin-like genes, type IV and VI secretion systems, fimbrial operons, prophages, invasines and cytotoxines (Waterfield *et al.* 2008). PYG broth was also used to study the effect of *P. aeruginosa* type III secretion system effectors on *A. castellanii* in which expression of ExoU completely restored PA103 virulence while partial restoration was achieved when ExoS and ExoT were expressed. Expression of ExoY or lack of exotoxin A production did not affect PA103 virulence against *A. castellanii* in this conditions (Abd *et al.* 2008).
Aims

*P. aeruginosa* genome is large and plastic due to several processes including acquisition of genomic islands through horizontal gene transfer. It is generally considered that genomic islands contribute to *P. aeruginosa* virulence in the human host but less is known about their contribution in bacterial survival in other environments in particular in presence of naturally occurring bacterivorous. Consequently, two organisms, a multicellular invertebrate and a unicellular amoeba that naturally pray on this bacterium, were adapted to screen *P. aeruginosa* genomic island deletion mutants created by homologues recombination and *P. aeruginosa* clinical isolates for which the presence of type-three secretion system effector *exoU*-bearing island was assessed alongside presence of other effector genes and site of isolation from patients. The accumulation of more information regarding both the predators, *C. elegans* and *Acanthamoeba*, and the prey, *P. aeruginosa*, may provide insights on how specific genetic elements are maintained and evolve in the natural environments and eventually effect clinical outcomes for opportunist pathogens. Specifically, the aims of this project were:

1. To test survival of *C. elegans* and *Acanthamoeba* spp. using previously made *P. aeruginosa* PA14 mutants lacking PAPI-1 and/or PAPI-2 genomic islands (Harrison *et al.* 2010) and compare them with PA14 wildtype and assess whether presence of these islands in PA14 affect survival of *C. elegans* and/or *Acanthamoeba* as they did for mice
2. To use *C. elegans* to discover previously unidentified *P. aeruginosa* genomic islands involved in virulence;
3. To use *C. elegans* to study virulence associated with clinical *P. aeruginosa* from different sources and genotypes;

4. To have a better understanding of *A. castellanii* and *A. polyphaga* non-nutrient agar assays as means to study virulence in *P. aeruginosa*. 
Chapter 2

Materials and Methods
Pseudomonas aeruginosa

Collection of clinical isolates

Blood or sputum samples were collected from patients referred to the diagnostic laboratory at the University Hospitals of Leicester (UHL) where isolation of P. aeruginosa was confirmed (Alleyne 2009). Bacterial cultures were frozen and subsequently retrieved at the diagnostic laboratory and streaked on P. aeruginosa-selective cysteine lactose electrolyte deficient agar (CLED) plates (Sandys 1960).

Maintenance of bacterial cultures

Upon receiving reference strains or isolates from UHL (Table 2-1), P. aeruginosa or E. coli cultures were streaked for single colonies on LA plates (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 40 μl 5 M NaOH, 15 g/l agar no. 1; Sambrook, Fritsch & Maniatis 1989) and incubated at 37°C overnight. Plates with cultures were kept in fridge at 4°C for short-term storage. Long-term storage was done by collecting bacterial lawn from LA plates using 30% w/v glycerol in brain-hearth infusion (BHI; Oxoid CM1135) broth and storing at -20°C or -80°C.

T3SS effector genes colony PCR assays

P. aeruginosa strains were collected from -20°C or -80°C frozen stock, streaked for single colonies on LA plates and grown overnight at 37°C. 30 μl of sterile TE (10 mM Tris-HCl, pH= 8.0; 5 mM EDTA, pH= 8.0) were aliquoted into 1.5 ml tubes and two to
four colonies from a strain were added to a tube. Tubes were heated at 100°C for 5 min and then spun at 13,000 × g for 2 min.

A PCR master mix was prepared by using 5 × GoTaq Green PCR Buffer, 10 mM dNTPs, 5% DMSO, 10 µM forward and reverse primer (Table 2-2) and 5 U/µl of GoTaq (Promega). Then 23 µl of master mix was transferred to a 0.2 ml PCR tube and 2 µl of colony DNA prep was added. Genomic DNA prepared from PAO1 and PA14 strains were used as controls. The PCR thermocycler was set up in the following way: 1 cycle at 95°C for 5 min; 25 cycles at 95°C for 30 sec, 51-54°C for 30 sec (depending on primers pair; Table 2-2), and 72°C for 30 sec; 1 cycle at 72°C for 10 min. An aliquot of 10 µl of PCR sample was then run in a 1% agarose gel with 5 µg/ml Ethidium bromide. PCRs were repeated twice for each strain using the same protocol. In case of discordant results, PCR was repeated a third time.

Association between effector genes prevalence and other categorical variables (i.e. source of isolation of strains or development of pigmentation) was tested using the Pearson χ² test as calculated by SPSS software (SPSS) and considered significant if P-value was below 0.05. If significant, the strength of the association was measured using Cramer V. The relationship was weak if the Cramer V coefficient was between 0 and 0.1, intermediate if between 0.1 and 0.5 or strong if above 0.5 (Field 2009).
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**Table 2-1: Bacterial strains used in this project**

List of the strains used in this project; all of them but *E. coli* (OP50), were *P. aeruginosa* strains. For each strain, the designation lab number, the alternative denomination if available, the source of isolation and the reference are indicated. “Lab” indicates that the strain was genetically engineered using homologous recombination; CF= from cystic fibrosis patient; UHL= University Hospitals of Leicester; *= these strains were kindly donated by Dr Winstanley at the University of Liverpool; *= unpublished data.
<table>
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<tr>
<td>KR1844</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
<tr>
<td>KR1845</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
<tr>
<td>KR1846</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
<tr>
<td>KR1847</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
<tr>
<td>KR1848</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
<tr>
<td>KR1849</td>
<td>/</td>
<td>Blood</td>
<td>UHL</td>
</tr>
</tbody>
</table>

**Table 2-1 (continued): Bacterial strains used in this project**

<table>
<thead>
<tr>
<th>Effector gene</th>
<th>Primers</th>
<th>Annealing T</th>
<th>Band size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>exoU</em></td>
<td>5’-GGAATACCTTTCCGGGAAGTT 5’-CGATCTCGGTGATAATGTGTT</td>
<td>51°C</td>
<td>428 bp</td>
<td>Allewelt <em>et al.</em> (2000)</td>
</tr>
<tr>
<td><em>exoT</em></td>
<td>5’-CGAGATCAAGCAGATGATG 5’-TTTACCTCGCTCTCTACCG</td>
<td>54°C</td>
<td>390 bp</td>
<td>Alleyne (2009)</td>
</tr>
<tr>
<td><em>exoS</em></td>
<td>5’-GAAAAGTACCTGGGGCAACAG 5’-TCTTGTAGTTGATATCCCG</td>
<td>53°C</td>
<td>385 bp</td>
<td>Alleyne (2009)</td>
</tr>
<tr>
<td><em>exoY</em></td>
<td>5’-CCTATACCCAGACCCGACCT 5’-ATATCTCCCTGCCCATA GA</td>
<td>53°C</td>
<td>388 bp</td>
<td>Alleyne (2009)</td>
</tr>
</tbody>
</table>

**Table 2-2: Primers used for T3SS effector genes PCR assays**
Caenorhabditis elegans assays

Maintenance of C. elegans

C. elegans N2 strain was obtained from Dr James Lonnen and Dr Simon Kilvington (University of Leicester, UK) and strain CF512 from Caenorhabditis Genetic Center (CGC, University of Minnesota, USA), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Nematodes were maintained on modified nematode growth medium plates (NGM; 3 g/l NaCl, 3.5 g/l bacteriological peptone, 17 g/l agar no. 1, 25 mM KPO₄ buffer [1 M KH₂PO₄, 1 M K₂HPO₄, pH= 6], 100 mM CaCl₂, 100 mM MgSO₄ and 5 µg/ml cholesterol; Tan, Mahajan-Miklos & Ausubel 1999) seeded with *Escherichia coli* OP50 as previously described (Stiernagle 2006). Briefly, 50 µl of *E. coli* overnight culture in LB (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 40 µl 5 M NaOH; Sambrook, Fritsch & Maniatis 1989) was plated on NGM agar and incubated at room temperature for 24 h or 8 h at 37°C. OP50 plates were kept at 4°C until needed. The nematodes were transferred to a new plate seeded with OP50 by moving a chunk of agar from an old plate. Plates with *C. elegans* were incubated at 16°C for 4-7 days until *E. coli* food was almost finished and a piece of agar moved to a new plate.

Slow-killing assay (SKA)

Protocol used at University of Leicester (UoL)

For this assay, *C. elegans* CF512 [fer-15(b26)II; fem-1(hc17)IV] was employed (Figure 2-1). The nematodes were synchronized by collecting them from 3-4 days old OP50
plates and treating them with 0.5 M NaOH and 1% NaClO for 2 min. This process kills all the nematodes both at adult and larval stage leaving eggs intact (Stiernagle 2006). Eggs were collected by spinning at 1,300 × g for 30 sec and washed twice with sterile water. Eggs were then transferred to M9 solution (3 g/l of KH₂PO₄, 6 g/l of Na₂HPO₄, 5 g/l NaCl and 100 mM MgSO₄) and incubated for 16-18 h at 20°C while shaking at 220 rpm. During this time, the eggs hatch and stop at the first larval stage (L1) because of lack of food (Stiernagle 2006). L1 larvae were pulled together by cooling down the M9 solution on ice for 15 min, removing the top layer and then spinning at 1,200 × g for 5 min. L1 larvae were plated on NGM with OP50 until they reached young adult stage (2-2.5 days at 25°C).

*P. aeruginosa* strains were recovered from frozen stock, streaked on LA plates and incubated overnight at 37°C. One colony was used to inoculate 5 ml of King’s B broth (20 g/l of bacteriological peptone, 1.5 g/l of K₂HPO₄, 1.5 g/l of MgSO₄, 1% w/v glycerol; King, Ward & Raney 1954) and incubated at 37°C for 16 h while shaking at 200 rpm; 10 µl of overnight culture was plated on NGM agar plates, spread to cover the centre of the plate and incubated at 37°C for 24 h followed by 24 h at room temperature. *P. aeruginosa* plates were used immediately and nematodes were manually transferred to them. The assay was carried out at 25°C (in a humidified chamber) because CF512 is unable to produce offspring at this temperature (Hsin 2007). The plates were observed every 10-12 h and number of nematodes found alive, dead, dried on the plate plastic wall or missing was recorded. Nematodes were recognized as dead if they did not move when touched.
Figure 2-1: SKA at UoL

Schematic representation of the slow-killing assay protocol carried out at the University of Leicester.
Protocol used at Universiti Kabangsaan Malaysia (UKM)

A different strain of nematode sterile at 25°C (rrf-3;glp-4) was used for these assays and maintained on OP50 plates at 16°C until needed (Figure 2-2). *C. elegans* were collected from a crowded plate in 5 ml of sterile dH2O and transferred to a 15 ml conical tube. The same volume of lysis solution (40% household bleach, 1 M NaOH) was added in order to rupture the nematodes. Eggs were recovered using centrifugation and washed with sterile water twice. After the second wash, eggs were transferred to a fresh NGM plate with OP50 and incubated at 25°C for 3 days.

*P. aeruginosa* bacterial cultures were prepared by streaking single colonies on LA plates followed by overnight incubation at 37°C. One colony for each strain was picked with a sterile tip and used to inoculate 3 ml of King’s B broth with 100 µg/ml rifampicin. When OP50 was used as negative control, a single colony was used to inoculate 3 ml of LB. Bacterial cultures were incubated for 16-18 h at 37°C while shaking at 250 rpm. Then, 10 µl of overnight culture was plated on modified NGM plates and spread to cover the entire surface of the plate. Plates were left to dry, incubated at 37°C for 24 h and then left at room temperature for ~ 24 h. *P. aeruginosa* plates were used immediately and 50 to 80 nematodes were manually added to each plate. Then, they were incubated at 25°C and number of nematodes found alive, dead, dried on the plate plastic wall or missing was recorded twice a day (every 8-15 h).

The protocol of this assay done at Universiti Kabangsaan Malaysia was similar to the assay done at University of Leicester but there were some differences regarding the preparation of the NGM plates (Table 2-3A), of King’s broth (Table 2-3B) and nematodes synchronization (Table 2-3C).
Figure 2-2: SKA at UKM

Schematic representation of the slow-killing assay protocol carried out at the Universiti Kabangsaan Malaysia
### A. NGM plates

<table>
<thead>
<tr>
<th></th>
<th>University of Leicester</th>
<th>Universiti Kabangsaan Malaysia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>Added after autoclaving</td>
<td>Added before autoclaving</td>
</tr>
<tr>
<td>Phosphate buffer stock solution</td>
<td>$1 \text{ M } \text{KH}_2\text{PO}_4 + 1 \text{ M } \text{K}_2\text{HPO}_4$</td>
<td>$1 \text{ M } \text{KH}_2\text{PO}_4$</td>
</tr>
<tr>
<td>Plate diameter</td>
<td>60 mm</td>
<td>35 mm</td>
</tr>
<tr>
<td>Bacteria plating</td>
<td>10 µl placed in the centre of plate</td>
<td>10 µl spread on entire surface</td>
</tr>
</tbody>
</table>

### B. King’s broth

<table>
<thead>
<tr>
<th></th>
<th>University of Leicester</th>
<th>Universiti Kabangsaan Malaysia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1%</td>
<td>1.5%</td>
</tr>
<tr>
<td>MgSO\textsubscript{4}</td>
<td>Added before autoclaving</td>
<td>Added after autoclaving</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Not used</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Culture volume</td>
<td>5 ml</td>
<td>3 ml</td>
</tr>
</tbody>
</table>

### C. Age synchronization and nematodes

<table>
<thead>
<tr>
<th></th>
<th>University of Leicester</th>
<th>Universiti Kabangsaan Malaysia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs</td>
<td>Hatched in M9 solution, L1 larvae collected and plated on OP50/NGM</td>
<td>Plated directly on OP50/NGM</td>
</tr>
<tr>
<td>Strain</td>
<td>Temperature-sterile CF512 $[\text{fer-15}(\text{b26})\text{ll}; \text{fem-1}(\text{hc17})\text{ll}]$</td>
<td>Temperature-sterile $[\text{rrf-3}(\text{pk1426}); \text{glp-4}(\text{bn2ts})]$</td>
</tr>
</tbody>
</table>

**Table 2-3: Differences between UoL and UKM SKA protocols**

Table shows the differences between the slow-killing assay protocols carried out at the University of Leicester and at the Universiti Kabangsaan Malaysia regarding (A) preparation of NGM plates, (B) preparation of King’s broth and (C) preparation of eggs to synchronize nematodes at the same age.
Fast-killing assay (FKA)

Nematodes were collected from 4-7 day old OP50 plates and treated with 0.5 M NaOH and 1% NaClO for 2 min. Eggs were collected by centrifugation, washed twice with sterile dH2O and plated on OP50/NGM plates for 3 days at 15°C.

Each *P. aeruginosa* strain was prepared by inoculating 5 ml of King’s broth with a single colony from LA plates while a single colony of *E. coli* OP50 was used to inoculate 5 ml of LB. Liquid cultures were incubated at 37°C for 16 h while shaking at 200 rpm. Afterwards, 10 µl of the bacterial culture was plated on PGS agar (10 g/l of peptone, 10 g/l of glucose, 27.3 g/l of sorbitol, 17 g/l of agar no. 1; Mahajan-Miklos, Rahme & Ausubel 2000) and incubated at 37°C for 24 h followed by 8-24 h at room temperature. *P. aeruginosa* PGS plates were used immediately and 10 or 20 worms were manually transferred to them followed by incubation at 25°C in a humidified chamber. Plates were observed every 2-6 h in the first 2 days and then every 10-14 h until the end of the experiment and number of nematodes found alive, dead or missing was recorded at each examination.

Survival analysis

At the end of slow-killing or fast-killing assays, the data collected was used to perform survival analysis considering that nematodes that went missing during the experiments would be omitted (or censored). The data was tabulated to indicate whether each nematode survived, died or was censored and the time at which any of these events occurred. SPSS software was used to perform this analysis using the Kaplan-Meier method to compare survival of nematodes between different bacterial strains or species and between mutants and isogenic wildtype (SPSS). The comparison was carried out
using the Log-rank statistical test. This analysis allowed calculating the TD\textsubscript{m} or average time-to-death for the nematodes and TD\textsubscript{50} or the time required to kill 50% of the nematodes. Graphical representations were obtained using the GraphPad software (GraphPad).

**Hightthroughput assay (HTA)**

For this assay, *C. elegans* N2 wildtype strain was employed because it produces offspring at 25°C (Figure 2-3). The nematodes were synchronized by collecting them from 3-4 days old OP50 plates followed by treatment with 0.5 M NaOH and 1% NaClO for 2 min. Eggs were collected by spinning at 1,300 \( \times \) g for 30 sec and washed twice with sterile water. Eggs were then transferred to M9 solution and incubated for 16-18 h at 20°C while shaking at 220 rpm. L1 larvae were pulled together by cooling down the M9 solution on ice for 15 min, removing the top layers and then spinning at 1,200 \( \times \) g for 5 min. The L1 larvae were then plated on NGM with OP50 until they reached the young adult stage (2 days at 25°C).

In the highthroughput assay, 24-well plates were employed instead of Petri dishes. NGM agar was prepared as described above and distributed in 24-well plates (2 ml/well). *P. aeruginosa* strains were recovered from frozen stock, streaked on LA plates and incubated overnight at 37°C. One colony was used to inoculate 5 ml of King’s B broth and incubated at 37°C for 16 h while shaking at 200 rpm; 5 µl/well of overnight culture was plated on NGM agar and 4 wells for each strain were prepared. 24-well plates were incubated at 37°C for 24 h followed by 24 h at room temperature. These plates were used immediately and 4 nematodes were manually transferred to each well. The 24-well plates were incubated at 25°C in a humidified chamber for 4 days and a semi-quantitative score was assigned to each well based on the amount of nematodes
present in comparison with PAO1 and PA14 strains (Table 2-4). The final HTA score assigned to each strain resulted by calculating the average of scores from 4 wells.

Correlation analysis between HTA scores and SKA $TD_{50}$ was carried out by calculating the Pearson correlation using the SPSS software. $P$-value was considered significant if below 0.05 and relationship was considered weak if the Pearson coefficient was between 0 and 0.1, intermediate if between 0.1 and 0.5 or strong if above 0.5 (Field 2009).

Since the HTA scores distribution deviates from normality, non-parametric tests were used to compare HTA score mean values between two or more groups of strains based of their categorization (i.e. source of isolation, presence of T3SS effector genes or development of pigment). When three or more groups were compared, Kruskal-Wallis test was used to evaluate the difference between all groups. If $P$-value was significant ($< 0.05$), post-hoc test were carried out by calculating the Mann-Whitney U and considered significant if $P$-value was below 0.05. If two groups were compared, the Mann-Whitney test was carried out as first choice. All statistical tests were performed using the SPSS software while graphical representations were obtained using the GraphPad software.
Figure 2-3: Hightthroughput assay

Schematic representation of the hightthroughput assay protocol carried out at the University of Leicester.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Typical image</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No <em>C. elegans</em> were present in the well</td>
<td><img src="image0.png" alt="Typical image 0" /></td>
</tr>
<tr>
<td>1</td>
<td>Very few <em>C. elegans</em>, mainly L1 or L2 larvae at the edge of the bacterial lawn, were present in the well</td>
<td><img src="image1.png" alt="Typical image 1" /></td>
</tr>
<tr>
<td>2</td>
<td>Few <em>C. elegans</em> from larval stages to adult were present in the well</td>
<td><img src="image2.png" alt="Typical image 2" /></td>
</tr>
<tr>
<td>3</td>
<td>Several <em>C. elegans</em>, from larval stages to adult, were present in the well</td>
<td><img src="image3.png" alt="Typical image 3" /></td>
</tr>
</tbody>
</table>

**Table 2-4: Scoring system for HTA**

The high-throughput assay was scored on a scale from 0 to 6. Each level was associated with a description and an image exemplifying the well content.
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Typical image</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Many <em>C. elegans</em>, from larval stage to adult, were present in the well but bacterial lawn was still clearly visible</td>
<td>bacterial lawn</td>
</tr>
<tr>
<td>5</td>
<td>Many <em>C. elegans</em>, from larval stages to adult, were present in the well and bacterial lawn was consumed almost completely</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Many <em>C. elegans</em>, from larval stages to adult, were present in the well and bacterial lawn was consumed completely</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-4 (continued): Scoring system for HTA
**Acanthamoeba spp. assays**

**Maintenance of Acanthamoeba cultures**

*A. castellanii* (strain ATCC50370) and *A. polyphaga* (strain ATCC30871) were obtained from Dr James Lonnen and Dr Simon Kilvington (University of Leicester, UK). *Acanthamoeba* spp. were maintained as trophozoites in 25 cm² tissue culture flasks using the *Acanthamoeba* axenic growth medium, Ac⁶ (Kilvington, Price 1990). When the amoeba were near to reached confluence with more than 70% of the flask surface covered, media was discarded and 10 ml of fresh Ac⁶ was added. The cells were dislodged by tapping the flask and transferring 2 ml to a new flask with 8 ml of fresh media. *Acanthamoeba* flasks were incubated at room temperature in the dark and passaged once a week.

**Non-nutrient agar (NNA) assay**

*P. aeruginosa* were streaked for single colonies, 3 to 10 colonies were picked for each strain and grown overnight on tryptone soya agar (TSA) plates at 37°C (Figure 2-4). A bacterial lawn was collected for each colony separately in sterile dH₂O and diluted to OD₆₀₀= 0.1 (corresponding to ~ 10⁸ cells/ml in *E. coli*). Then 1 ml of each bacterial suspension was plated on a non-nutrient agar plate (NNA; 1.2 mg/l of NaCl, 0.2 mg/l MgSO₄, 1.42 mg/l of Na₂HPO₄; 1.36 mg/l KH₂PO₄; 0.04 mg/l CaCl₂; 15 g/l of agar No.1), and spread to cover the entire surface and let dry completely (Fenner et al. 2006). NNA plates covered with *P. aeruginosa* were used immediately.
Acanthamoeba spp. were grown as explained previously in Ac#6. Trophozoites were harvested, washed with Phosphate Buffer Saline-Tween solution (PBS-Tween; 8 g/l of NaCl, 0.2 g/l of KCl, 1.15 g/l of Na₂HPO₄, 0.2 g/l KH₂PO₄ at pH= 7.3 with 0.05% Tween 80) and counted using a haemocytometer chamber. Then, 5 µl of amoebae suspension in PBS-Tween containing $5 \times 10^5$ cells were spotted at the centre of the NNA plates covered with P. aeruginosa. Plates were incubated at 30°C and expanding amoeba film was measured with a ruler (Figure 2-5). Two or three measurements were taken and average used.

Serial dilution was performed from the original bacterial suspension (OD₆₀₀= 0.1) prepared for each colony using sterile dH₂O. 20µl of $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were spotted on LA, let dry and incubate overnight at 37°C. The number of colonies was counted and CFU (colony forming unit)/plate calculated (number of colonies $\times 10^4$ or $10^5$ or $10^6 \times 50$).

To assess whether there were significant differences in the ability of Acanthamoeba spp. film to expand when plated on different P. aeruginosa strains or mutants, the Kruskal- Wallis test was carried out at each time point. If the $P$-value was below 0.05, pair-wise comparisons were carried out by calculating the Mann-Whitney U and considered significant if $P$-value was below 0.05. The same statistical tests were used when comparing CFU/plate between strains. All statistical analyses were carried out using SPSS software while graphical representation of the Acanthamoeba film expansion and CFU/plate were done using GraphPad software.
Figure 2-4: NNA assay

Schematic representation of the non-nutrient agar assay protocol carried out at the University of Leicester.
Figure 2-5: Measurement of amoeba film on NNA plates

Images showing the same non-nutrient agar plate after 3 days and after 9 days from the beginning of the experiment where PA14 was layer on NNA plate and Acanthamoeba added at the centre of the plate (white spot). Amoeba move from the centre forming a film with a defined edge and the diameter of the amoeba film is measured with a ruler.
Use of *Acanthamoeba* spp. and *C. elegans* infection models revealed the involvement of PAPI-2 but not PAPI-1 in *P. aeruginosa* virulence
Introduction

PAPI-1 and PAPI-2 islands

*Pseudomonas aeruginosa* pathogenicity island I (PAPI-1) and PAPI-2 were identified by comparative genomics of *P. aeruginosa* strain PA14 and the historical reference strain, PAO1 (He *et al.* 2004). Both islands are inserted within the 3’end of a tRNA<sup>lys</sup> and have been classified as intact (PAPI-I) and defective (PAPI-2) integrative and conjugative elements (ICEs) related to pKLC102 plasmid (Wurdemann, Tummler 2007) but also displaying additional distinctive characteristics.

**Features of PAPI-1**

PAPI-1 is a large modular genomic island (~108 kb) which is completely absent in PAO1 (He *et al.* 2004). This island is predicted to carry more than 100 orfs (Morales-Espinosa *et al.* 2012), many of which have not been characterized yet. In PA14, PAPI-1 is inserted at the tRNA<sup>lys47</sup> (PA14_60150), it is flanked by 58 bp direct repeats and it has a G+C content lower than the core genome (PAPI-1 = 59.7 %; core genome = 66.6 %).

PAPI-1 carries several genes involved in island mobilization such as *int*, encoding a XerC/XerD-like integrase, *soj*, encoding a chromosome-partitioning protein (Qiu, Gurkar & Lory 2006), and the *pilL2*, -N2, -O2, -P2, -Q2, -R2, -S2, T2, -V2 and -M2 cluster, encoding type IVb pili (Carter, Chen & Lory 2010). It has been shown that only a small percentage of cells produces the PilS2/PilV2 pili required for the transfer of the island through conjugation (Carter, Chen & Lory 2010) and it has been hypothesized that this same sub-set of cells is likely to carry the circular version of the island (Qiu, Gurkar & Lory 2006). PAPI-1 encodes the major type IVb pili subunit and the prepilin
PilS2, but the latter requires a chromosomally-encoded pre pilin peptidase, PilD, to be transformed in its mature version. This interplay between island and core chromosome is expected to restrict island dissemination to strains or species able to provide a suitable pre pilin peptidase (Carter, Chen & Lory 2010).

Beside mobilization-related genes, PAPI-1 carries putative virulence factors such as secreted effectors and bacteriocin/immunity protein systems, such as pyocin S5/PA14_59230 (He et al. 2004). Of the ORFs with predicted function, many show the highest degree of similarity to proteins found in both human and plant bacterial pathogens, supporting the hypothesis that this island contributes to the broad-host range of P. aeruginosa (He et al. 2004). PAPI-1 carries a chaperon-usher pathway (Cup) fimbrial cluster named CupD. This cluster is additional to the CupA, CupB, CupC and CupE clusters which are common to both PAO1 and PA14 (Winsor et al. 2011). CupD promotes biofilm formation and is antagonistically regulated by two distinct two-component systems, PvrSR and RcsBC, encoded by genes residing adjacent to cupD (Mikkelsen et al. 2009). RcsB downregulates PAPI-1 encoded type IV pili by upregulating PvrR under certain growth conditions prompting the dispersal of biofilm (Mikkelsen et al. 2013). Microarray analysis showed that RcsB also affects expression of several chromosomally-encoded genes. In particular, RcsB downregulates expression of type III secretion systems (T3SS) effectors, ExoT and ExoY, and of the genes that code for the T3SS translocation machinery, other Cup fimbriae and some adhesins (Mikkelsen et al. 2013). RcsB also increases expression of several known virulence factors such as phospholipase C (PlcB), flagella precursor (FliF), elastase (LasA) and the alginate synthesis regulator (AlgR; Mikkelsen et al. 2013). The pleiotropic effects of RcsB hint at complex core chromosome-island interplays, and emphasize the role this
island plays in virulence both through its cargo genes and through influencing expression of genes outside the island.

**Features of PAPI-2**

In contrast with PAPI-1, PAPI-2 is a smaller island that spans ~11 kb and is inserted at the tRNA\(^{Lys10}\) in PA14 (PA14_51660). Like many other *P. aeruginosa* genomic islands, it has a lower G+C content than the core genome (56.4% and 66.6%, respectively). Several putative proteins are predicted to play a role in DNA mobility such as a XerC/XerD-like integrase, a relaxase and two transposases. However, it is the presence of the genes which code for the type III secretion system effector, ExoU, and its chaperon, SpcU, that denote PAPI-2 as pathogenicity island (He *et al.* 2004). ExoU is a powerful phospholipase exerting a strong cytotoxic effect on *in vitro* grown epithelial cells (Engel, Balachandran 2009) and its presence is associated with *P. aeruginosa* strains isolated from patients with more severe disease (Hauser *et al.* 2002).

In PAO1, the same locus is occupied by an 8.9 kb genomic island named PA0977-0987 (Kiewitz *et al.* 2000, He *et al.* 2004). Six *orfs*, five of which reside at the tRNA\(^{Lys}\)-proximal end, are homologous to the *orfs* in PAPI-2 but the PA0977-0987 cluster lacks the *exoU*/spcU gene pair and eight other PAPI-2-specific predicted genes (He *et al.* 2004). Among the *orfs* shared by PA0977-0987 and PAPI-2, are three putative transposases.

**Contribution of PAPI-1 and PAPI-2 to PA14 virulence**

In order to study the overall contribution to virulence of PAPI-1 and/or PAPI-2 in both *in vitro* and *in vivo* assays, these islands were deleted from PA14 both individually and in combination using homologous recombination techniques (Harrison *et al.* 2010). The PAPI-1-minus mutant was designated KR572 and in this project will be referred to as
PA14ΔPAPI-1. The PAPI-2-minus mutant was designated KR573 and will be referred to as PA14ΔPAPI-2. The mutant lacking both islands, designated KR608, was derived from KR573 and will be referred to as PA14Δ1Δ2.

By *in vitro* assays, it was shown that PA14Δ1Δ2 did not display any differences in growth in liquid media, biofilm formation, pyocyanin production and motility when compared to the original PA14 wildtype strain. However, PA14ΔPAPI-2 and PA14Δ1Δ2 showed supposed increased invasion of cultured human lung carcinoma cells but decreased cytotoxicity towards these same cells when compared with wildtype or PA14ΔPAPI-1 (Harrison *et al.* 2010). This is consistent with the absence of ExoU-specific cytotoxic activity in the mutants lacking PAPI-2 (Engel, Balachandran 2009, Evans *et al.* 1998).

Using an *in vivo* murine acute pneumonia model (Harrison *et al.* 2010), it was shown that mice infected with PA14Δ1Δ2 exhibited a marked reduction in *P. aeruginosa* CFUs recovered from nasopharynx, lung and blood compared to the mice infected with PA14. Of the single island deletion mutants, only PA14ΔPAPI-2 showed a slight reduction in CFUs recovered from mice, while recovered CFU numbers of PA14ΔPAPI-1 were comparable to those of PA14. Infection with the mutant lacking both islands showed ~10 times less monocytes and macrophages in the lung tissue compared to PA14 wildtype and the single mutants (PA14ΔPAPI-1 and PA14ΔPAPI-2), potentially hindering dissemination of bacteria from the lung to the bloodstream and prompting the conclusion that these islands have a synergistic effect in the acute pneumonia mouse model (Harrison *et al.* 2010).
Aims of this chapter

The general aim of this chapter was to identify simpler organisms that may be used to study the effect of genomic island deletion. Interestingly, deletion of PAPI-2 had a modest effect while the deletion of both PAPI-1 and PAPI-2 had the biggest effect allowing longer mice survival and prompting to hypothesize the presence of a synergistic effect between these islands. It would be useful to identify easier to handle organisms in which similar phenomenon occur to allow for further elucidation of the processes involved. Specifically, the contribution of PAPI-1 and/or PAPI-2 to PA14 virulence was assessed using the following non-mammalian model organisms:

1. Test the survival of *C. elegans* when seeded on mutants lacking PAPI-1 and/or PAPI-2 and compare it with survival of the nematode when seeded on wildtype strain using slow- and fast-killing assays;

2. Test changes in *A. castellanii* and *A. polyphaga* grazing in a non-nutrient agar assay with PA14 wildtype and PA14 mutant strains as a food source.
Results

Survival of *C. elegans* using the slow-killing assay

Preliminary evidence of PAPI-2 involvement in virulence as demonstrated by the slow-killing assay

In order to test whether PAPI-1 and PAPI-2 contribute to PA14 virulence against *C. elegans*, the island-deletion mutants (PA14ΔPAPI-1, PA14ΔPAPI-2 and PA14Δ1Δ2) were compared against the wildtype strain using the slow-killing assay (SKA). In a preliminary experiment, ten nematodes per plate were seeded for the *P. aeruginosa* strains and five nematodes per plate for *E. coli* OP50, with three plates being prepared for each strain. *P. aeruginosa* PAO1 strain was used as a virulence-attenuated control because it had been shown to allow longer survival of nematodes by this assay (Lee et al. 2006). OP50 was used as negative control because *C. elegans* normally survives 10-15 days when seeded on this *E. coli* strain (Altun, Hall 2009).

SKA was carried out by growing *P. aeruginosa* strains in King’s B broth and spotting 5 µl of culture medium (~ 5 × 10^7 CFU) on modified nematode growth medium (NGM). In parallel, CF512 nematodes were synchronized, transferred as young adults to test plates and incubated at 25°C until the end of the experiment. The plates were observed at ~ 12 h intervals and the numbers of nematodes alive, dead, missing and found dried on a plate wall were recorded. *C. elegans* CF512 is derived from N2 wildtype strain and it does not lay eggs at 25°C, allowing the same cohort of nematodes to be followed without the cofounding effect of progeny.
When the percentage of surviving nematodes was plotted over time (Figure 3-1), it was evident that nematodes seeded on either PAO1 or OP50 survived much longer than nematodes seeded on PA14. When the experiment was terminated at 168 h, more than half of the *C. elegans* on PAO1 and all those on *E. coli* were still alive while all the accounted for *C. elegans* seeded on PA14 were dead by 108 h. On average, nematodes seeded on PA14 died ~ 3.5 days sooner than nematodes seeded on PAO1, and in shorter time than OP50-seeded nematodes (Table 3-1). Consequently, the difference in survival of *C. elegans* seeded on PA14 compared to PAO1 or OP50 was confirmed to be statistically significant (Table 3-1).

*C. elegans* seeded on all three mutants appeared to survive longer than those seeded on PA14 but with observable differences between the mutants. Nematodes seeded on PA14ΔPAPI-1 survived longer in the mid-period of the experiment compared to nematodes seeded on PA14 wildtype (Figure 3-1) returning an average time-to-death for nematodes maintained on the mutant ~ 7 h longer than that of wildtype. However, the difference in survival was not significant (Table 3-1).

A higher survival rate of *C. elegans* seeded on PA14ΔPAPI-2 or PA14Δ1Δ2 was first observable at ~ 48 h and then continued to become more apparent right up to the end of the experiment (Figure 3-1). On average, nematodes seeded on PA14ΔPAPI-2 or PA14Δ1Δ2 survived ~ 1 day longer than those seeded on PA14 (Table 3-1) with these mutants showing a delayed *C. elegans*-directed killing effect compared to that of the wildtype for which the last nematodes died sooner (Figure 3-1). Survival analysis confirmed a significant difference in *C. elegans* survival when exposed to PA14 compared to either PA14ΔPAPI-2 or PA14Δ1Δ2 (Table 3-1).
Figure 3-1: *C. elegans* survival in the small-scale slow-killing assay

Plot showing the survival of nematodes (CF512) over time using 10 nematodes/plate for *P. aeruginosa* strains (PA14, PA14 mutant strains and PAO1) and 5 nematodes/plate for *E. coli* strain (OP50) and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and the average ± standard deviation values shown. Survival of nematodes fed with PA14 wildtype strain was compared to that of other bacteria using the Log-Rank test within the Kaplan-Meier survival analysis (Table 3-1) and significant *P*-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- ○ = *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- ▼ = *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- ▲ = *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- ▭ = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- ● = *P. aeruginosa* PAO1 (KR124)
- ✤ = *E. coli* OP50
### Table 3-1: Survival analysis of *C. elegans* CF512 in small scale slow-killing assay

The survival of *C. elegans* CF512 seeded on different bacterial strains was analysed using the Kaplan-Meier method.*Because the experiment ended when many of the nematodes seeded on PAO1 and all the nematode seeded on OP50 were still alive, the percentage of censored nematodes was very high.

**Table headings legend:**

- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TD_m (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD_{50} (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank \( \chi^2 \); P-value** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- \( \chi^2 \) = Log-Rank test value calculated by the software with P-value significant if below 0.05 (underlined)
- **n.a.** = not available

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TD_m (h ± SE)</th>
<th>TD_{50} (h ± SE)</th>
<th>Log-Rank ( \chi^2 ); P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>30 (50%)</td>
<td>70.2 ± 5.0</td>
<td>72 ± 3</td>
<td>/</td>
</tr>
<tr>
<td>PA14ΔPAPI-1</td>
<td>30 (40%)</td>
<td>77.5 ± 4.8</td>
<td>84 ± 5</td>
<td>0.659; 0.417</td>
</tr>
<tr>
<td>PA14ΔPAPI-2</td>
<td>30 (30%)</td>
<td>95.3 ± 7.4</td>
<td>84 ± 13</td>
<td>4.823; 0.028</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>30 (40%)</td>
<td>89.4 ± 5.4</td>
<td>84 ± 5</td>
<td>4.840; 0.028</td>
</tr>
<tr>
<td>PAO1</td>
<td>30 (80%*)</td>
<td>157.7 ± 6.1</td>
<td>n.a.</td>
<td>28.083; &lt;0.001</td>
</tr>
<tr>
<td>OP50</td>
<td>15 (100%*)</td>
<td>105.7 ± 4.4</td>
<td>n.a.</td>
<td>20.551; &lt;0.001</td>
</tr>
</tbody>
</table>
The PA14Δ1Δ2 mutant showed attenuated virulence in a large-scale slow-killing assay

When survival analysis is applied to human trials, subjects can be “censored” when they are still alive at the end of the study, the information regarding the survival time is lost or they withdraw from the trial (Kleinbaum 1996). Accordingly, nematodes may be excluded from the study (censored) either because they are found dried on the plate wall, are unaccounted for or are still alive when the experiment reached its end. During the small-scale SKA, the percentage of censored nematodes was very high for all four PA14-related strains (Table 3-1). The high percentages of censored nematodes recorded for PAO1 and OP50 were due to the experiment ending when many of the nematodes were still alive and very few were unaccounted for. Since these initial experiments used a maximum of 30 nematodes per strain, the corresponding survival analysis was based on relatively few “subjects”. Consequently, a large-scale experiment was carried out to increase the numbers of *C. elegans* used and thus reduce the statistical impact of the missing ones. This experiment was executed as described for the small-scale SKA, but using plates with a larger diameter (100 mm instead of 60 mm plates) and by plating 30-50 nematodes per plate onto 3 separate plates for each *P. aeruginosa* strain.

The use of larger plates was five times more effective in reducing the percentages of unaccounted nematodes (Table 3-2). However, because of the experimentally-demanding nature of this assay only PA14 and the PA14Δ1Δ2 were compared, along with PAO1 as virulence-attenuated control.

The *C. elegans* survival on PA14 and PAO1 was very similar up to 48h from the start of the experiment (Figure 3-2). After that, nematodes fed with PA14 started to die in higher numbers than the nematodes fed with PAO1. The average time-to-death for nematodes on PAO1 was more than twice that of PA14 wildtype where PAO1-fed *C.
*C. elegans* survived almost four days longer than the PA14-fed *C. elegans* and the difference in survival was again significant (Table 3-2).

The decline in survival for nematodes seeded on PA14Δ1Δ2 was very similar to that of PA14 wildtype (Figure 3-2). The survival analysis returned an identical time needed to kill 50% of the nematodes (TD50 in Table 3-2) but this was a slightly longer average time-to-death value for nematodes fed with PA14Δ1Δ2 (TDm in Table 3-2). The latter difference may indicate only a minor ability to survive longer on mutants lacking both PAPI-1 and PAPI-2 compared to PA14 wildtype but the difference was still significant (Table 3-2).

![Figure 3-2: C. elegans survival in the large-scale slow-killing assay](image)

**Figure 3-2: C. elegans survival in the large-scale slow-killing assay**

Plot showing the survival of nematodes (CF512) over time done using 30-50 nematodes/plate and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± the standard deviation values shown. Survival of nematodes fed with PA14 wildtype strain was compared to that of the other *P. aeruginosa* strains using the Log-Rank test within the Kaplan-Meier survival analysis (Table 3-2) and significant *P*-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- **●** = *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- **■** = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- **◆** = *P. aeruginosa* PAO1 (KR124)
Table 3-2: Survival analysis of *C. elegans* CF512 in large scale slow-killing assay

The survival of *C. elegans* CF512 seeded on different bacterial strains was analysed using the Kaplan-Meier method.

**Table headings legend:**

- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TD<sub>m</sub> (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD<sub>50</sub> (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **χ²; P-value** = Log-Rank test value calculated by the software with P-value significant if below 0.05 (underlined)
- **n.a.** = not available

<table>
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<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TD&lt;sub&gt;m&lt;/sub&gt; (h ± SE)</th>
<th>TD&lt;sub&gt;50&lt;/sub&gt; (h ± SE)</th>
<th>Log-Rank χ²; P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>130 (11%)</td>
<td>72.2 ± 2.2</td>
<td>72 ± 2</td>
<td>/</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>130 (8%)</td>
<td>79.6 ± 2.8</td>
<td>72 ± 3</td>
<td>5.521; 0.019</td>
</tr>
<tr>
<td>PAO1</td>
<td>130 (15%)</td>
<td>166.8 ± 5.7</td>
<td>168 ± 6</td>
<td>170.956; &lt;0.001</td>
</tr>
</tbody>
</table>

The survival of *C. elegans* CF512 seeded on different bacterial strains was analysed using the Kaplan-Meier method.
Changes to slow-killing assay protocol erased the difference between PA14 wildtype and PA14Δ1Δ2

The PA14Δ1Δ2 mutant and its isogenic wildtype were retested at Prof. Nathan’s lab at the Universiti Kabangsaan Malaysia (UKM). The slow-killing assay protocol done in Prof. Nathan’s laboratory was similar to the protocol set up at the University of Leicester but there were a few experimental differences, in particular the use of a different \( C. \ elegans \) strain (\( rrf-3; glp-4 \)) and different synchronization and bacterial growth protocols (see Material and Methods, Chapter 2).

The vast majority of \( C. \ elegans \) seeded onto the \( E. \ coli \) OP50 negative control strain survived up to 168 h experimental end-point (Figure 3-3). Nematodes fed with \( E. \ coli \) survived significantly longer (~ 3.5 days) than the ones fed with PA14 wildtype as expected (Table 3-3).

The decline of survival of \( C. \ elegans \) on PA14 and PA14Δ1Δ2 was again very similar, with the most obvious difference being evident between 48 h and 96 h during which a lower percentage of nematodes seeded on PA14Δ1Δ2 were scored as dead (Figure 3-3) leading to a higher \( TD_{50} \) and \( TD_{m} \) values than wildtype (Table 3-3). However, the difference in nematode survival measured over the entire experiment was not significant and PA14Δ1Δ2 appeared to be as virulent as the wildtype under these experimental conditions.

The addition of PAPI-1 did not increase PA01 virulence

In order to establish whether PAPI-1 contributes to virulence against \( C. \ elegans \) in a non-PA14 genetic background, the engineered strain PA01::PAPI-1 (KR911, kindly provided by Dr Ewan Harrison) was tested in a slow-killing assay and compared to the isogenic PA01 wildtype strain. The addition of PAPI-1 did not enhance \( C. \ elegans \)
killing by PAO1 (Figure 3-4) with survival of nematodes seeded on PAO1::PAPI-1 not significantly different to that seen with the wildtype PAO1 (Log-Rank $\chi^2 = 0.035$; $P$-value = 0.852).

Figure 3-3: *C. elegans* survival in slow-killing assay done at Universiti Kabangsaan Malaysia

Plot showing the survival of nematodes (*rrf3*;*glp-4*) over time using ~50 nematodes/plate and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± the standard deviation values shown. Survival of nematodes fed with PA14 wildtype strain was compared to that of the other bacteria using the Log-Rank test within the Kaplan-Meier survival analysis (Table 3-3) and significant $P$-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- **=** *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- **=** *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- **=** *E. coli* OP50
<table>
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<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TDₘ (h ± SE)</th>
<th>TD₅₀ (h ± SE)</th>
<th>Log-Rank χ²; P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>150 (59%)</td>
<td>81.0±5.6</td>
<td>72 ± 5</td>
<td>/</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>150 (61%)</td>
<td>89.5±4.9</td>
<td>96 ± 8</td>
<td>0.118; 0.731</td>
</tr>
<tr>
<td>OP50</td>
<td>154 (93%*)</td>
<td>168.8±2.0</td>
<td>n.a.</td>
<td>196.962; &lt;0.001</td>
</tr>
</tbody>
</table>

**Table 3-3: Survival analysis of *C. elegans* in slow-killing assay done at Universiti Kabangsaan Malaysia**

The survival of *C. elegans rrf-3:glp-4* seeded on different bacterial strains was analysed using the Kaplan-Meier method. *Because the experiment ended when many of the nematodes seeded on PAO1 and all the nematode seeded on OP50 were still alive, the percentage of censored nematodes was very high.*

**Table headings legend:**
- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TDₘ (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD₅₀ (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **χ² =** Log-Rank test value calculated by the software with P-value significant if below 0.05 (underlined)
- **n.a.** = not available
Figure 3-4: *C. elegans* survival on PAO1::PAPI-1 and PAO1

Plot showing the survival of nematodes (CF512) over time using 10 nematodes/plate and two plates for PAO1::PAPI-1 and 30-50 nematode/plate and three plates for PAO1. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± the standard deviation values shown. There was no significant difference in survival of nematodes between the two strains.

**Bacterial strains on which nematodes were seeded:**
- **=** *P. aeruginosa* PAO1 with PAPI-1 (PAO1::PAPI-1; KR911)
- **=** *P. aeruginosa* PAO1 wildtype (KR124)
Survival of *C. elegans* as measured by the fast-killing assay

The fast-killing assay (FKA) was used to establish whether low-molecular weight toxins associated with the presence of PAPI-1 and/or PAPI-2 had a role in killing *C. elegans*. The FKA was carried out analogously to the slow-killing assay, but with a few changes. Peptone-glucose-sorbitol (PGS) agar was used instead of NGM as describe previously (He *et al.* 2004). *P. aeruginosa* strains were grown in King’s B broth and plated on PGS agar while either CF512 or N2 nematodes were synchronized and transferred to test plate. Ten or 20 nematodes were seeded on a PGS agar carrying either a *P. aeruginosa* strain or OP50. The number of dead nematodes was recorded every few hours in the first 2 days and less frequently after that. Two independent experiments were run for each *C. elegans* strains and the results were combined.

**Fast-killing assay using *C. elegans* N2**

*C. elegans* N2 is known to be particularly sensitive to the toxin-mediated killing exerted by *P. aeruginosa* PA14 under the FKA conditions (Mahajan-Miklos *et al.* 1999), but it was expected to survive much better on *E. coli* OP50. Therefore, the *E. coli* strain was used a as virulence-attenuated control. All *P. aeruginosa* strains tested killed nematodes very quickly in the FKA when compared to OP50 (Figure 3-5). Very few *C. elegans* were dead at the end of the experiments when fed with OP50, dying on average more than four days later than PA14-seeded nematodes (Table 3-4).

The number of dead *C. elegans* found on *P. aeruginosa*-bearing plates increased very quickly within 12 h from the beginning of the experiment for all strains tested (Figure 3-5). However, the rate of death on PAO1 appeared slightly lower than that on PA14. Consequently, the average time-to-death of nematodes from the PAO1 plates was
higher than that of PA14 plates with the former nematodes surviving 6 h longer (Table 3-4). The observed difference between *C. elegans* survival on PAO1 and on PA14 by the FKA was borderline significant with a *P*-value equal to 0.05 (Table 3-4).

The percentages of surviving nematodes on PA14ΔPAPI-1, PA14ΔPAPI-2 and PA14Δ1Δ2 were very similar to those on PA14 wildtype. All showed a very rapid decline in the percentages of surviving nematodes within 12 h from the beginning of the experiment but some minor differences of questionable significance were observed after the 24 h time-point (Figure 3-5). Notwithstanding these variations, no significant differences between the nematode survival on PA14 and the deletion mutants could be demonstrated by the FKA (Table 3-4).

**Fast-killing assay using *C. elegans* CF512**

In the FKA with the CF512 *C. elegans* strain, the survival of nematodes was 3 to 6 times longer than with N2. Furthermore, a much higher degree of variability between experiments was observed as indicated by widely separated standard deviation values (Figure 3-6). OP50 was used as a negative control for the assay and only few nematodes died when seeded on this strain. In fact, the only significant difference recorded was between *C. elegans* survival on *P. aeruginosa* strains and on *E. coli* OP50 (Table 3-5). The survival of nematodes was very similar when these were seeded on any *P. aeruginosa* strains (Figure 3-6) and no significant differences were noted (Table 3-5), though the high degree of inter-experiment variability is likely to have masked potential minor differences.
Figure 3-5: *C. elegans* N2 survival in fast-killing assay

Plot showing the survival of nematodes over time using 10 or 20 nematodes/plate for *P. aeruginosa* strains and one plate per experiment; two distinct experiments were combined here. At each time point, the percentage/plate of nematodes that were alive was calculated for each plate and average ± the standard deviation values shown. Survival of nematodes fed with PA14 wildtype strain was compared to that of the other bacteria using the Log-Rank test within the Kaplan-Meier survival analysis (Table 3-4) and significant or borderline *P*-values (≤0.05) are shown.

**Bacterial strains on which nematodes were seeded:**
- = *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- = *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- = *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- = *P. aeruginosa* PAO1 (KR124)
- = *E. coli* OP50
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<th>$TD_{50}$ (h ± SE)</th>
<th>Log-Rank $\chi^2$; $P$-value</th>
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<td>PA14</td>
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<td>9.9 ± 2.7</td>
<td>2 ± 0</td>
<td>/</td>
</tr>
<tr>
<td>PA14ΔPAPI-1</td>
<td>30 (3%)</td>
<td>11.0 ± 3.5</td>
<td>5 ± 2</td>
<td>0.292; 0.589</td>
</tr>
<tr>
<td>PA14ΔPAPI-2</td>
<td>30 (0%)</td>
<td>6.3 ± 1.0</td>
<td>2 ± 0</td>
<td>0.580; 0.446</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>30 (0%)</td>
<td>6.5 ± 1.0</td>
<td>2 ± 0</td>
<td>0.531; 0.466</td>
</tr>
<tr>
<td>PAO1</td>
<td>30 (10%)</td>
<td>16.0 ± 3.8</td>
<td>10 ± 2</td>
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<tr>
<td>OP50</td>
<td>20 (95%*)</td>
<td>115.3 ± 3.5</td>
<td>n.a.</td>
<td>42.642; &lt;0.001</td>
</tr>
</tbody>
</table>

**Table 3-4: Survival analysis of *C. elegans* N2 in fast-killing assay**

The survival of *C. elegans* N2 seeded on different bacterial strains was analysed using the Kaplan-Meier method. *Because the experiment ended when many of the nematodes seeded on PAO1 and all the nematode seeded on OP50 were still alive, the percentage of censored nematodes was very high.

**Table headings legend:**

- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **$TD_m$ (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **$TD_{50}$ (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- $\chi^2$ = Log-Rank test value calculated by the software with $P$-value significant if below 0.05 (underlined)
- **n.a.** = not available
Figure 3-6: *C. elegans* CF512 survival in fast-killing assay

Plot showing the survival of nematodes over time using 10 or 20 nematodes/plate for *P. aeruginosa* strains and one plate per experiment; two distinct experiments were combined here. At each time point, the percentage/plate of nematodes that were alive was calculated for each plate and average ± the standard deviation values shown. Survival of nematodes fed with PA14 wildtype strain was compared to that of the other bacteria using the Log-Rank test within the Kaplan-Meier survival analysis (Table 3-5) and significant *P*-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- **= *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- ▼ = *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- ▲ = *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- ■ = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- ◆ = *P. aeruginosa* PAO1 (KR124)
- ○ = *E. coli* OP50
The survival analysis of *C. elegans* CF512 in fast-killing assay

The survival of *C. elegans* CF512 seeded on different bacterial strains was analysed using the Kaplan-Meier method. *Because the experiment ended when many of the nematodes seeded on PAO1 and all the nematode seeded on OP50 were still alive, the percentage of censored nematodes was very high.*

**Table headings legend:**

- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TDₘ (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD₅₀ (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **χ²; P-value** = Log-Rank test value calculated by the software with *P*-value significant if below 0.05 (underlined)
- **n.a.** = not available

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<th>Strain</th>
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<th>TD₅₀ (h ± SE)</th>
<th>Log-Rank χ²; P-value</th>
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</thead>
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<td>PA14</td>
<td>30 (13%)</td>
<td>42.7 ± 6.5</td>
<td>47 ± 15</td>
<td>/</td>
</tr>
<tr>
<td>PA14ΔPAPI-1</td>
<td>30 (13%)</td>
<td>48.6 ± 7.2</td>
<td>58 ± 24</td>
<td>0.447; 0.504</td>
</tr>
<tr>
<td>PA14ΔPAPI-2</td>
<td>30 (13%)</td>
<td>33.8 ± 5.1</td>
<td>29 ± 12</td>
<td>1.374; 0.241</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>30 (23%)</td>
<td>42.5 ± 7.3</td>
<td>47 ± 23</td>
<td>0.027; 0.869</td>
</tr>
<tr>
<td>PAO1</td>
<td>30 (10%)</td>
<td>45.6 ± 7.7</td>
<td>29 ± 9</td>
<td>0.100; 0.752</td>
</tr>
<tr>
<td>OP50</td>
<td>18 (89%*)</td>
<td>124.9 ± 9.4</td>
<td>n.a.</td>
<td>27.042; &lt;0.001</td>
</tr>
</tbody>
</table>

Table 3-5: Survival analysis of *C. elegans* CF512 in fast-killing assay
Grazing of *A. castellanii* and *A. polyphaga* on *P. aeruginosa*

In order to examine the potential contribution to virulence against *Acanthamoeba* spp. of PAPI-1 and/or PAPI-2 in a *P. aeruginosa* PA14 background, two media were initially tested: peptone-yeast extract-glucose (PYG) and non-nutrient agar (NNA). The growth of *P. aeruginosa* strains on PYG prevented the grazing of either *A. castellanii* or *A. polyphaga* impeding the use of this medium in the virulence assay (data not shown). However, NNA was found to be suitable for grazing assays as the bacteria were unable to grow on this medium and thus produced no visible colonies or lawn on the surface of the plate. Ten independent colonies for each *P. aeruginosa* strain were grown on tryptone soya agar (TSA) plates and a bacterial suspension of OD$_{600nm}$ = 0.1 was prepared in sterile water. One ml of the bacterial suspension was spread evenly on NNA plates and allowed to dry. Either *A. castellanii* or *A. polyphaga* were collected from maintenance flasks, washed with PBS-tween, concentrated and spotted at the centre of the bacteria-covered NNA plate and the plates incubated at 30°C. During the experiment, the amoeba growth film expanded from the centre of the plates forming a defined border at the bacteria-amoebae interface. Therefore, it was possible to infer the rate of expansion of the amoebae growth film by measuring the diameter of the area covered by amoebae. For the *A. castellanii* assay, the amoebae growth film diameter was measured at days 3, 6, 9 and 12 while for the *A. polyphaga* assay it was measured at days 3, 6 and 9. The number of bacteria initially plated was determined by CFU counts obtained after the bacterial suspension was used.

*A. castellanii* growth film expanded faster on PA14 mutants

The behaviour of *A. castellanii* plated on PAO1 and on PA14 was very different (Figure 3-7). The diameter of the growth film was greater on PAO1 than PA14 from day 3
onwards with the difference becoming progressively greater at days 6 and 9 (Figure 3-7; Appendix A, Table A-1). Consequently, the *A. castellanii* growth film diameters from PAO1 plates were significantly higher than those from PA14 plates at each time point (Table 3-6).

*A. castellanii* was able to expand on PA14ΔPAPI-1 extremely fast with the amoeba reaching the edge of the plates after 6 days while the diameter measured from PA14 wildtype plates at this same stage was below 20 mm, corresponding to ~5 times growth increment (Figure 3-7). By contrast *A. castellanii* expanded on PA14ΔPAPI-2 and PA14Δ1Δ2 at very similar rates throughout, and at an equivalent pace to amoeba on PA14 as assessed at days 3 and 6 displaying diameters only marginally larger (2-4 mm) that wildtype. Minor differences between the two mutants and PA14 may have begun to emerge by days 9 and 12 where the measured growth film in the PA14ΔPAPI-2 and in the PA14Δ1Δ2 plates was ~10-12 mm larger than PA14 (Figure 3-7). The diameter of *A. castellanii* on PA14 lacking PAPI-1 and/or PAPI-2 was significantly larger than that on PA14 wildtype at each of these time point (Table 3-6). Most surprisingly PA14ΔPAPI-1 exhibited a markedly increased growth film diameter than those of PA14, PA14Δ1Δ2 and PA14ΔPAPI-2 particularly when assayed at days 9 and 12.

In order to plate similar amounts of bacteria, the OD_{600} of each single colony suspension was adjusted to 0.1. Subsequently, serial dilution was performed to measure the CFU/plate. The amount of bacteria plated for PA14 wildtype, PA14ΔPAPI-2 and the PAO1 was similar (Figure 3-8) and there was no significant difference between them (Table 3-6). On the contrary, the CFU/plate of PA14Δ1Δ2 was significantly greater than wildtype, while CFU/plate for PA14ΔPAPI-1 was significantly fewer than PA14 (Table 3-6).
A. polyphaga growth film expansion was similar between P. aeruginosa strains

The difference of A. polyphaga grazing on PA14 and PAO1 was not as clear as the expansion recorded for A. castellanii. At day 3, A. polyphaga growth film diameter on PAO1 was on average smaller than that of amoebae on PA14, at day 6 it was as large while at day 9 it was wider (Figure 3-9; Appendix A, Table A-2). The diameter of A. polyphaga growth film on PAO1 plates was significantly smaller than PA14 at day 3 while it was significantly larger at day 9 after changing its trend (Table 3-7).

A. polyphaga growth film expanded on lawns of PA14ΔPAPI-1 and on PA14ΔPAPI-2 in a very similar manner (Figure 3-9) with the measured diameter going from 1-2 mm larger for the mutants at day 3 to 5 mm larger at day 9. The diameter measured was significantly larger than for wildtype at all the time points for PA14ΔPAPI-2 and at day 6 and 9 for PA14ΔPAPI-1 (Table 3-7). However, the expansion of the A. polyphaga growth film on PA14Δ1Δ2 was analogous to the expansion on the PA14 wildtype (Figure 3-9; Appendix A, Table A-2) with no significant difference observed (Table 3-7).

The amount of bacteria plated for the A. polyphaga experiment was similar for all the PA14-derived strains (Figure 3-10) with no significant difference between wildtype PA14 and any of the mutant strains (Table 3-7). On the contrary, the amount of PAO1 plated was significantly greater than the amount of PA14 (Figure 3-10; Table 3-7).
Figure 3-7: Grazing of *A. castellanii* on *P. aeruginosa*

Plot showing the diameter of *A. castellanii* growth film on NNA plates layered with *P. aeruginosa* strains at days 3, 6, 9 and 12. At each time point, diameter was measured for 10 plates corresponding to 10 single colonies for each strain and average ± standard deviation values shown.

*A. castellanii* was seeded on the following strains:

- ▲ = *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- ▲ = *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- ▲ = *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- ▲ = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- ▲ = *P. aeruginosa* PAO1 (KR124)
Table 3-6: Statistical analysis for *A. castellanii* grazing on *P. aeruginosa*

Non-parametric statistical tests were used to compare *A. castellanii* film diameter and CFU/plate. Comparison between all groups was performed using the Kruskal-Wallis test. If significant, PA14 wildtype measurements were compared with each of the other strain using Mann-Whitney test. Difference was considered significant when *P*-value was < 0.05 (underlined).
Figure 3-8: Amount of bacteria plated for A. castellanii NNA assay

Plot showing the numbers of P. aeruginosa estimated to have been plated on non-nutrient agar plates. In this experiment, 10 single colonies for each strain were selected and bacterial suspensions prepared. Serial dilutions of each bacterial suspension were plated and colony forming unit (CFU)/plate calculated. Each box symbolizes the interquartile range of 10 colonies, while the black line represents the median and the whiskers represent the maximum and the minimum values. Difference in the amount of bacteria plated for PA14 was compared to that of the other P. aeruginosa strains using the Mann-Whitney test and significant P-values (<0.05) are shown (Table 3-6).

Legend:
- = P. aeruginosa PA14 wildtype (PA14 WT; KR125)
- = P. aeruginosa PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- = P. aeruginosa PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- = P. aeruginosa PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- = P. aeruginosa PAO1 (KR124)
Figure 3-9: Grazing of *A. polyphaga* film on *P. aeruginosa*

Plot showing the diameter of *A. polyphaga* growth on NNA plates layered with *P. aeruginosa* strains at days 3, 6 and 9. At each time point, the diameter was measured for 10 plates corresponding to 10 single colonies for each strain and average ± standard deviation values shown.

*A. polyphaga* seeded on the following strains:

- ● = *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- ▼ = *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- ▲ = *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2; KR573)
- ■ = *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- ◆ = *P. aeruginosa* PAO1 (KR124)
Table 3-7: Statistical analysis for *A. polyphaga* grazing on *P. aeruginosa*

Non-parametric statistical tests were used to compare *A. polyphaga* film diameter and CFU/plate. Comparison between all groups was performed using the Kruskal-Wallis test. If significant, PA14 wildtype measurements were compared with each of the other strain using Mann-Whitney test. Difference was considered significant when *P*-value was < 0.05 (underlined).
Figure 3-10: Amount of bacteria plated for *A. polyphaga* NNA assay

Plot showing the numbers of *P. aeruginosa* estimated to have been plated on non-nutrient agar plates. In this experiment, 10 single colonies for each strain were selected and bacterial suspension prepared. Serial dilutions of each bacterial suspension were plated and colony forming unit (CFU)/plate calculated. Each box symbolizes the interquartile range of 10 colonies, while the black line represents the median and the whiskers represent the maximum and the minimum values. Difference in the amount of bacteria plated for PA14 was compared to that of the other *P. aeruginosa* strains using the Mann-Whitney test and significant *P*-values (<0.05) are shown (Table 3-7).

Legend:

- **= *P. aeruginosa* PA14 wildtype (PA14 WT; KR125)
- **= *P. aeruginosa* PA14 without PAPI-1 (PA14ΔPAPI-1; KR572)
- **= *P. aeruginosa* PA14 without PAPI-2 (PA14ΔPAPI-2, KR573)
- **= *P. aeruginosa* PA14 without both PAPI-1 and PAPI-2 (PA14Δ1Δ2, KR608)
- **= *P. aeruginosa* PAO1 (KR124)
Conclusions and Discussion

PAPI-2 is not consistently involved in *C. elegans* virulence

When this project started, the slow-killing assay was the most commonly used assay to study the *C. elegans/P. aeruginosa* interaction followed by the fast-killing assay. Based on the previous literature, it was appropriate to use *P. aeruginosa* strain PAO1 as an attenuated virulence control and PA14 as a highly virulent strain (Lee *et al.* 2006) and collectively was a good starting point to test any attenuation in the PA14 island deletion mutants.

In all the slow-killing assays, nematodes seeded on to PA14 died faster than nematodes seeded on to PAO1. This relative difference in survival of *C. elegans* seeded on these two strains was maintained at a qualitative level regardless of whether few nematodes (10 nematodes/plate and three plates per strains in the small-scale experiment) or many nematodes were used (30-50 nematodes/plate and three plate per strain in the large-scale experiment), and even when a different *C. elegans* strain was employed (Chapter 4, Figure 4-5B). These observations prompted the conclusion that PA14 was generally more virulent than PAO1 under slow-killing assay conditions, confirming previous reports (Lee *et al.* 2006).

When the island-deletion mutants were compared with the isogenic wildtype the differences were less dramatic than between PA14 and PAO1. In the small-scale slow-killing assay, the survival of nematodes were generally similar to the survival on wildtype, with nematodes seeded on either PA14ΔPAPI-2 or PA14Δ1Δ2 living slightly but significantly longer than those exposed to PA14. In contrast to previous results
based on deletion of selected individual PAPI-1 genes (Lee et al. 2006, He et al. 2004), loss of this entire ICE appeared to not affect virulence as assessed by the *C. elegans* SKA model. In support of this finding was the observation that introduction of PAPI-1 into PAO1 had no detectable impact on PAO1 virulence as measured by this same assay.

The large numbers of nematodes unaccounted for (censored) in the assay when using PA14 wildtype and derived strains was indicative of nematode-associated pathogen avoidance behaviour (Zhang, Lu & Bargmann 2005). Indeed, this is consistent with the finding that fewer nematodes exposed to the attenuated PAO1 strain were censored. Accordingly, the small-scale slow-killing assay derived survival curves were based on relatively few nematodes, potentially skewing the analysis. A large-scale experiment was performed in which survival on PA14 and PA14Δ1Δ2 were close but significantly different. In contrast, in the experiment run at the Universiti Kabangsaan Malaysia which utilised minor variations of the original protocol, the survival of nematodes seeded on the PA14Δ1Δ2 was not significantly different when compared with the survival of nematodes seeded on PA14.

Based on these observations, it may be inferred that the major observed difference in survival of nematodes in the slow-killing assay when seeded on PA14 and on PAO1 was mostly due to features specific to each of the two *P. aeruginosa* strains. In contrast, the contribution, if any, of PAPI-2 to PA14 virulence appeared minor and was highly dependent on the specific conditions of the assay rather than on virulence functions specified by the island.

Regarding the fast-killing assay, as expected, nematodes generally died faster than in the slow-killing assay (Tan, Mahajan-Miklos & Ausubel 1999). Interestingly, the difference in survival of nematodes on PAO1 and PA14 was markedly reduced.
compared to that observed with the slow-killing assay. In fact, the difference wasorderline significant with the N2 nematode strain and not significant with the CF512
nematode strain based assay. Survival of nematodes seeded on each of the PA14
mutants was very similar to that on PA14 with no significant difference detected for any
comparison. It seems that the conditions of the fast-killing assay masked most, if not all,
of the SKA-defined virulence-associated difference between PAO1 and PA14, and that
neither PAPI-1 nor PAPI-2 contributed significantly to virulence in this assay.

**A. castellanii growth film expanded faster on PA14 when**

**PAPI-2 was deleted**

Examination of the *P. aeruginosa* strains using the Acanthamoeba-based non-nutrient
assay, demonstrated that amoeba growth films expanded faster on PAO1 than on PA14,
although the effect was more conspicuous with *A. castellanii*. The area covered by the
*A. castellanii* growth film on PAO1 was larger than the area covered on PA14
throughout the entire experiment. By contrast, the difference in diameter of the *A.
polyphaga* growth films between PAO1- and PA14-fed amoeba varied over the course
of the experiment with PAO1 inoculated plates exhibiting a smaller growth film
diameter than PA14 plates at the beginning of the experiment but a larger diameter by
the end of the experiment. Therefore under these conditions, *A. castellanii* had a more
consistent grazing behaviour than *A. polyphaga* with regards to these prey bacteria.

The usefulness of *A. castellanii* to measure the effect of *P. aeruginosa* virulence factors
was confirmed when PAPI-1 and PAPI-2 deletion mutants were tested. *A. castellanii*
growth films on all mutants expanded faster compared to that on PA14, supporting the
idea that these island-minus mutants were less virulent than PA14 for this amoeba
species. However, a surprising difference between the mutants was observed. *A.
growth film on PA14ΔPAPI-2 and PA14Δ1Δ2 expanded in an indistinguishable manner and remained below that of the PAO1-associated growth film. In contrast, the rate of grazing on PA14ΔPAPI-1 as judged by the expansion of the ameoba film was extremely fast compared to those of the other mutants and was even comparable to that of the control virulence-attenuated PAO1 strain. The very large decrease in virulence seen with the mutant lacking only PAPI-1 was initially ascribed to a difference in the actual number of bacteria plated since CFU/plate for this mutant were significantly lower than CFU/plate for PA14 wildtype for the assays in question. Additionally, the number of PA14Δ1Δ2 plated was significantly greater than that of PA14, while the number of PA14ΔPAPI-2 compared to PA14 plated was not statistically different. Notwithstanding this difference, the PA14ΔPAPI-2 and PA14Δ1Δ2 associated growth film expansion trend lines were very nearly indistinguishable. Moreover, it will be shown that plating fewer bacteria on NNA is not a major factor in determining the rate of expansion of the *A. castellanii* growth film (Appendix F, Figure F-1).

The basis of the difference in the *A. castellanii* NNA-defined phenotypes between PA14ΔPAPI-1 and PA14Δ1Δ2 remained uncertain. However, in 2011 it was demonstrated that the PA14ΔPAPI-1 strain, designated as KR572, had undergone a further, previously undetected deletion event resulting in the loss of the Pf5 prophage as well (Harrison, Deyell & Rajakumar; unpublished data). Considering that PA14Δ1Δ2 was derived from the PA14ΔPAPI-2 strain by deletion of PAPI-1 and that the expansion of these two mutants on *A. castellanii* was very similar, it can be inferred that the PA14ΔPAPI-1 phenotype may be due to loss of the Pf5 prophage and not loss of PAPI-1 itself, and that only PAPI-2 and not PAPI-1 is involved in protecting *P. aeruginosa* from *A. castellanii* predation.
The *A. polyphaga* growth film expansion on PA14Δ1Δ2 was indistinguishable from that on the wildtype strain, while expansion on the single mutants was slightly faster. It appeared that losing either PAPI-1 or PAPI-2 marginally affected the virulence against *A. polyphaga*, but loss of both islands caused the bacteria to revert back to the wildtype virulence phenotype. Since the slightly faster expansion on PA14ΔPAPI-1 or on PA14ΔPAPI-2 was not repeated on the expansion of PA14Δ1Δ2, it is doubtful that the differences recorded have any biological significance.

The difference of the diameter recorded for *A. polyphaga* on *P. aeruginosa* strains was narrower than that of *A. castellanii* (Figure 3-7 and 3-9) showing an intrinsic difference between the two *Acanthamoeba* species in their capacity to be affected by *P. aeruginosa* virulence factors.

When PA14 island-deletion mutants were tested in an acute pneumonia mouse model, the loss of both PAPI-1 and PAPI-2 rendered PA14 less virulent to a higher degree than the loss of each island by itself. This prompted the hypothesis that the two islands made a synergistic contribution to virulence at least in the model examined (Harrison et al. 2010). No similar synergistic effect was observed in any of the *C. elegans* or *Acanthamoeba* spp. assays used in this study.

The presence of PAPI-2 conferred a reproducibly negative impact on the ability of *A. castellanii* to graze on PA14 in the non-nutrient agar assay and a more subtle and condition-dependent virulence-enhancing effect as measured by the *C. elegans* slow-killing assay. It is known that type III secretion system (T3SS) effectors are involved in protection against *A. castellanii*, although previous experiments were performed under different assay conditions (Abd et al. 2008, Matz et al. 2008). Therefore, it is plausible that the observed effect of PAPI-2 on *A. castellanii* was exerted via ExoU. On the contrary despite the induction of the components of T3SS in the *C. elegans* slow-killing
assay conditions (Wareham, Papakonstantinopoulou & Curtis 2005), ExoU did not contribute significantly to *C. elegans* killing during this assay (Miyata *et al.* 2003). As consequence, the effect of PAPI-2, if any, on *C. elegans* was exerted by factors other than ExoU. Consistent with this idea, a possible association of non-*exoU* PAPI-2 *orfs* to virulence was shown in a *Galleria mellonella* survival assay where infection by PA14ΔPAPI-2 resulted in significantly delayed killing compared to infection by PA14 or PA14ΔexoU (Harrison *et al.* 2010).

The influence on virulence exerted by PAPI-1 cannot be truly tested using KR572 because of the deletion of Pf5 in this specific strain. However it can be inferred by comparison of data obtained from PA14Δ1Δ2 and PA14ΔPAPI-2. In all the relevant assays applied in this study the PA14 strain lacking only PAPI-2 (PA14ΔPAPI-2) behaved in an indistinguishable manner to the PA14 strain lacking both PAPI-1 and PAPI-2 (PA14Δ1Δ2). Consequently, it can be concluded that the contribution of PAPI-1 to virulence was negligible or below the detection threshold of the assays used.
Chapter 4

Identification of genomic islands involved in *P. aeruginosa* virulence by a newly established *C. elegans* highthroughput assay
Introduction

Horizontal gene transfer, through alteration of the accessory genome, is a pivotal mechanism for shaping the evolution of bacteria and the virulence of pathogens (Gal-Mor, Finlay 2006, Groisman, Ochman 1996). The relationship between accessory elements and virulence has been reinforced by the demonstration that genomic islands (GIs) of pathogenic bacteria contain a significantly higher proportion of virulence-related genes than the rest of the genome (Ho Sui et al. 2009). Virulence traits can be classified in different categories according to the role they sustain during infection as “offensive” (i.e. enable bacterial invasion or damage of the host), “defensive” (i.e. evasion from immune response), “nonspecific” and “regulatory” (Chen et al. 2012). Interestingly, genomic islands are more likely to be associated with “offensive” or “nonspecific” virulence traits than with “defensive” factors (Ho Sui et al. 2009).

Genomic islands associated with tRNAs in P. aeruginosa

tRNAs represent hotspots for foreign DNA integration in the bacterial chromosome in particular by integrative and conjugative elements (Williams 2002) and prophages (Dobrindt et al. 2004). P. aeruginosa possess 20 regions of genome plasticity (RGPs) associated with tRNAs, many of which are involved in integration of pKLC102-related or PAGI-2-related genomic islands (Klockgether et al. 2011).

Association between tRNA-integrated accessory elements and virulence

Little is known about the contribution to virulence of tRNA-associated genomic islands as a whole but links has been established for some cargo genes. For example,
The interruption of genes residing in $tRNA^{Lys}$-associated PAPI-1 and PAPI-2 showed increased $C. elegans$ survival (He et al. 2004). Moreover, deletion of PAPI-2 decreased bacterial recovery from lungs and blood of mice in an acute pneumonia infection model (in particular in conjugation with PAPI-1 deletion), increased survival of $Galleria mellonella$ ($G. mellonella$) larvae (Harrison et al. 2010) and increased expansion of $A. castellanii$ film on non-nutrient agar layered with PA14ΔPAPI-2 strain (Chapter 3 in this thesis) compared with the wildtype strain. Association between NR-I and NR-II regions found in PAGI-5 $tRNA^{Lys}$-integrated island and virulence was established in an acute pneumonia mouse model (Battle et al. 2008). The prevalence of virulence-related traits in the accessory genome and the association of some $tRNA^{Lys}$-related genomic islands with survival in several models of infection have led to the hypothesis that islands associated with other $tRNAs$ may contribute to $P. aeruginosa$ virulence in both animal models of infection and in humans.

**Genomic islands associated with $tRNA^{Pro21}$ (RGP28)**

Integration at the $tRNA^{Pro21}$ site occurs at extremely high frequency. A panel of 37 $P. aeruginosa$ isolates with different origins (from China or from UK) was tested by PCR and all strains showed integrated elements at this site (Harrison 2009). *In silico* analysis of completely or nearly-completely sequenced $P. aeruginosa$ genomes revealed the presence of strain-specific inserted elements larger than 10kb in 27 out of 31 (87%) strains with an average size of ~ 30 kb (range= 11.4-116.0 kb). Of the remaining strains, two presented elements smaller than 10 kb (7.5 kb and 2.5 kb from RP73 and YL84 strains, respectively) while for the remaining two strains, downstream flanking regions could not be identified (Appendix B, Table B-1).

To assign a role to the $tRNA^{Pro21}$ elements has been difficult due to the deficient level of annotation of the open reading frames (ORFs) predicted inside accessory elements. In
fact, 54% of identified coding regions have been classified as hypothetical. The best described coding regions are the ones with functions related to DNA mobility (such as transposases, integrases and DNA helicases) and related to restriction/modification systems. Some have been identified as phage tail proteins pointing toward the foreign origin of the island. Moreover, efflux systems, DNA sulphur modification protein, cell division protein, alkaline phosphatase and many transcriptional regulators have been putatively identified (Appendix B, Table B-1) that might directly or indirectly influence the virulence of the strain that carries the island.

The genomic islands present at this integration hotspot appeared very diverse with the exception of the islands associated with LES-like strains. Therefore the accessory elements present in this hotspot appear to contribute to both plasticity of *P. aeruginosa* genome and diversity between strains. Because of the great variability of islands inserted at this hotspot and the lack of more informative annotation, it is difficult to predict any association with virulence.

**Genomic islands associated with tRNA^{Gly}_{19} (RGP27)**

*tRNA^{Gly}_{19}* is one of the preferred integration sites for *P. aeruginosa* genomic island (PAGI)-2-related elements (Klockgether *et al.* 2011). In the present study of the 31 completely or nearly-completely sequenced *P. aeruginosa* strains, almost half of them (47%) reported an “empty site” with no accessory elements inserted. Of the remaining strains, downstream flanking region could not be identified in one of them; ten strains showed an inserted element larger than 10 kb (31%) and six consisted of an element smaller than 10 kb (19%). The average size of genomic island inserted at this hotspot was 104 kb (Appendix B, Table B-2).

The genomic islands found in the LES-like strains and in the DK2 strain exhibited several transcriptional regulators, heavy metal detoxification systems and efflux
systems similar to other PAGI-2 related elements (Larbig et al. 2002) as well as integrases, transposases and transcriptional regulators. Interestingly, in the Larbig et al. study it was found that the DK2 $tRNA^{Gly_{19}}$ island carries a component of the type IV secretion system not present in the island carried by LES-like strains. While it is smaller than the elements found in LES-like and DK2 strains, the genomic island found in PA96 similarly carries many transposases, a phage-related integrase and a disrupted type IV secretion system component (Appendix B, Table B-2).

Integration at $tRNA^{Gly_{19}}$ site occurs seldom in *P. aeruginosa* strains but usually leads to the integration of very large elements. They might improve the ability of a strain to survive in environments with high levels of heavy metals as it was postulated for PAGI-2 (Larbig et al. 2002) and other PAGI-2 like elements (Klockgether et al. 2011).

**Genomic islands associated with $tRNA^{Ser_{11}}$ (RGP8)**

Although $tRNA^{Ser_{11}}$ has been included among the regions of genome plasticity, the presence of genetic elements at this site is rare. Of the 31 completely or nearly-completely sequenced strains, 26 (84%) did not show any genetic element integrated at this site, while for one strain the downstream flanking region could not be identified. Of the remaining four strains, NCGM 1984 and NCGM 1900 carry an identical 54.5 kb island while PES305 and DK2 carry an islet (Appendix B, Table B-3). The data shows that almost three-quarters of the recognized ORFs (71%) are indicated in NCBI database as hypothetical. Among the annotated ORFs, many are similar to bacteriophages proteins (in particular integrases and structural proteins) and transposases while several were annotated as kinase, flavodoxin, efflux-related proteins and undefined lytic enzyme. Because of the paucity of information, it is difficult to predict any association between presence of an island at this hotspot and virulence.
Aims of this chapter

There is a high number of *orfs* associated with the assessor genome, in particular genomic islands, that remain hypothetical and for which no function can be identified. Therefore, it was hypothesized that by creating *P. aeruginosa* mutants lacking genomic islands and using different assays both *in vitro* and *in vivo*, it would be possible to identify phenotypes associated with specific islands and consequently match them to specific *orfs*. The overall aim of this chapter was to identify *tRNAs*-associated genomic islands influencing the pathogenicity of *P. aeruginosa* using *C. elegans* as infection model. The specific aims were:

1. To establish a modified *C. elegans* slow-killing assay to more quickly screen *P. aeruginosa* strains for their virulence potential;

2. To test survival of *C. elegans* when seeded on mutants lacking *tRNAPro21*- or *tRNAGly19*- or *tRNASer11*-associated islands and compare it with survival on the isogenic wildtype strains;

3. To test attenuation of selected *P. aeruginosa* deletion mutants using the slow-killing assay.
Results

Validation of a new *C. elegans* highthroughput assay

The *C. elegans* traditional slow-killing assay (SKA) is a time-consuming procedure and with low-throughput results. In order to more quickly evaluate *P. aeruginosa* ability to compromise the survival of nematodes, I sought to modify the SKA by using a multi-well plate system.

To set up a multi-well highthroughput *C. elegans* assay

*C. elegans* assays based on the use of multi-well plates have been described previously and employed to predict the attenuation in virulence of a library of PA14 mutants generated by random transposition using 96-well plates (Tan *et al.* 1999) or 6-well plates (Lee *et al.* 2006). These assays were based on the observation that more offspring were present when the mutant tested was attenuated. Therefore, I postulated that the “attenuated bacterial mutant = more *C. elegans* progeny” concept could be extended to comparison between *P. aeruginosa* strains, with the size of progeny depending on the specific virulence of each strain.

It is known that *P. aeruginosa* PA14 strain is more virulent than PAO1 in many models of infection, including in the *C. elegans* slow-killing assay (Lee *et al.* 2006). More generally, *P. aeruginosa* strains demonstrated diverse capacity in preventing plaque formation on agar when co-cultured with *Dictyostelium discoideum* (Janjua *et al.* 2012) and in hindering the survival of nematodes in both fast-killing (Janjua *et al.* 2012) and slow-killing assays (Lee *et al.* 2006). This phenomenon was also confirmed when
several clinically relevant isolates were tested using the slow-killing assay at the University of Leicester (Figure 4-1A and 4-1B).

The *C. elegans* multi-well plate assay protocol used is based on previously published work (Tan *et al.* 1999, Lee *et al.* 2006) and is essentially a modified version of the slow-killing assay. The major differences with the slow-killing assay were the use 24-well plates and fertile *C. elegans* N2 in the multi-well assay instead of 60 mm culture dishes and temperature sterile *C. elegans* CF512 in the slow-killing assay (see Materials and Methods). The 24-well plates were preferred to 96-well plates because the difference in *C. elegans* survival between PAO1 and PA14 was comparable to that of the slow-killing assay. In fact, wells plated with PA14 and seeded with N2 showed few L1 or L2 larvae after 4 days of incubation at 25°C while wells plated with PAO1 showed many nematodes at different developmental stages (Figure 4-2). Based on the fact that 35 *P. aeruginosa* strains could be screened at once in a shorter time, this assay was named high-throughput assay (HTA).

The output of the high-throughput assay has been termed “HTA score” and it is based on a discretionary scale. During HTA, four wells for each *P. aeruginosa* strain tested were plated and seeded with *C. elegans* N2 synchronized young adults. The 24-well plates were incubated at 25°C for 4 days and a score was assigned to each well. Wells with PA14 and PAO1 were assigned arbitrary scores based on the amount of offspring visible, with a low value indicating the presence of few nematodes while a high value indicated the presence of many nematodes (Chapter 2, Table 2-4). The score for the other wells was defined by visual comparison with PAO1 and PA14. For each strain, the HTA score was calculated as the average of scores from four individual wells.
Figure 4-1: Slow-killing assay for *P. aeruginosa* clinical isolates

Plot showing the survival of nematodes (CF512) over time in slow-killing assay performed with (A) eight or (B) four *P. aeruginosa* clinical isolates and PAO1 and PA14 as virulence-attenuated and highly virulent control strains, respectively. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± the standard deviation values shown. These experiments were performed using 10 nematodes/plate and three plates for each strain.
Figure 4-2: *C. elegans* brood size when seeded on PA14 or PAO1

Images of wells plated with PA14 (highly virulent strain) or PAO1 (attenuated strain), seeded with *C. elegans* N2 followed by 4 days incubation at 25°C obtained using a 10× objective in bright field from two representative experiments (Exp. 53 and Exp. 51). The presence of nematodes at L1/L2 larval stage in PA14 wells is indicated by dotted circles while nematodes at various developmental stages are indicated by arrows for PAO1.
To equate the high-throughput assay with the slow-killing assay

In order to establish whether the *P. aeruginosa* virulence phenotype assessed with high-throughput assay is comparable to the phenotype obtained with the classical slow-killing assay, the *P. aeruginosa* strains with established SKA phenotype (Figure 4-1A and 4-1B) were tested in the HTA. For each strain, the HTA score was plotted against the time required to kill 50% of the nematodes (TD$_{50}$) and calculated using the Kaplan-Meier survival analysis after SKA (Figure 4-3A). Statistical analysis confirmed the presence of a strong positive and significant correlation between HTA score and SKA TD$_{50}$ (Spearman correlation coefficient = 0.805; $P$-value = 0.002).

Furthermore, in the classical slow-killing assay (Figure 4-1A and 4-1B), the *P. aeruginosa* isolates may be divided into three groups based on how quickly they kill nematodes in comparison with PA14 and PAO1 (Table 4-1): strains with high virulence, strains with intermediate virulence and strains with low virulence. After HTA scores were obtained for the same groups of strains, the isolates were also divided into the same categories (Table 4-1). Only three strains out of twelve were grouped differently and they were either in the intermediate or the low virulence category. Strains belonging to the high virulence and intermediate category were never or seldom, respectively, classified differently by the high-throughput assay compared to the slow-killing assay.

Reproducibility of the high-throughput assay

Since the high-throughput assay score was assigned empirically by comparison against *P. aeruginosa* control strains (PAO1 and PA14) while the slow-killing assay TD$_{50}$ was calculated as part of the survival analysis, it was necessary to assess the reliability of the HTA score. Over a long period of time (one year), several experiments were performed and nine *P. aeruginosa* strains were independently tested two or three times. The scores reported in different experiments for these strains were very consistent showing a
narrow standard deviation (Table 4-2). The only exception was KR129 with one of the values (1.6 in Exp. 58) lower than the other two values (2.8 in Exp. 53 and 2.9 in Exp. 65).

---

**Figure 4-3: Relationship between high throughput assay (HTA) score and slow-killing assay (SKA) TD$_{50}$**

A. Scatterplot showing the time needed to kill 50% of the nematodes (TD$_{50}$) against the score obtained from *C. elegans* high throughput assay (HTA score) for twelve *P. aeruginosa* strains. The TD$_{50}$ was calculated by the Kaplan-Meier survival analysis after slow-killing assay was performed while the HTA score was calculated as average of 4 wells after high throughput assay was performed. The black line represents the linear regression between TD$_{50}$ and HTA score calculated by GraphPad software.

B. Equation describing the mathematical relationship between TD$_{50}$ and HTA score calculated by GraphPad software.

\[
TD_{50} = \frac{HTA \text{ score} + 1.086}{0.02866}
\]
Table 4-1: Virulence phenotype assigned to *P. aeruginosa* strains

*P. aeruginosa* clinical isolates were divided into three groups (high, intermediate and low virulence) by comparing the survival of CF512 nematodes during slow-killing assays with survival on the highly virulent PA14 strain (KR125) and the attenuated PAO1 strain (KR124). Based on the HTA scores, the strains were also divided into three groups: strains with HTA score from 0.1 to 0.5 (high virulence), strains with HTA score from 2.3 to 3.5 (intermediate virulence) and strains with HTA score from 4.5 to 5.8 (low virulence).

<table>
<thead>
<tr>
<th>Virulence level</th>
<th>Slow-killing assay</th>
<th>Hightthroughput assay</th>
</tr>
</thead>
</table>
| HIGH (As virulent as PA14 or more virulent) | K6 (KR133)  
KR378  
KR381 | K6 (KR133)  
KR378  
KR381 |
| INTERMEDIATE (Between PA14 and PAO1) | Clone C (KR127)  
LES431 (KR129) | Clone C (KR127)  
LES431 (KR129)  
LESB58 (KR130) |
| LOW (As attenuated as PAO1 or more attenuated) | LES400 (KR128)  
LESB58 (KR130) | LES400 (KR128)  
KR419  
C3719 (KR479) |

### Table 4-2: Reproducibility of HTA scores

*C. elegans* N2 highthroughput assay (HTA) score assigned to *P. aeruginosa* strains tested in more than one experiment. The last column shows the average and the standard deviation (St. Dev.) of scores reported from each experiment. Exp. = original experiment number; / = strain was not tested in the experiment.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Exp. 49</th>
<th>Exp. 51</th>
<th>Exp. 53</th>
<th>Exp. 54</th>
<th>Exp. 58</th>
<th>Exp. 65</th>
<th>Average ± St. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR129</td>
<td>/</td>
<td>/</td>
<td>2.8</td>
<td>/</td>
<td>1.6</td>
<td>2.9</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>KR133</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>KR373</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6 ± 0.0</td>
</tr>
<tr>
<td>KR378</td>
<td>/</td>
<td>/</td>
<td>0.5</td>
<td>/</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>KR822</td>
<td>0.3</td>
<td>/</td>
<td>0.3</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>KR836</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>0.1</td>
<td>0.0</td>
<td>/</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>KR842</td>
<td>/</td>
<td>/</td>
<td>0.0</td>
<td>0.5</td>
<td>/</td>
<td>/</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>KR877</td>
<td>/</td>
<td>/</td>
<td>2.8</td>
<td>/</td>
<td>2.9</td>
<td>/</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>KR1175</td>
<td>/</td>
<td>0.8</td>
<td>/</td>
<td>0.5</td>
<td>/</td>
<td>/</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>
Screening of *P. aeruginosa* wildtype/deletion mutants pairs

Since previously established highthroughput assay were used to screen *P. aeruginosa* mutant libraries to pinpoint attenuated mutants, I sought to employ the *C. elegans* highthroughput assay to screen *P. aeruginosa* mutants lacking a *tRNA*-associated genomic island by determining their HTA scores and comparing them with that of isogenic wildtype strains. It was estimated that a difference in HTA score of 1 corresponded to a TD$_{50}$ difference of 35 h in the slow-killing assay (Figure 4-3B). Therefore, a score difference of 1 was used as cut-off to define a “substantial” difference between *P. aeruginosa* wildtype and genomic island mutant and to select the pairs to be tested further.

**Deletion of *tRNA*$_{Pro21}$-, *tRNA*$_{Gly19}$- and *tRNA*$_{Ser11}$-inserted genomic islands and preliminary growth tests**

In order to establish the relevance of *tRNA*-associated genomic islands in both *in vivo* and *in vitro* assays, these accessory elements were knocked-out by homologous recombination, similarly to the technique used to create ΔPAPI-1 and ΔPAPI-2 from PA14 (Harrison et al. 2010). In particular, studies of the effects of deletion of islands associated with *tRNA*$_{Pro21}$, *tRNA*$_{Ser11}$ or *tRNA*$_{Gly19}$ were pursued. Because the *tRNA*$_{Pro21}$ site is very often occupied while *tRNA*$_{Ser11}$ or *tRNA*$_{Gly19}$ sites are seldom occupied, deletion of the *tRNA*$_{Pro21}$ island was achieved in 15 strains (Harrison 2009; Rieck, Yacub & Rajakumar, unpublished data), while deletion of *tRNA*$_{Ser11}$ island or *tRNA*$_{Gly19}$ island was achieved in three and two strains, respectively (Rieck, Yacub & Rajakumar, unpublished data).
In preliminary tests, changes due to the deletion of genomic islands were assayed by measuring *P. aeruginosa* growth in rich medium. In doing so, several different phenotypes were revealed in comparison with the original wildtype strain. In some instances, deletion of the island resulted in a reduction of the growth rate and/or the overall density reached (Appendix C, Figure C-2, C-5 and C-6), while more rarely, the absence of the island increased the bacterial growth (Appendix C, Figure C-7). In other cases (Appendix C, Figure C-1, C-3 and C-4), no growth difference was recorded between the *P. aeruginosa* wildtype and the deletion mutants. These preliminary results are an indication of the phenotype diversity attributable to this group of genomic island.

**Screening of *P. aeruginosa* mutants with deletion of tRNAPro21 genomic island**

Seventeen wildtype/tRNAPro21-deletion mutant pairs were tested in the high-throughput assay (Figure 4-4: wildtype= red column, ΔPro21= blue column; Appendix D, Table D-1). For two pairs out of seventeen, the HTA score of the wildtype strain was the same as the score of the deletion mutant indicating that the absence of the island had no effect on brood size and therefore no effect on the virulence of the *P. aeruginosa*. For six pairs, the score recorded for wildtype *P. aeruginosa* was higher than the score recorded for the mutants. Consequently, it appears that the loss of the island in these six mutants decreased the size of the progeny of the seeded nematodes. However, the difference was minimal for all mutant pairs but one (MID9245/KR976) for which it was equal to the cut-off of 1 and therefore considered substantial.

For the nine remaining pairs, the score of the mutants were higher than the score of the corresponding wildtype (Figure 4-4; Appendix D, Table D-1). In this group, two mutants were derived from PA14 and two from KR378. Although created independently, the mutants showed similar trends when compared to their respective
wildtype strain. Out of seven pairs, four reported substantial increase of the HTA score associated with the mutant compared to the score of the isogenic wildtype (difference above the cut-off of 1). These pairs included two mutants derived from PA14, one from PA103 and one from the clinical isolate KR877. Deletion of \( tRNA^{Pro21} \)-associated genomic island from K6, KR378, KR822 and KR836 did not seem to markedly alter the progeny formation and consequently the virulence of the strain, while deletion from PA103, PA14 and KR877 appeared to considerably increase brood size and therefore reduce \( P. aeruginosa \) virulence toward \( C. elegans \).

**Screening of \( P. aeruginosa \) mutants with deletion of the \( tRNA^{Gly19} \) genomic island**

Because of the infrequent presence of genomic islands at \( tRNA^{Gly19} \), only two strains could be used to create deletion mutants (Figure 4-4: wildtype= red column, \( \Delta \)Gly19= purple column; Appendix D, Table D-2). In one instance, the mutant without the \( tRNA^{Gly19} \)-associated island (KR1236) showed a lower virulence score than the corresponding wildtype (the clinical isolate KR352) implicating the island in increasing the progeny size and therefore decreasing the virulence toward nematodes. On the contrary, the deletion of the \( tRNA^{Gly19} \)-associated island from LES431 increase the HTA score of the mutant compared to the wildtype. In this case, the \( tRNA^{Gly19} \) island appeared to reduce the brood size and be implicated in increasing the overall virulence of the strain.

**Screening of \( P. aeruginosa \) mutants with deletion of \( tRNA^{Ser11} \) genomic island**

Because there were few strains that had a genomic island associated with \( tRNA^{Ser11} \), this could be deleted only from three strains (Figure 4-4: wildtype= red column, \( \Delta \)Ser11= 
For one pair (KR352/KR1274), the score of the mutant was lower than that of the wildtype but far from the cut-off point indicating the lack of effect this island had on progeny formation and presumably in survival of nematodes. The other two wildtype/deletion mutant pairs tested showed a positive difference with the mutants scoring higher than the wildtype. Since the difference was well above the cut-off of 1, it can be assumed that the presence of $tRNA^{Ser11}$ island in these strains contribute to reduce the brood size of the nematodes and consequently increase $P. aeruginosa$ virulence toward $C. elegans$.

![Figure 4-4: HTA scores for wildtype and mutants strains](image)

**Legend:**
- $\text{red}$ = HTA score for wildtype
- $\text{dark green}$ = HTA score for $\Delta Pro21$ mutant lacking the $tRNA^{Pro21}$ genomic island
- $\text{purple}$ = HTA score for $\Delta Gly19$ mutant lacking the $tRNA^{Gly19}$ genomic island
- $\text{light green}$ = HTA score for $\Delta Ser11$ mutant lacking the $tRNA^{Ser11}$ genomic island
Slow-killing assay for selected \textit{P. aeruginosa} wildtype/mutant pairs

Based on the initial tests, some of the mutants that returned a score substantially higher than the isogenic wildtype strain (above the cut-off of 1) were re-tested using the classical slow-killing assay. Three mutants lacking the \textit{tRNA}^{Pro21}\textit{-associated island}, one mutant lacking the \textit{tRNA}^{Ser11}\textit{-associated island} and one lacking the \textit{tRNA}^{Gly19}\textit{-associated island} were selected.

Testing PA14 wildtype and derived deletion mutants

According to the highthroughput assay, both independent \textit{tRNA}^{Pro21}\textit{-associated island} deletion mutants derived from PA14 (KR1034 and KR1035) showed an attenuated virulence phenotype exhibited as an increase in \textit{C. elegans} brood size. These were compared with the original PA14 strain in the slow-killing assay both at the University of Leicester and at the Universiti Kabangsaan Malaysia. In both places, PAO1 was used as an attenuated control to gauge the level of attenuation of the mutants.

In the slow-killing assay performed at the University of Leicester (Figure 4-5A), the survival of nematodes seeded on PA14 wildtype was significantly shorter than that of the other strains tested (Table 4-3A). In fact, \textit{C. elegans} seeded on \textit{P. aeruginosa} mutants lacking \textit{tRNA}^{Pro21}\textit{ island} or on PAO1 survived ~ 1-1.5 days or 4 days longer, respectively, than nematodes seeded on PA14. In the slow-killing assay performed at the Universiti Kabangsaan Malaysia (Figure 4-5B), the nematodes seeded on PAO1 survived on average almost 2 days longer than on PA14 (Table 4-3B). This difference was reduced compared to that recorded at the University of Leicester but was still significant (Table 4-3B). Notwithstanding a narrowed difference between PAO1 and PA14, \textit{C. elegans} seeded on either \textit{P. aeruginosa} deletion mutant showed a significantly
longer survival (~ 1 day) compared to nematodes seeded on PA14 wildtype (Table 4-3A and Table 4-3B).

**Testing LES431 wildtype and derived deletion mutant**

Two deletion mutants were created from LES431, a *P. aeruginosa* strain isolated from a parent of a cystic fibrosis patient that demonstrated LasR overproduction (Salunkhe *et al.* 2005). The $tRNA^{Pro21}$ genomic island was deleted in one of the mutants (LES431$\Delta$Pro21) while $tRNA^{Gly19}$ genomic island was deleted in the other mutant (LES431$\Delta$Gly19). According with the highthroughput assay results, the lack of the former island did not seem to have a major impact on virulence, while the lack of the latter appeared to decrease LES431 virulence towards *C. elegans*. Therefore, the mutant without the $tRNA^{Gly19}$-associated island was compared with isogenic LES431 wildtype in the slow-killing assay (Figure 4-6). PA14 and PAO1 were used as virulent and attenuated controls, respectively. LES431 wildtype strain showed a virulence level intermediate between PA14 and PAO1 with *C. elegans* seeded on LES431 surviving significantly longer (~ 2.5 days) that those seeded on PA14 and significantly shorter (~ 1 day) that those seeded on PAO1 (Table 4-4). The survival of nematodes seeded on LES431$\Delta$Gly19 was very similar to that of the wildtype strain with *C. elegans* dying on average ~10 h earlier. The difference in *C. elegans* survival between the wildtype and mutant was minimal and consequently not significant (Table 4-4).
Figure 4-5: PA14-related strains assessed with slow-killing assay

Plot showing the survival of nematodes over time in slow-killing assay (A) done at University of Leicester by seeding 18-20 nematodes/plate using *C. elegans* CF512 and three plates for each strain or (B) done at Universiti Kabangsaan Malaysia by seeding 50-90 nematodes/plate using *C. elegans rrf-3; glp-4* and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± standard deviation values shown. Survival of nematodes fed with PA14 wildtype was compared to that of ∆Pro21 mutants using the Log-Rank test within the Kaplan-Meier survival analysis (Table 4-3) and significant *P*-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- = PA14 wildtype (PA14 WT; KR125)
- = PA14 without *tRNA<sub>Pro21</sub>* island (PA14ΔPro21, KR1034)
- = PA14 without *tRNA<sub>Pro21</sub>* island (PA14ΔPro21, KR1035)
- = PAO1 (KR124); = *E. coli* OP50
Table 4-3: Survival analysis of *C. elegans* seeded on PA14-related strains

Survival analysis performed using the Kaplan-Meier method for slow-killing assays (A) done at the University of Leicester by using *C. elegans* CF512 or (B) done at Universiti Kabangsaan Malaysia by using *C. elegans* rrf-3;glp-4.

Table headings legend:
- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TD<sub>m</sub> (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD<sub>50</sub> (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank χ<sup>2</sup>; P-value** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **χ<sup>2</sup>** = Log-Rank test value calculated by the software with *P*-value significant if below 0.05 (underlined)
- **n.a.** = not available

### A.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TD&lt;sub&gt;m&lt;/sub&gt; (h ± SE)</th>
<th>TD&lt;sub&gt;50&lt;/sub&gt; (h ± SE)</th>
<th>Log Rank χ&lt;sup&gt;2&lt;/sup&gt;; P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14 WT</td>
<td>57 (54%)</td>
<td>85.5 ± 3.6</td>
<td>86 ± 5</td>
<td>/</td>
</tr>
<tr>
<td>PA14ΔPro21 (KR1034)</td>
<td>56 (55%)</td>
<td>131.6 ± 4.8</td>
<td>123 ± 8</td>
<td>69.086; &lt;0.001</td>
</tr>
<tr>
<td>PA14ΔPro21 (KR1035)</td>
<td>56 (32%)</td>
<td>114.1 ± 4.4</td>
<td>110 ± 9</td>
<td>34.377; &lt;0.001</td>
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<tr>
<td>PAO1</td>
<td>60 (32%)</td>
<td>177.6 ± 7.6</td>
<td>172 ± 8</td>
<td>16.033; &lt;0.001</td>
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</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TD&lt;sub&gt;m&lt;/sub&gt; (h ± SE)</th>
<th>TD&lt;sub&gt;50&lt;/sub&gt; (h ± SE)</th>
<th>Log Rank χ&lt;sup&gt;2&lt;/sup&gt;; P-value</th>
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<tbody>
<tr>
<td>PA14 WT</td>
<td>250 (50%)</td>
<td>66.6 ± 2.6</td>
<td>55 ± 2</td>
<td>/</td>
</tr>
<tr>
<td>PA14ΔPro21 (KR1034)</td>
<td>240 (36%)</td>
<td>95.2 ± 3.2</td>
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<td>PA14ΔPro21 (KR1035)</td>
<td>240 (34%)</td>
<td>94.7 ± 3.0</td>
<td>96 ± 0</td>
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</tr>
<tr>
<td>PAO1</td>
<td>180 (38%)</td>
<td>112.3 ± 4.1</td>
<td>120 ± 9</td>
<td>60.672; &lt;0.001</td>
</tr>
</tbody>
</table>
Figure 4-6: LES431-related strains assessed with slow-killing assay

Plot showing the survival of nematode over time in slow-killing assay done at the University of Leicester by seeding 18-20 nematodes/plate using *C. elegans* CF512 and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± standard deviation values shown. Survival of nematodes fed with LES431 wildtype was compared to that of ΔGly19 mutant and the other strains using the Log-Rank test within the Kaplan-Meier survival analysis (Table 4-4) and significant P-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**
- ● = PA14 wildtype (PA14 WT; KR125)
- ▢ = LES431 wildtype (KR129)
- ▲ = LES431 without tRNA\(^{Gly19}\) island (LES431ΔGly19, KR1243)
- ◆ = PAO1 (KR124)
<table>
<thead>
<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>$T_D_m$ (h ± SE)</th>
<th>$T_D_{50}$ (h ± SE)</th>
<th>Log Rank $\chi^2$; $P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LES431 WT</td>
<td>59 (37%)</td>
<td>147.4 ± 4.7</td>
<td>146 ± 1</td>
<td>/</td>
</tr>
<tr>
<td>LES431(\Delta)Gly19 (K81243)</td>
<td>60 (32%)</td>
<td>136.8 ± 5.1</td>
<td>135 ± 6</td>
<td>2.097; 0.148</td>
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<tr>
<td>PA14 WT</td>
<td>57 (54%)</td>
<td>85.5 ± 3.6</td>
<td>86 ± 5</td>
<td>48.217; &lt;0.001</td>
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<tr>
<td>PAO1</td>
<td>60 (32%)</td>
<td>177.6 ± 7.6</td>
<td>172 ± 8</td>
<td>13.397; &lt;0.001</td>
</tr>
</tbody>
</table>

**Table 4-4: Survival analysis of *C. elegans* seeded on LES431-related strains**

Survival analysis performed using the Kaplan-Meier method for slow-killing assays done at the University of Leicester using *C. elegans* CF512.

**Table headings legend:**

- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **$T_D_m$ (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **$T_D_{50}$ (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **$\chi^2$** = Log-Rank test value calculated by the software with $P$-value significant if below 0.05 (underlined)
- **n.a.** = not available
Testing KR877 wildtype and derived deletion mutants

Similarly to LES431, two independent deletion mutants were created using KR877, a clinical isolate collected from sputum sample of a patient at the University Hospitals Leicester. According with highthroughput assay results, the deletion of either \( tRNA_{Pro21}^{KR877} \) (KR877\( \Delta \)Pro21) or \( tRNA_{Ser11}^{KR877} \)-associated island (KR877\( \Delta \)Ser11) reduced KR877 virulence. Therefore both mutants were tested against the wildtype strain in the slow-killing assay. As done previously, PAO1 and PA14 were used as controls (Figure 4-7). Similarly to LES431, KR877 had an intermediate virulence with the survival of nematodes being significantly different from both PAO1 and PA14 (Table 4-5). The seeding of nematodes on KR877\( \Delta \)Pro21 significantly extended their survival (~1 day) when compared to that of wildtype strain (Table 4-5). The survival of nematodes on the \( P. \ aeruginosa \) mutant without \( tRNA_{Ser11}^{KR877} \) was identical to survival on wildtype up to 108 h from the beginning of the experiment. At 120 h, the nematodes seeded on the mutant started to die less frequently, with the difference with the wildtype being maintained until the end of experiment (Figure 4-7). Overall, \( C. \ elegans \) seeded on KR877\( \Delta \)Ser11 (KR1278) died on average only ~10 h later than those seeded on wildtype but the difference was significant (\( P \)-value= 0.024; Table 4-5).
Plot showing the survival of nematode over time in slow-killing assay done at the University of Leicester by seeding 18-20 nematodes/plate using C. elegans CF512 and three plates for each strain. At each time point, the percentage/plate of nematodes that were alive was calculated and average ± standard deviation values shown. Survival of nematodes fed with KR877 wildtype was compared to that of mutants and the other strains using the Log-Rank test within the Kaplan-Meier survival analysis (Table 4-5) and significant P-values (<0.05) are shown.

**Bacterial strains on which nematodes were seeded:**

- **•** = PA14 wildtype (PA14 WT; KR125)
- **□** = KR877 wildtype
- **▲** = KR877 without tRNA\(^{Pro21}\) island (KR877ΔPro21, KR1228)
- **▼** = KR877 without tRNA\(^{Ser11}\) island (KR877ΔSer11; KR1278)
- **◆** = PAO1 (KR124)
<table>
<thead>
<tr>
<th>Strain</th>
<th>No. nematodes (% censored)</th>
<th>TDₘ (h ± SE)</th>
<th>TD₅₀ (h ± SE)</th>
<th>Log Rank χ²; P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR877 WT</td>
<td>60 (22%)</td>
<td>123.9 ± 3.0</td>
<td>122 ± 4</td>
<td>/</td>
</tr>
<tr>
<td>KR877ΔPro21 (KR1228)</td>
<td>61 (28%)</td>
<td>146.9 ± 5.1</td>
<td>145 ± 3</td>
<td>19.742; &lt;0.001</td>
</tr>
<tr>
<td>KR877ΔSer11 (KR1278)</td>
<td>60 (27%)</td>
<td>133.8 ± 4.4</td>
<td>135 ± 4</td>
<td>5.104; 0.024</td>
</tr>
<tr>
<td>PA14 WT</td>
<td>57 (54%)</td>
<td>85.5 ± 3.6</td>
<td>86 ± 5</td>
<td>42.380; &lt;0.001</td>
</tr>
<tr>
<td>PAO1</td>
<td>60 (32%)</td>
<td>177.6 ± 7.6</td>
<td>172 ± 8</td>
<td>48.181; &lt;0.001</td>
</tr>
</tbody>
</table>

**Table 4-5: Survival analysis of *C. elegans* seeded on KR877-related strains**

Survival analysis performed using the Kaplan-Meier method for slow-killing assays done at the University of Leicester using *C. elegans* CF512.

**Table headings legend:**
- **Strain** = bacterial strains on which nematodes were seeded
- **No. nematodes (% censored)** = total number of nematodes seeded on each strain and in parenthesis the percentage of these nematodes censored
- **TDₘ (h ± SE)** = average time-to-death for nematodes shown in hours ± standard error
- **TD₅₀ (h ± SE)** = time required to kill 50% of the nematodes seeded shown in hours ± standard error
- **Log-Rank** = statistical test used to compare survival on plates with PA14 wildtype against survival on plates with the other strains
- **χ²** = Log-Rank test value calculated by the software with P-value significant if below 0.05 (underlined)
- **n.a.** = not available
Conclusions and discussion

*C. elegans* highthroughput assay is an effective screen for *P. aeruginosa* virulence

A modified version of the slow-killing assay employing 24-well plates was devised to screen more *P. aeruginosa* strains in a shorter amount of time. *P. aeruginosa* strains showing little or no effect on *C. elegans* survival were assigned high value scores while *P. aeruginosa* strains able to compromise the survival of the nematodes were assigned low value scores. A strong association between the highthroughput assay (HTA) score and the slow-killing assay (SKA) TD$_{50}$ coupled with score reproducibility proved that the *C. elegans* highthroughput assay consistently classifies the virulence of different *P. aeruginosa* strains similarly to the slow-killing assay, but in a more useful shorter time frame.

The *C. elegans* highthroughput assay allowed the testing of a total of twenty-two wildtype/deletion mutant pairs in a relatively short period. Five pairs were subsequently tested in the slow-killing assay, and four mutants out of five showed significantly extended survivals of nematodes compared to the isogenic wildtype. The attenuated virulence phenotype reported with the highthroughput assay was confirmed in 80% of the mutants tested with slow-killing assay. It can be concluded that the new *C. elegans* 24-well highthroughput assay can be efficiently used to determine the virulence phenotype of *P. aeruginosa* strains and as preliminary screening tool for the slow-killing assay.
The development of *C. elegans* highthroughput assays have been reported also elsewhere. Highthroughput assays employing liquid medium demonstrated lack of congruity with the slow-killing assay. In the assay set up by Kirienko and colleagues, none of the mutants derived from PA14 and known to be attenuated in the slow-killing assay (including Δ*gacC*, Δ*lasR*, Δ*psqE*, Δ*mvfR*, Δ*kinB*, Δ*clpA*) showed attenuation in the liquid highthroughput assay (Kirienko *et al.* 2013). In the assay set up by Garvis and colleagues, twelve *P. aeruginosa* TBCF10839-derived mutants showed a high degree of attenuation compared to wildtype strain in the highthroughput assay but only seven (58%) were also attenuated in slow-killing assay (Garvis *et al.* 2009). Elsewhere, screenings were done using NGM agar in either 6-well plates or 35 mm culture dishes. Feinbaum and colleagues evaluated more than 5,000 PA14 mutants in these conditions for their ability to yield a larger progeny followed by slow-killing assays done with temperature-sterile worms in which 48 out of 52 selected mutants (93%) were confirmed to be avirulent (Feinbaum *et al.* 2012). The Feinbaum *et al.* results combined with the ones reported in this chapter indicate that highthroughput assay done using solid media, 6-well or 24-well plates and *C. elegans* N2 are likely to be the best proxy for slow-killing assay done with *C. elegans* CF512.

The highthroughput assay and the slow-killing assay done at the University of Leicester showed a strong correlation but also occasional differences. LES431ΔGly19 did not demonstrate a virulence-attenuated phenotype in the slow-killing assay compared to LES431, while it yielded a larger progeny than the wildtype in the highthroughput assay. The lack of complete overlap of results between HTA and SKA may be ascribed to the specific experimental conditions of each assay. The most relevant differences are the preparation of the bacterial lawn and the strain of nematode used. *P. aeruginosa* lawns often cover the entire agar surface in 24-well plates at the beginning of the
experiment, while untouched agar remains between the bacterial lawn and the edge of
the plate in the slow-killing assay. By spreading the bacterial lawn to reach the edge of
the plate (known as “big lawn”), the nematode-pathogen avoidance behaviour is
abolished affecting the survival of the C. elegans (Reddy et al. 2009). In the 24-well
highthroughput assay, the fertile C. elegans N2 strain was employed, while C. elegans
CF512 strain (sterile at 25°C) was used in the slow-killing assay to avoid the
confounding effect of progeny. Although the survival of N2 and CF512 nematodes
seeded on the attenuated PA14ΔgacA was comparably longer than when seeded on
PA14 wildtype (Troemel et al. 2006), it has been hypothesized that mutations that
render C. elegans sterile might influence other phenotypes including survival on
pathogens (TeKippe, Aballay 2010). Interestingly, pathogen avoidance and egg-laying
behaviours share the same biochemical pathway in the nematode nervous system
(Shivers et al. 2009) therefore they may be influenced by the same stimuli.

**Genomic islands involved in virulence against C. elegans**

Twenty-two wildtype/tRNA-associated deletion mutant pairs were assayed using the
highthroughput assay and seven mutants, corresponding to six genomic islands out of
twenty (30%), showed HTA score substantially higher than the corresponding wildtype.
In these mutants the absence of the genomic island led to an increase of the nematodes
brood size which was equated to a decrease in P. aeruginosa virulence. Since
complementation after deletion of unknown or large genomic islands has been difficult
to achieve, independently generated deletion mutants were created and their phenotype
tested. Two independent mutants were created by deletion of tRNAPro21 island from
PA14 or KR378. Both mutants from PA14 showed a substantial decrease in virulence
while both mutants from KR378 showed very little difference compared to the wildtype.
Therefore, it can be assumed that the phenotypes returned by the mutants were likely only due to deletion of the islands.

Three out of fifteen tRNA\textsuperscript{Pro21} genomic islands (20%), one out of two tRNA\textsuperscript{Gly19} genomic islands (50%) and two out of three tRNA\textsuperscript{Ser11} genomic islands (67%) substantially contributed to \textit{P. aeruginosa} virulence in the highthroughput assay. Subsequent analysis with the slow-killing assay showed that the deletion of tRNA\textsuperscript{Pro21} islands from PA14 and KR877 and of tRNA\textsuperscript{Ser11} island from KR877 increased \textit{C. elegans} survival compared to the respective wildtype but the same was not true for the deletion of tRNA\textsuperscript{Gly19} island from LES431. It could be hypothesized that while the genomic islands from PA14 and KR877 carry virulence factors affecting \textit{C. elegans} independently from specific assay conditions, genomic island in LES431 might carry virulence factors that are only relevant in the highthroughput assay (i.e. traits influencing pathogen-avoidance or egg-laying behaviours).

Although genomic islands contributing to virulence against \textit{P. aeruginosa} were sought after, at least one genetic element seemed to negatively affect virulence. In fact, the presence of tRNA\textsuperscript{Pro21} island from MID9245 substantially increased progeny formation appearing to dampen the virulence potential of the strain. No slow-killing assay was carried out to confirm this phenotype.

The screening to test virulence phenotypes is usually limited to mutants that do not show growth defect in either rich or minimal medium (Feinbaum \textit{et al.} 2012). In the case of KR877- (Appendix C, Figure C-1) or MID9245-related strains (Appendix C, Figure C-3), there was no difference in growth between the wildtype and deletion mutants. Consequently, the attenuated or increased virulence phenotype of the mutants could not be ascribed to changes in growth. On the other hand, the decreased virulence of KR873\textDeltaSer11 compared with the wildtype strain (Appendix C, Figure C-5) was due
at least partially to the growth defect displayed by the deletion mutant. Interestingly, KR352ΔGly19 was unable to grow as fast as and to reach the same density as the wildtype strain (Appendix C, Figure C-6) but the lack of the island did not compromise the virulence of KR352 mutant in the highthroughput assay. Although in vitro growth curves are an easy and effective way to assess general changes in phenotype in mutants, they do not always translate to an in vivo phenotype. Consequently, discarding growth-defective mutants for subsequent virulence screening as done in previous work (Feinbaum et al. 2012) should be considered carefully.

There has been little characterization of the factors associated with tRNAPro21, tRNAGly19 and tRNAser11 genomic islands in P. aeruginosa and consequently of their contribution to virulence. A notable exception is represented by the tRNAPro21 island found in PA14. In fact, the survival of G. mellonella larvae increased when injected with PA14ΔPro21 compared to wildtype (Harrison 2009). The extended survival of G. mellonella combined with the extended survival of C. elegans (both at University of Leicester and at Universiti Kabangsaan Malaysia) upon deletion of this island may indicate the presence of factor/factors affecting conserved innate immunity pathways and/or influencing the adaptability of the bacterium to different environments. However, it cannot be excluded that the process allowing G. mellonella larvae to live longer is independent from the process allowing C. elegans to survive longer.

A more in-death bioinformatics analysis of the ORFs associated with the tRNAPro21 island in PA14 showed the presence of factors likely to influence the bacterium adaptability. An hybrid toxin-antitoxin system (PA14_28780-PA14_28790) was found in this island with the toxin harbouring a PIN (PilT N-terminal) domain reminiscent of the one present in VapC-FitB toxin family (vap= virulence associated protein) and the antitoxin harbouring a domain similar to the one found in VagC belonging to the
antitoxin-MazF superfamily. Toxin-antitoxin systems like MazEF and VapBC may trigger cell death as a response to stress factors (Engelberg-Kulka, Hazan & Amitai 2005) such as presence of antibiotics (Sat et al. 2001, Engelberg-Kulka et al. 2004), increased temperature (Cooper et al. 2009) and DNA damage (Sat, Reches & Engelberg-Kulka 2003, Hazan, Sat & Engelberg-Kulka 2004). In general, they seem to be involved in helping *P. aeruginosa* to adapt to changed environmental conditions (McKenzie et al. 2012, Cook et al. 2013) and in particular VapBC has been involved in intracellular growth adaptation (Hopper et al. 2000). Another predicted ORF (PA14_28800) contains a Fic (filamentation-induced by cAMP) domain that in other proteins have been proved to modify host Rho GTPases by AMP addition (Yarbrough et al. 2009) and cause cytotoxicity (Worby et al. 2009). PA14_28820 showed a high degree of homology (98%) at protein level with the tellurite resistance protein TerB found in *P. aeruginosa* VRFP05. Finally, several helicases (PA14_28810 and PA14_28840) and transposases (PA14_28860 and PA14_28870) has been identified (Harrison 2009), as expected in a mobile genetic element.

A wide range of phenotypes ascribable to genomic islands is not surprising since virulence of *P. aeruginosa* is both combinatorial and multifactorial (Lee et al. 2006) relying on interactions between cargo genes and chromosomal genes. Since the cargo genes associated with genomic islands can be extremely varied (Appendix B) in particular for *tRNAPro21* islands, a wide range of virulence phenotypes is expected when screening with highthroughput assay. However, few genomic islands in particular for the one associated with *tRNAPro21* were involved in virulence against *C. elegans*; this is unexpected considering the association between genomic islands and virulence (Ho Sui et al. 2009).
The lack of $tRNA^{Gly19}$ island from LES431 did not have any impact on *C. elegans* survival in slow-killing assay. LES431 genome sequence has become recently available and its $tRNA^{Gly19}$ island is very similar to that of the other LES-like strains (Appendix B, Table B-2). Therefore, it is reasonable to assume that in general this group of islands does not affect *C. elegans* survival in the slow-killing assay conditions. KR877 is a clinical isolate and no information regarding the cargo genes carried by the $tRNA^{Pro21}$ island or $tRNA^{Ser11}$ island is available. However, both islands were implicated in reducing *C. elegans* survival.

The use of homologues recombination technique coupled with a high throughput assay mimicking infection conditions may allow identification of novel genomic island (or other relatively large section of the genomes) associated with virulence and/or assign function to poorly annotated portions of genomes. These may highlight islands or section of interest and help to direct future work to focus on them.
Chapter 5

Investigation of the links between survival of *C. elegans* and *P. aeruginosa* source of isolation, type III secretion system effectors and pigment production
Introduction

The *P. aeruginosa* genome is larger (Klockgether *et al.* 2011) and more plastic than that of many other bacteria (Shen *et al.* 2006, Finnan *et al.* 2004) due to the occurrences of horizontal gene transfer, mutations, chromosomal inversions and deletions (Romling, Schmidt & Tummler 1997, Kresse *et al.* 2003). These genetic processes gives raise to metabolically versatile *P. aeruginosa* strains (Klockgether *et al.* 2011) explaining their adaptability (Campa, Bendinelli & Friedman 1993, Kung, Ozer & Hauser 2010) to very diverse environments. This can range from surviving in mammalian tissues (Pier 2012) and in presence of bacterivorous predators (Matz, Kjelleberg 2005, Irazoqui, Ausubel 2010) to living on plastic surfaces (Pier 2012). The environmental surroundings of a strain may also mould its genetic and phenotypic characteristics adapting it to thrive in apparently unrelated conditions (Dobrindt *et al.* 2004).

**Phenotypes associated with *P. aeruginosa* clinical isolates**

*P. aeruginosa* represents a frequent source of hospital-acquired infections (Gaynes, Edwards & National Nosocomial Infections Surveillance System 2005) because of the high level of colonization of hospitalized patients by this bacterium (Cortes *et al.* 2001). However, only some colonized patients develop a *P. aeruginosa*-related illness depending on bacterial factors, specific host factors and/or underlining conditions (Talon *et al.* 1998).

Attempts have been made to define the bacterial factors involved in progression from colonisation to acute infection in humans. It has been previously noted that *P.*
aeruginosa strains isolated from different body fluids or sites possessed characteristic phenotypes. For example, strains obtained from blood produced in vitro higher amounts of proteases, exotoxin A and phospholipase C while strains isolated from lungs of patients with pneumonia produced higher amount of elastase when compared with strains from other sites (Woods et al. 1986). Differences were also observed between strains from patients colonized by P. aeruginosa and strains causing an active infection with the latter more likely to express type III secretion system effectors (Ledizet et al. 2012).

**Type III secretion system effectors and virulence**

Secretion systems are of paramount importance for bacteria to permit controlled and specific release of intracellular material. The type III secretion system (T3SS) forms a needle-like structure to transfer effectors, ExoU, ExoS, ExoY and ExoT, from the bacterial cytosol to the eukaryotic cell cytosol. In order for the translocation to occur, the bacterial cell must be in direct contact with the eukaryotic target cell (Bleves et al. 2010). P. aeruginosa possess very few T3SS effectors compared to other bacteria (Hauser 2009). Interestingly, while the proteins forming and regulating the translocation machinery are placed within a single locus, the effectors are present in different loci spread around the P. aeruginosa genome (Bleves et al. 2010).

**Contribution of T3SS effectors to P. aeruginosa virulence**

In general, the presence of a functional T3SS has been shown to enhance the virulence of P. aeruginosa strains in many conditions such as acute pneumonia (Finck-Barbancon et al. 1997), bacteremia (Vance, Rietsch & Mekalanos 2005), burn infections (Holder, Neely & Frank 2001) and gut-derived sepsis (Koh, Priebe & Pier 2005). However, the mode of action and relative contribution to virulence are specific to each effector.
ExoU is considered the most potent among the four T3SS effectors being 100 times more cytotoxic than ExoS (Lee et al. 2005). In *in vitro* assays, ExoU caused rapid lysis of cell cultures (Sato, Frank 2004, Lin et al. 2006) while *in vivo*, it was able to cause damage in an animal model for sepsis following lung injury without the intervention of other effectors (Allewelt et al. 2000, Shaver, Hauser 2004). In epidemiological studies, the mortality risk of patients infected with an *exoU*-positive strain was increased compared to patients infected with *exoU*-negative strains (Roy-Burman et al. 2001). Moreover, the presence of *exoU* was linked to higher levels of antibiotic resistance through an unknown mechanism (Garey et al. 2008).

Albeit less powerful than ExoU, ExoS has also been implicated in *P. aeruginosa* colonization and invasion during infections (Lee et al. 2005); this effector also presented anti-phagocytic properties both *in vitro* (Bleves et al. 2010) and *in vivo* in a mouse model of pneumonia (Rangel, Logan & Hauser 2014). It also has been shown that ExoS may bind to TLR-2 and TLR-4 and modify the host innate immune response (Epelman et al. 2004).

ExoT displays a high level of amino acid identity with ExoS (Hauser 2009) and therefore it has been shown to interfere with phagocytosis but also with wound healing (Cowell et al. 2000) probably following ExoS intervention (Shafikhani, Morales & Engel 2008). In a mouse infection model, the contribution of ExoT to cause disease was less relevant than both ExoU and ExoS (Shaver, Hauser 2004). Moreover, evidences have suggested that ExoT might protect cultured cells from lysis induced by other T3SS effectors as it curbs the toxic effect of ExoS in a PAK strain (Lee et al. 2005). ExoY has been known to increase lung permeability (Sayner et al. 2004) and demonstrates cytotoxic effect in *in vitro* cell culture (Lin et al. 2006) but little involvement in causing *P. aeruginosa*-derived pneumonia in a mouse model (Lee et al. 2005).
Exclusiveness of exoU and exoS

Most of the *P. aeruginosa* strains tested to date carry both *exoY* and *exoT* but they seem to have either *exoU* or *exoS*, not both (Hauser 2009). In fact, only a small percentage of the isolates when analysed were found to test positive (1.6%-5%) or negative (2.5%) for both genes (Garey *et al.* 2008, Bradbury *et al.* 2010), with the overall prevalence among the clinical strains at 28-42% for *exoU* and at 58-72% for *exoS* (Hauser 2009).

Remarkably, both *exoU* and its chaperon (*spcU*) and *exoS* and its chaperon (*spcS*) appear to be part of the accessory genome. The *exoU/spcU* gene pair was found in genomic islands or islets associated with *tRNA^{Lys}^{10}* known as PAPI-2 in PA14 (He *et al.* 2004) or ExoU-A, ExoU-B and ExoU-C in three other *P. aeruginosa* strains (Kulasekara *et al.* 2006). These elements possess the hallmarks of integrative and conjugative elements related to the pKLC102 plasmid (Kung, Ozer & Hauser 2010) including a XerC/XerD-like integrase and 15-20bp direct repeats at each end of the element (Klockgether *et al.* 2011). *exoS* is found in a genomic locus conserved between strains and lacking DNA mobility-related genes (Wolfgang *et al.* 2003). Nonetheless the *exoS* locus is surrounded by 10bp direct repeats and has been classified as a degenerated accessory element (Kung, Ozer & Hauser 2010). Kulasekara *et al.* noted a partial homology between repeats surrounding the *exoS* locus and *exoU/spcU* elements and hypothesized that the machinery instigating the integration of *exoU*-carrying island might simultaneously induce the excision of *exoS* locus, providing a molecular explanation of the exclusiveness of these effector genes (Kulasekara *et al.* 2006).

The inverse relationship between *exoU* and *exoS* has also been associated with different *P. aeruginosa* phenotypes and with isolation from different infection sites. *exoU*-positive/*exoT*-positive strains (such as PA103 and PA14) have been defined as “cytotoxic” while *exoS*-positive/*exoT*-positive strains (such as PAO1 and PAK) have
been defined more as “invasive” based on the different phenotypes exerted by ExoU and ExoS (Bleves et al. 2010). Moreover, an ExoU expressing strain was shown to require a significantly lower infective dose to cause pneumonia in a mouse model compared to the isogenic strain expressing ExoS (Shaver, Hauser 2004). Interestingly, it has been found that bloodstream clinical isolates from patients were prevalently exoU-positive/exoS-negative in some hospital settings (Wareham, Curtis 2007). Feltman and colleagues compared the prevalence of exoU and exoS in strains from sputum of cystic fibrosis patients or mechanically ventilated non-cystic fibrosis patients with strains obtained from blood culture. They found that exoU prevalence was highest in blood-derived isolates and lowest in sputum of CF patients with sputum from non-CF patient having an intermediate prevalence. Distribution of exoS was diametrically opposite, being highest in strains collected from CF patients and lowest from strains collected from blood (Feltman et al. 2001). Bradbury and colleagues also reported that a significantly higher number of strains isolated from intensive care and neurosurgical unit wards were exoU-positive/exoS-negative (Bradbury et al. 2010).
Aims of this chapter

The observations that exoU and exoS appear mutually exclusive led to hypothesize that presence of both ExoU and ExoS may be disadvantageous for a strain and that particular environmental conditions (for example extensive use of disinfectant in ICU wards) might drive the exchange between exoU-carrying island and exoS locus effectively selecting ExoU against ExoS (Bradbury et al. 2010). Therefore it was assessed whether presence of exoU-bearing genomic islands may confer an advantage to P. aeruginosa against natural predators such as C. elegans and whether this may be connected to the original source of isolation of the bacterium. The specific aims of this chapter were:

1. To collect clinical isolates obtained from culturing blood of patients or sputum of patients since they may present different phenotypes in term of overall virulence and ultimately different genotype;
2. To survey clinical isolates for T3SS effector genes prevalence using PCR assays and confirm the exclusivity between exoU and exoS;
3. To determine the overall virulence of clinical isolates in a non-mammalian model organism using a C. elegans highthroughput assay in order to assess their phenotypic diversity.
Results

Association between *P. aeruginosa* isolation source and virulence in *C. elegans*

It has been observed that some bacterial traits are associated with acute infection while others are more prominent in chronic infections and that in general *P. aeruginosa* strains isolated from acute infections express a higher number of virulence factors (Gellatly, Hancock 2013). The presence of *P. aeruginosa* in the bloodstream has been considered a sign of acute infection while its presence in the lungs could be transient and such strains have been considered to be less virulent (Alleyne 2009). Therefore, it was assumed that strains isolated from blood would be genotypically and phenotypically different from strains isolated from sputum. A *C. elegans* model of infection was employed to test whether a difference in virulence phenotype could be detected and associated with *P. aeruginosa* taken from a particular isolation site.

*P. aeruginosa* bloodstream isolates were more virulent against *C. elegans* than isolates from sputum

As seen previously (Lee *et al.* 2006, Janjua *et al.* 2012), *P. aeruginosa* strains showed a wide variation in their ability to kill *C. elegans* ranging from highly virulent strains such as PA14 to attenuated strains such as PAO1 as well as strains possessing an intermediate phenotype (Chapter 4, Figure 4-1 and Table 4-1). This wide range of phenotypes was also reproduced when tested with the highthroughput assay (HTA;
Therefore, I employed the HTA to assess the level of virulence exhibited by clinical isolates. A total of 138 clinical isolates were tested. Of these, 132 strains were obtained from the University Hospitals Leicester between 2005 and 2010 from culturing either the blood or the sputum of patients. The six remaining strains, LES400, LES431, LESB58, MID9246 (Salunkhe et al. 2005), PA103 (Vallis et al. 1999) and PA2192 (Mathee et al. 2008), were reference strains. All the clinical isolates were tested in the high-throughput assay and an HTA score was assigned to each. The score was indicative of the level of virulence demonstrated toward *C. elegans* in comparison with PA14 and PAO1. High value scores were assigned to strains that had little or no effect on *C. elegans* survival while low value scores were assigned to strains that impacted on *C. elegans* survival.

Of the 138 isolates tested, 71 were obtained from blood culture while 67 from sputum culture (Figure 5-1). The blood-derived strains showed an average HTA score of 2.5 ± 1.8 while the sputum-derived strains reported a higher average score (3.3 ± 1.8). Although the standard deviation showed overlap, the HTA score of strains isolated from blood had a significant tendency to be lower than the score of strains isolated from sputum (Mann-Whitney U= 1807.500; *P*-value= 0.014).

**Prevalence of *P. aeruginosa* T3SS effector genes and virulence in *C. elegans***

PCR assays were used to assess the presence of genes codifying for T3SS effectors by employing four pairs of effector gene-specific primers. Assays were carried out twice but the in the case of discordant results, they were repeated a third time. A strain was judged to be effector gene-positive if the expected band size appeared in the agarose gel. In the absence of expected band, the strain was deemed effector gene-negative.
**exoU and exoY are associated with strains exhibiting lower HTA scores**

The presence of T3SS effector genes was investigated for 131 clinical isolates. Since exoU and exoS were inversely linked, very few strains carried both effectors (2%) or neither effector (5%). The bulk of the strains were exoU-negative/exoS-positive (70%) while the remaining strains exhibited presence of exoU but absence of exoS (23%). As expected, most of the strains were also exoY-positive (89%) and almost all of the strains were exoT-positive (94%). Because of the high prevalence of exoT in these clinical isolates, the contribution of this effector gene was not analysed further.

When the clinical isolates were divided based on the presence/absence of exoU and exoS (Figure 5-2A), the exoU-positive/exoS-negative strains reported the lowest average HTA score (2.1 ± 1.5) followed by strains that were exoS-positive and either exoU-positive (2.8 ± 3.9) or exoU-negative (2.9 ± 1.8). The group of strains with neither effector gene showed the highest HTA average score (4.1 ± 1.8). Statistical overall comparison between the four groups revealed a significant difference between them (Kruskal-Wallis $\chi^2$= 8.545, degree of freedom= 3, $P$-value= 0.036). Pairwise comparisons demonstrated that the exoU-positive/exoS-negative strains had a score significantly lower than either exoU-negative/exoS-positive strains (Mann-Whitney U= 1035.000, $P$-value= 0.039) or strains lacking both effectors (Mann-Whitney U= 36.000, $P$-value= 0.005).

As mentioned above, most of the strains carried exoY with only 14 strains resulted exoY-negative (Figure 5-2B). Nonetheless, this group had an average score (3.8 ± 1.7) higher than the exoY-positive group (2.7 ± 1.8) and the difference was statistically significant (Mann-Whitney U= 539.000, $P$-value= 0.036).
P. aeruginosa strains were classified based on their source of isolation (either from blood or from sputum of a patient) and HTA score was determined for 138 isolates using the high-throughput assay described in Chapter 4. The difference in HTA scores between the two groups was assessed using the Mann-Whitney test returning a significant P-value (Mann-Whitney = 1807.500; P-value = 0.014).

Figure 5-1: HTA scores for P. aeruginosa clinical isolates grouped by their source of isolation

P. aeruginosa strains were classified based on their source of isolation (either from blood or from sputum of a patient) and HTA score was determined for 138 isolates using the high-throughput assay described in Chapter 4. The difference in HTA scores between the two groups was assessed using the Mann-Whitney test returning a significant P-value (Mann-Whitney = 1807.500; P-value = 0.014).
A. 

Figure 5-2: HTA scores for *P. aeruginosa* clinical isolates characterized by the presence of T3SS effector genes

*P. aeruginosa* strains were assessed for the presence of the type 3 secretion system (T3SS) effector genes (A) *exoU* and *exoS* or (B) *exoY* by colony PCR and HTA scores were determined for 131 clinical isolates using the highthroughput assay described in Chapter 4.

(A) The overall difference between all groups was assessed using the Kruskal-Wallis test (Kruskal-Wallis $\chi^2 = 8.545$, degree of freedom= 3, $P$-value= 0.036) while pairwise comparison were done using the Mann-Whitney test and comparison with significant $P$-value are shown.

(B) The difference between the two groups was assessed using the Mann-Whitney test returning a significant $P$-value (Mann-Whitney $U= 539.000$; $P$-value= 0.036).
Lack of association between isolation source and presence of T3SS effector genes

Because the presence of *exoU*, in particular in *exoS*-negative strains, was linked to a lower HTA score and the absence of *exoY* was linked to higher HTA score, I investigated whether the prevalence of these effector genes was linked to the original source of isolation of the *P. aeruginosa* strains. Among the isolates from blood, 29% were *exoU*-positive/*exoS*-negative and 64% were *exoU*-negative/*exoS*-positive (Table 5-1A). In the isolates obtained from sputum, the prevalence of *exoU*-positive/*exoS*-negative was lower (16%) while the prevalence of *exoU*-negative/*exoS*-positive was higher (77%). The prevalence of strains with both and neither effector gene was very similar between blood-derived and sputum-derived strains. Although the strains collected from blood seemed more likely to have *exoU*, the distribution was not significantly different from what would be expected by chance (Pearson \( \chi^2 = 3.285 \), degree of freedom= 3, \( P \)-value= 0.369). The prevalence of *exoY*-positive and *exoY*-negative strains was very similar between blood- and sputum-derived strains (Table 5-1B) and therefore not significantly different (Pearson \( \chi^2 = 0.126 \), degree of freedom= 3, \( P \)-value= 0.783).

Analysis of the *exoU* locus

It has been shown from previous research that production of type three secretion system effectors, including ExoU from *P. aeruginosa* strain PA14 has no effect on the survival of *C. elegans* in a slow-killing assay (Miyata *et al.* 2003, Wareham, Papakonstantinopoulou & Curtis 2005). Therefore, analysis of the genetic contest of *exoU/spcU* was performed in order to acquire more information about virulence traits associated with this locus.
A.

<table>
<thead>
<tr>
<th>Presence of exoU and exoS</th>
<th>Isolation Blood N (%)</th>
<th>Isolation Sputum N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoU-positive/exoS-negative</td>
<td>20 (29%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td>exoU-negative/exoS-positive</td>
<td>44 (64%)</td>
<td>48 (77%)</td>
</tr>
<tr>
<td>exoU-negative/exoS-negative</td>
<td>4 (6%)</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>exoU-positive/exoS-positive</td>
<td>1 (1%)</td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Presence of exoY</th>
<th>Isolation Blood N (%)</th>
<th>Isolation Sputum N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exoY-positive</td>
<td>8 (12%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>exoY-negative</td>
<td>61 (88%)</td>
<td>56 (90%)</td>
</tr>
</tbody>
</table>

Table 5-1: Prevalence of T3SS effector genes in *P. aeruginosa* clinical isolates derived from blood or sputum

*P. aeruginosa* clinical isolates were tested by colony PCR to establish the prevalence of (A) exoU and exoS or (B) exoY among blood-derived or sputum-derived strain. Pearson $\chi^2$ was used to test whether the prevalence was significantly different from what expected by chance.

(A) Pearson $\chi^2 = 3.285$, degree of freedom $= 3$, $P$-value $= 0.369$; see Appendix E, Table E-3 for more details.

(B) Pearson $\chi^2 = 0.126$, degree of freedom $= 1$, $P$-value $= 0.783$; see Appendix E, Table E-4 for more details.
**exoU/spcU pair is always part of genetic element associated with tRNA^{Lys10}**

Of the all completely or nearly completed sequenced *P. aeruginosa* genomes available in NCBI (November 2014), 22% of the strains (7 out of 32) were *exoU*-positive. In these strains, *exoU* was always followed by *spcU* even though occasionally it has not been annotated as such. Interestingly, in all the *exoU/spcU*-positive strains, these genes were found in a mobile genetic element integrated at the *tRNA^{Lys10}* site, the same integration site for PAPI-2, the *exoU/spcU* bearing island from PA14 (He et al. 2004).

In general, 31% of the sequenced strains lacked any genetic elements integrated at the *tRNA^{Lys10}* site (empty site). Of the remaining strains, 25% (8 out of 32) presented an islet with the average size of 9.1 kb and 44% (14 out of 32) presented an island with an average size of 74.0 kb. Moreover, only one of the islets (13%) and six of the islands (43%) included *exoU/spcU*.

**Comparison between *exoU/spcU* carrying genetic elements**

The *exoU/spcU* genetic elements vary in size from 4.3 kb from MTB-1 strain to 81.8 kb from NCGM2.S1, NCGM 1984 and NCGM 1900 strains. Because genomic islands from the latter three strains are practically identical, the island from NCGM 1984 was used as model. By comparing the nucleotide sequence of these genetic elements containing *exoU* (Figure 5-3), it appeared that they were very diverse not only in size but also in content. All the genetic elements contain *exoU*, *spcU* and a putative transposase just upstream *exoU*. These three coding regions with the addition of an *orf* labelled as thioesterase formed the islet from MTB-1. In the larger genomic islands, including PAPI-2, the transposase-*exoU-specU* coding regions were always located at the 5’ end of the island, the farthest away from the *tRNA*. The first *orf* of PAPI-2 just downstream the *tRNA* is annotated as integrase and it is conserved in all the genomic islands. PAPI-2 was the smallest island with only few other *orfs* (all annotated as
hypothetical) in common with the island from B136-33 strain. Most of regions of this island that were missing in PAPI-2 were found in both VRFP04 island and NCGM 1984 island. Many orfs were annotated as hypothetical but the presence of a putative relaxase, plasmid stabilization protein and NikR transcriptional regulator were reported. The island found in VRFP04 seemed a shorter version than the island found in NCGM 1984 and contained a putative protein disulphide isomerase, conjugal transfer proteins, type three Hop protein, helicase and dTDP-glucose 4,6-dehydrogenase alongside many hypothetical orfs. Notwithstanding the common features, these islands also show strain specific orfs increasing their variability between strains.
Figure 5-3: Sequence comparison between genetic elements containing exoU/spcU

The image shows the comparison at the nucleotide level of genetic elements carrying exoU from five P. aeruginosa strains. Sequences were downloaded from NCBI and included the conserved upstream (2 kb) and downstream (1 kb) flanking regions of the accessory elements inserted at tRNA^{Lys}_{11}. Sequences homologues at 95% or above are indicated in red if collinear or in blue if inverted. The sequences comparison was done using the ACT (Artemis comparison tool) webtools and the ACT 14.0.0 software was used to visualize the alignment.
Production of pigment by *P. aeruginosa* and survival of *C. elegans*

During the highthroughput assay, it was noticed that some *P. aeruginosa* strains produced pigments and their presence was recorded for the 115 isolates. In King’s B broth, most of the strains do not change the natural colour of the medium (yellow) but 24% developed a green hue most likely due to the production of pyoverdine, a green fluorescent siderophore known previously as fluorescin (King, Ward & Raney 1954). When grown on nematode growth medium (NGM), development of green-blue (likely due to pyocyanin production), red or brown hues were more frequently observed with 59% of the strains showing this phenotype. Since both pyoverdine and phenazine compounds such as pyocyanin are considered bacterial factors involved in virulence in mammalian (Lau *et al.* 2004) and *C. elegans* (Zaborin *et al.* 2009, Cezairliyan *et al.* 2013), it was tested whether the appearance of pigmentation during *P. aeruginosa* growth affected the score in highthroughput assay.

**Association between production of pigmented compounds and HTA score**

In order to simplify the analysis, the clinical isolates were divided in to three groups: strains that did not produce any pigment (38%), strains that produced pigment during growth either in King’s broth or on NGM (40%) and strains that produced pigments during growth both in King’s broth and on NGM (22%). Interestingly, almost all the strains (25 out of 28) that change King’s broth colour to green yielded pigmentation when plated on NGM and were significantly more likely to do so than strains that did not change King’s broth colour (Pearson $\chi^2 = 13.927$, degree of freedom= 1, $P$-value$< 0.001$; Appendix E, Table E-5).
The strains that produced no pigment had on average the highest HTA score (4.0 ± 1.8) while the strain that produced pigment in both media showed on average the lowest score (1.4 ± 1.2; Figure 5-4). Interestingly, the strains that produced pigment either in King’s broth or on NGM reported an intermediate HTA score (2.9 ± 1.6). The statistical comparison between all groups was significant (Kruskal-Wallis $\chi^2 = 31.103$, degree of freedom= 2, $P$-value< 0.001) as it was each pairwise comparison. In fact, the HTA score associated with pigment-less strains was significantly higher than that of strains producing pigmentation in one medium (Mann-Whitney U= 626.500, $P$-value= 0.002) or in both (Mann-Whitney U= 145.500; $P$-value< 0.001) as it was the score of strains developing pigmentation in one type of medium compared to strains that produced pigmentation in both media (Mann-Whitney U= 271.500; $P$-value< 0.001).

**Investigation of the association between production of pigment and source of isolation or the presence of T3SS effector genes**

Since the production of pigment was linked to lower HTA scores similar to what was observed for strains that had been isolated from the blood of patients, I tested whether there was an association between pigment production and source of isolation (Table 5-2A and Appendix E, Table E-1). Interestingly, half of all the strains isolated from sputum (50%) did not develop pigmentation while only one-quarter of the strains derived from blood (26%) had the same phenotype. Conversely, almost half of the strains from blood developed pigmentation in at least a medium (48%) and the remaining (26%) in both media. Statistical analysis proved that the frequencies observed were significantly different from those expected by chance in particular for the over-representation of pigment-less strains in the sputum-derived category and their under-representation in the blood-derived category (Pearson $\chi^2 = 7.366$, degree of freedom= 2, $P$-value= 0.027). Moreover, the relationship between source of isolation
and presence of pigment proved to have an intermediate strength ($P$-value $= 0.027$, Cramer’s $V = 0.253$).

The presence of T3SS effector genes $exoU$ and $exoY$ was also associated with a lower HTA score. Therefore, I assessed whether presence of these T3SS effector genes were related to production of pigments. $exoY$-negative strains were equally distributed between isolates that did not develop pigmentation (50%) and isolates that develop it either in King’s broth or on NGM (50%). On the contrary, 25% of the $exoY$-positive strains produced pigments both in King’s broth and on NGM (Table 5-2C) but the prevalence was not significantly different from that expected by chance (Pearson $\chi^2 = 3.946$, degree of freedom $= 2$, $P$-value $= 0.128$).

Remarkably, production of pigment appeared to be related to presence of $exoU$ (Table 5-2B). The $exoU$-positive strains showed a strong tendency to develop pigmentation both in King’s broth and on NGM with almost half of all $exoU$-positive strain falling in this category (44%). Moreover, more than three-quarter of the $exoU$-negative strains did not develop pigmentation or produced pigments in one medium only (39% and 45%, respectively). Consequently, the observed distribution was significantly different from what expected by chance (Pearson $\chi^2 = 8.994$, degree of freedom $= 2$, $P$-value $= 0.012$) in particular for the over-representation of $exoU$-positive strains among those producing pigments both in King’s broth and on NGM. Moreover, the relationship between presence of $exoU$ and production of pigments proved to have an intermediate strength ($P$-value $= 0.012$, Cramer’s $V = 0.289$).
Pigment production by *P. aeruginosa* strains

Figure 5-4: HTA scores for *P. aeruginosa* clinical isolates characterized by the production of pigmented compounds

*P. aeruginosa* strains were classified based on their production of pigment when growing in King’s B broth or on NGM during high-throughput assay. The overall difference between all the groups was assessed using the Kruskal-Wallis test (Kruskal-Wallis $\chi^2 = 31.103$, degree of freedom= 2; $P$-value< 0.001) while pairwise comparison were done by employing the Mann-Whitney test and significant $P$-value are shown.
A.

<table>
<thead>
<tr>
<th>Pigment production by <em>P. aeruginosa</em> strains</th>
<th>Isolation</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood N (%)</td>
<td>Sputum N (%)</td>
</tr>
<tr>
<td>Pigment-negative</td>
<td>14 (26%)</td>
<td>30 (50%)</td>
</tr>
<tr>
<td>Presence of pigment either in King’s broth or on NGM</td>
<td>27 (48%)</td>
<td>19 (32%)</td>
</tr>
<tr>
<td>Presence of pigment both in King’s broth and on NGM</td>
<td>14 (26%)</td>
<td>11 (18%)</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Pigment production by <em>P. aeruginosa</em> strains</th>
<th>Presence of <em>exoU</em> (N (%))</th>
<th>of <em>exoU</em> (N (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exoU-negative</td>
<td>exoU-positive</td>
</tr>
<tr>
<td>Pigment-negative</td>
<td>32 (39%)</td>
<td>7 (28%)</td>
</tr>
<tr>
<td>Presence of pigment either in King’s broth or on NGM</td>
<td>38 (45%)</td>
<td>7 (28%)</td>
</tr>
<tr>
<td>Presence of pigment both in King’s broth and on NGM</td>
<td>13 (16%)</td>
<td>11 (44%)</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>Pigment production by <em>P. aeruginosa</em> strains</th>
<th>Presence of <em>exoY</em> (N (%))</th>
<th>of <em>exoY</em> (N (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exoY-negative</td>
<td>exoY-positive</td>
</tr>
<tr>
<td>Pigment-negative</td>
<td>6 (50%)</td>
<td>33 (34%)</td>
</tr>
<tr>
<td>Presence of pigment either in King’s broth or on NGM</td>
<td>6 (50%)</td>
<td>39 (41%)</td>
</tr>
<tr>
<td>Presence of pigment both in King’s broth and on NGM</td>
<td>0 (0%)</td>
<td>24 (25%)</td>
</tr>
</tbody>
</table>

Table 5-2: Distribution of *P. aeruginosa* strains based on their source of isolation or presence of T3SS effector gene among pigment-producing isolates

The production of pigment in King’s B broth and on NGM from *P. aeruginosa* clinical isolates was recorded during highthroughput assay and tabulated versus (A) the source of isolation of the strain, (B) the presence of *exoU* or (C) the presence of *exoY*. Pearson $\chi^2$ was used to test whether the prevalence was significantly different from what expected by chance.

(A) Pearson $\chi^2$ = 7.366, degree of freedom= 2, $P$-value= 0.027, Cramer’s V= 0.253 (intermediate effect); see Appendix E, Table E-1 for more details.

(B) Pearson $\chi^2$ = 8.994, degree of freedom= 2, $P$-value= 0.012, Cramer’s V= 0.289 (intermediate effect); see Appendix E, Table E-2 for more details.

(C) Pearson $\chi^2$ = 3.946, degree of freedom= 2, $P$-value= 0.128; see Appendix E, Table E-6 for more details.
Conclusions and discussion

*C. elegans* as model organisms to gauge the virulence of *P. aeruginosa* clinical isolates

The presence of microorganisms in the bloodstream is treated as a serious hazard (Reimer, Wilson & Weinstein 1997) and it has been associated with high rate of mortality in the case of *P. aeruginosa* (Aksamit 1993, Fick 1993). The presence of this opportunist pathogen in the blood of a patient is due to both host factors, such as patient underlying conditions (Ventre *et al.* 2007), and bacterial factors, including a functional type III secretion system (Vance, Rietsch & Mekalanos 2005) and production of exotoxin A (Schumann, Bluethmann & Tiegs 2000), pyocyanin (Cheluvappa *et al.* 2010) and alkaline protease (Salyers, Whitt 1994).

It has been speculated that strains isolated from bloodstream of a patient were usually more virulent than strains isolated from other sites and in particular from sputum where presence of *P. aeruginosa* might indicate respiratory track colonization or chronic pulmonary disease (Alleyne 2009). Using the *C. elegans* high throughput assay, clinical isolates derived from blood cultures showed a significant tendency toward lower scores than isolates derived from sputum culture meaning that blood-derived strains were more virulent for *C. elegans*. On the contrary, no significant association between source of strain isolation and slow-killing assay (Lee *et al.* 2006) or fast-killing assay (Janjua *et al.* 2012) was found in previous reports. Using the slow-killing assay, eighteen strains isolated from various infection sites from humans and from the environment were compared to PAO1 and PA14 but no association was found between the original
sources of a strain with the level of virulence against *C. elegans*. It is possible that too few strains were used to show a clear trend and it is also probable that the conditions of the fast-killing assay did not allow for relevant virulence factors to act.

The higher virulence of blood-derived strains to *C. elegans* in slow-killing assay-like conditions contributed to better understand the nematode as model of infection. In fact, *C. elegans* appeared affected by *P. aeruginosa* strains involved in more serious acute infections of human since recovery of this bacterium in the blood of patients has been categorised as an acute infection (Feltman *et al.* 2001). However, the comparison with sputum-derived strains may be tainted by the fact that they are likely to be a heterogeneous group. No particular discrimination between patients was applied at the time of collection of the samples and little effort was made to define whether they had ongoing infection or were colonized by *P. aeruginosa*.

The concept that blood-derived *P. aeruginosa* strains were generally more virulent came from the clinical practice including the observation that patients were extremely ill (Samiy, Smith & Wyngaarden 1981) and that infections were difficult to treat due to high level of antibiotic resistance of the infecting strain (Hanberger *et al.* 1999, Fluit, Verhoef & Schmitz 2000). However, these remarks are more empirical than experimental. Determining that blood-derived strains have a tendency to be more virulent to the nematodes than sputum-derived strains may strengthen the observations obtained from the human clinical setting and may provide a framework to test why these strains are more dangerous.
**exoU as a marker for genomic islands affecting *C. elegans* survival**

The linkage between *exoU/spcU* and the accessory genome was observed previously in at least four other strains (He *et al.* 2004, Kulasekara *et al.* 2006) and it has been confirmed by bioinformatics analysis in this current study (Figure 5-3). The *exoU/spcU* gene pair was found in a subset of sequenced *P. aeruginosa* strains and in all the cases it was located in a genomic island or islet integrated at the $tRNA_{Lys10}^A$. The first genomic island carrying *exoU* and *spcU* identified at this integration site was PAPI-2 (He *et al.* 2004). The elements identified recently from the NCBI strains collection were either smaller or larger versions of PAPI-2 with the smallest element predicted to contain two coding regions beside *exoU* and *spcU* (similarly to ExoU-C islet; Chapter 1, Table 1-2). The larger elements were predicted to contain PAPI-2 integrase gene at the 3’end of the island and *exoU/spcU* at the 5’end of the island (similarly to ExoU-A and ExoU-B islands; Chapter 1, Table 1-2). These genomic islands possess a modular structure as described for other genetic elements (Kung, Ozer & Hauser 2010) because larger elements appear to possess most of the coding regions associated with smaller elements interspaced by strain specific regions.

Screening of T3SS effector genes revealed higher prevalence of *exoU*-positive/*exoS*-negative strains among blood-derived isolates and of *exoU*-negative/*exoS*-positive strains among the sputum-derived isolates. Although the prevalence was not significantly linked to the source of isolation of the strain, it showed a tendency similar to what reported previously (Wareham, Curtis 2007). Prevalence of *exoY* and *exoT* (data not shown) showed no difference between the blood-derived and the sputum-derived strains.
Presence of some T3SS effector genes was associated with reduced *C. elegans* survival. *exoY*-negative strains had a propensity toward higher HTA scores than *exoY*-positive strains meaning that strains without *exoY* were less virulent toward *C. elegans*. This phenomenon was probably linked to the high incidence of strains lacking other effector genes among the *exoY*-negative isolates. In fact, 93% of *exoY*-negative strains were also *exoU*-negative and 43% were negative for all effectors genes. Moreover, strains with an *exoU*-positive/*exoS*-negative genotype were associated with reduced *C. elegans* survival in comparison with strains with *exoU*-negative/*exoS*-positive genotype. The presence of *exoU* appeared to lessen *C. elegans* survival compared to *exoS* confirming that the inverse relationship between these effectors has physiological implications for the virulence of *P. aeruginosa*.

The attribution of higher level of virulence against *C. elegans* for *exoU*-positive strains was unexpected. The involvement of T3SS effectors in virulence against *Galleria mellonella* was confirmed using PA14 *exoU* and/or *exoT* deletion mutants while *exoY* did not seem to intervene (Miyata *et al.* 2003). The use of the same targeted deletion mutants showed the lack of involvement of PA14 T3SS effectors in killing of *C. elegans* in both slow- and fast-killing assays and in *Arabidopsis thaliana* infection assay (Miyata *et al.* 2003). This occurred even though the effectors were expressed during slow-killing assay condition (Wareham, Papakonstantinopoulou & Curtis 2005). A more recent report showed that ExoU was injected almost exclusively in the alveolar macrophages in the initial phases of infection and in the neutrophils and monocytes in subsequent phases in an acute pneumonia mouse model (Diaz, Hauser 2010). This might explain why T3SS effectors compromised insects but not nematodes survival since *C. elegans* does not possess professional phagocytes (Irazoqui, Ausubel 2010) while *G. mellonella* does (Bergin *et al.* 2005).
The lack of a direct effect of ExoU on *C. elegans* may indicate that genetic traits associated with *exoU* accessory elements may be responsible for the outcome. Deletion of PAPI-2 in PA14 as a whole had a very modest and assay condition-dependent effect (Chapter 3 in this thesis). Consequently, it may be speculated that genes associated with *exoU* in genomic islands larger than PAPI-2 may be involved in virulence against *C. elegans*. The poor annotation of coding region within the tRNA<sup>lys10</sup>-associated genomic islands rendered difficult to speculate which genes or function might be responsible for virulence against *C. elegans*. It should be noted that the lack of involvement of ExoU in virulence against *C. elegans* could be a PA14-specific phenomenon and T3SS effectors might augment bacterial virulence against *C. elegans* in other strains.

**Production of pigment by *P. aeruginosa* as predictor of virulence against *C. elegans***

The *P. aeruginosa* capacity to produce pigments has been established since the first half of the 20<sup>th</sup> century (Allin 1941) enabling the recognition of this bacterium after isolation (King, Ward & Raney 1954). When growing in King’s B broth, some *P. aeruginosa* strains developed a green hue that was historically associated with production of the siderophore pyoverdine, previously named fluorescin (King, Ward & Raney 1954). The colour presented by *P. aeruginosa* strains when plated on nematode growth medium (NGM) were more various ranging from dark yellow and green to red or brown although the majority of the strains (69%) developed a green-blue shade. The phenazine pyocyanin, a secondary metabolite, confers to the bacterium growing on agar its classic green-blue colour. *P. aeruginosa* has been shown to produce other pigmented compounds conferring different colours to the medium such as the red-brown pyorubin.
(King, Ward & Raney 1954) and the dark brown-black pyomelanin (Rodriguez-Rojas et al. 2009).

The participation of pyoverdine (Takase et al. 2000a, Takase et al. 2000b) and phenazines (Mahajan-Miklos et al. 1999, Lau et al. 2004) in virulence has been recognized in general as it is their involvement in iron scavenging (Cornelis, Dingemans 2013). When *C. elegans* assay were used, pyoverdine was essential to reduce nematodes survival in a phosphate-depleted slow-killing assay (Zaborin et al. 2009) while pyocyanin was required to mediate death in fast-killing assay (Cezairliyan et al. 2013). Regarding other pigments, pyomelanin was linked to increased virulence in a mouse infection model (Rodriguez-Rojas et al. 2009).

In this study, clinical isolates producing pigment while growing either in King’s broth or on NGM were more virulent against *C. elegans* than pigment-less strains. Furthermore, when a *P. aeruginosa* strain was able to develop pigmentation both in King’s broth and on NGM, its effect on *C. elegans* survival was even more pronounced. Remarkably, clinical isolates obtained from sputum were over-represented in the pigment-less category while *exoU*-positive strains were over-represented among isolates producing both pyoverdine and pigment on NGM.

Association between pyoverdine production in King’s broth and *C. elegans* demise could find explanation in the pyoverdine-induced expression of other virulence factors such as the protease PrpL and exotoxin A (Visca, Imperi & Lamont 2007, Cornelis, Dingemans 2013). Since the iron is sequestered by circulating proteins (Cornelissen, Sparling 1994), blood could be considered an iron-depleted environment where production of pyoverdine is essential for survival. It can be speculated that at least a sub-group of blood-derived strains was more apt to produce pyoverdine which in turn might produce factors affecting *C. elegans* survival. Incidentally, it has been reported
that *P. aeruginosa* isolated from blood produced higher levels of proteases and exotoxin A (Woods *et al.* 1986). The involvement of exotoxin A in *C. elegans* survival in slow-killing assay was reported earlier (Mahajan-Miklos, Rahme & Ausubel 2000) but questioned more recently at least for *C. elegans* N2 strain (McEwan, Kirienko & Ausubel 2012). However, preliminary data showed a link between strains able to produce proteases tested *in vitro* and lower HTA score (data not shown).

The involvement of pigments such as pyocyanin and pyorubin in *C. elegans* survival in slow-killing assay-like conditions has not been reported previously and it could be linked again to iron uptake and/or to the *Pseudomonas* quinolone signal quorum sensing system (PSQ; Chapter 1). In the slow-killing assay, survival of *C. elegans* was increased after interruption of *psqE* (Feinbaum *et al.* 2012) which is part of the operon involved in the synthesis of HHQ and PSQ (Gallagher *et al.* 2002, Deziel *et al.* 2004), the mediators of PSQ quorum sensing. The exact role of PsqE is still under scrutiny but the overexpression of its gene in PAO1 led to increased production of pyocyanin and lectin A and up regulation of genes codifying for type II secretion system proteins (Bradbury *et al.* 2010), involved in proteases secretion (Williams, Camara 2009). Moreover, the overexpression of *psqE* in a PAO1 Δ*psqA-*Δ*psqE* mutant restored the virulence against *C. elegans* to the level of wild-type and caused reduction in fertility of nematodes (Rampioni *et al.* 2010). It could be speculated that variation in *psqE* expression in clinical isolates might cause differential production of pigments and other virulence factors affecting the survival of *C. elegans*.

There has been no information available about strain-to-strain variability of *psqE* sequence and/or expression. Most of the work on PSQ quorum sensing system has been done using two reference strains, PAO1 and PA14, which show differences in behaviour and regulation of this system (Jimenez *et al.* 2012). It is conceivable that
these differences may be also present in other strains. Hypothetically, blood-derived strains may be more apt to produce pyoverdine because of the innate iron limitation of blood, which in turn may regulate expression of other virulence factors. In these strains, PsqE could work concurrently or consecutively pyoverdine to activate further factors including pyocyanin, proteases and traits present in exoU-related genomic islands. One of the outcomes could be a higher resistance toward the bacterivorous predator C. elegans. Vice-versa, a P. aeruginosa strain might have encountered the nematode as a predator and adapt to survive it. In this process, it might have also acquired characteristics that enhanced its ability to cause damages in a mammalian host leading to bacteremia.
Chapter 6

Final conclusions and future work
In order to characterize and identify mobile genetic elements involved in *P. aeruginosa* virulence, non-mammalian organism models were chosen and two main molecular approaches were used: creation of deletion mutants lacking genomic islands and association studies of genomic island-related virulence traits.

**Evidences supporting the use of *A. castellanii* as model organism to study *P. aeruginosa* virulence factors**

The use of the amoeba *A. castellanii* highlighted the involvement of the PAPI-2 pathogenicity in *P. aeruginosa* virulence. However, the same was not true for *A. polyphaga*. Subsequent assays showed that growing PA14 or PAO1 on different media (M9 minimal medium, LA and TSA) before layering on NNA agar influenced the ability of *A. castellanii* (Appendix F, Figure F-2) but not *A. polyphaga* (Appendix G, Figure G-2) growth film to expand on these bacteria. This may be because different strains growing in different conditions may have expressed different virulence-associated factors which would influence the ability of *A. castellanii* to graze on them.

It can be inferred that the factors affecting *A. castellanii* were expressed by the bacteria before plating on NNA. On the contrary, *A. polyphaga* was able to successfully graze on the *P. aeruginosa* strains in a manner independent from the type of strain, bacterial growth conditions and amount of bacteria plated. Consequently, while *A. castellanii* appear a suitable organism to model *P. aeruginosa* infection, *A. polyphaga* does not appear to be useful.

The use of PAPI-2 island deletion mutants suggested that some of the virulence factors affecting *A. castellanii* grazing were encoded or depended on the presence of this island. Initially, it was assumed that the lack of *exoU* was the major cause of attenuated virulence. However, NNA assays done using PA14Δ*exoU* strain, lacking only *exoU*, did
not show a reduction of virulence compared to PA14 wildtype (Appendix F, Figure F-3). It can be assumed that functions other than ExoU in PAPI-2 affected *A. castellanii* grazing. Interestingly, this phenomenon was also observed in *G. mellonella* survival in which the larvae injected with PA14ΔexoU survived longer that those injected with PA14 wildtype but shorter than those injected with PA14ΔPAPI-2 (Harrison et al. 2010). Since that both *G. mellonella* and *A. castellanii* are affected by the presence of PAPI-2, it could be inferred that it might enhance anti-phagocytic properties or bacterial survival within the amoeba/phagocytic cell. Single gene deletion followed by NNA assays and *G. mellonella* survival assays could clarify which functions are required in order for PAPI-2 to exert its effect.

**Genomic islands involved in *P. aeruginosa* virulence toward *C. elegans***

The creation of deletion mutants in several reference and clinical *P. aeruginosa* strains required the comparison with the original wildtype strain to assess any difference(s) in virulence phenotype. The deletion of genomic islands inserted at the *tRNA* Pro21 from several *P. aeruginosa* strains revealed that few islands were involved in virulence toward *C. elegans*, a phenomenon that is likely due the high level of genetic variability ascribed to these elements. However, at least one island, the *tRNA* Pro21 genomic island from PA14, was proved to increase *P. aeruginosa* virulence toward another model of infection, *G. mellonella* (Harrison 2009). This may be an indication that some of the *tRNA* Pro21 islands may express general virulence factors affecting more than one organism and possibly mechanisms conserved across invertebrate and vertebrate. It would be interesting to test deletion mutants with other models of infection to group the islands based on the affected organism/s and compare the islands within each group, in
order to find definitive conserved genetic traits. Ultimately, single genetic traits could also be deleted to test their individual contribution to virulence.

The deletion of genetic elements found in \( tRNA^{\text{Ser}11} \) had a greater impact since two-thirds of these islands affected virulence toward \( C. \) elegans. This is an initial indication that these genetic elements should be classified as PAIs and could be further characterized as proposed for \( tRNA^{\text{Pro21}} \) islands.

On the opposite, deletion of PA14-specific PAPI-1 located at \( tRNA^{\text{Lys47}} \) had no impact on \( C. \) elegans survival while deletion of PAPI-2 located at \( tRNA^{\text{Lys10}} \) had only a marginal impact on \( P. \) aeruginosa virulence. It appears that \( C. \) elegans is not a universally suitable model to study virulence related to these islands. However, interruption of several \textit{orfs} present in PAPI-1 was associated with reduced virulence towards \( C. \) elegans (He et al. 2004). The modular nature of large genomic islands, such as PAPI-1, means that genetic elements are continuously added (or deleted) potentially changing the fitness conferred to bacterial cells. It would be interesting to test whether the deletion of specific modules within PAPI-1 would increase \( C. \) elegans survival and other models of infection providing insights in how large genomic islands may evolve.

Genomic islands inserted at \( tRNA^{\text{Lys10}} \) and carrying \textit{exoU} were inferred to be associated with virulence toward \( C. \) elegans despite PAPI-2 having a modest effect. These islands were also associated with production of pyoverdine, phenazine and/or other pigments by \( P. \) aeruginosa. Whether the reduced survival is solely due to production of pyoverdine and/or other pigments or due to other functions encoded by the island, it is unclear. It is plausible that different islands will affect survival through different means.

The \textit{exoU}-bearing genomic islands in particular proved to be very diverse and more work is therefore required to define any direct involvement in virulence toward \( C. \) elegans and development of pigmentation. Creation of selected island-deletion mutants
coupled with sequence analysis of the islands and use of several models of infections should provide a comprehensive overview of these genetic elements and might help to identify new virulence factor or refine the function of known ones. The use of non-mammalian models of infections may provide a convenient framework where most of the preliminary *in vivo* work could be done reducing costs and use of expensive and slow to analyse mammalian models.

Highthroughput sequencing of bacterial genomes has become fast and affordable but a bottleneck is represented by the annotation of coding regions. This problem seems to particularly affect horizontally acquired elements such as genomic islands. Throughout this project, it was found that most of the identified open reading frames associated within genomic island were annotated as hypothetical or with too broad definition to be useful. Moreover, sequencing genomes does not provide insight on gene expression involved in responding to particular events (Kroger *et al.* 2013). It would be very informative to compare transcriptomes (i.e. using RNA-seq) between wildtype strains and mutant strains lacking genomic islands with and without presence of bacterivorous predators to gain a deeper understanding on the role played by genomic islands in bacteria.

*Are* *P. aeruginosa* and *C. elegans* competing for acquisition of iron?

The link between strains obtained from culture of blood of a patient and higher virulence toward *C. elegans* was surprising because human blood and nematodes represent very different environments. Interestingly, blood-derived isolates were also linked to production of pyoverdine and other pigments which presence affected directly *C. elegans* survival. Pyoverdine and pyocyanin, the green-blue phenazine pigment, are
involved in *P. aeruginosa* iron scavenging and recruitment and are known to affect *C. elegans* survival in other assay conditions but not in slow-killing assay-like conditions. It is also known that *C. elegans* iron sequestration pathways are activated by the nematode in response to exposure to pathogenic bacteria such as *S. aureus* and *P. aeruginosa* (Irazoqui et al. 2010). It could be hypothesized that the presence of *P. aeruginosa* iron scavenging compounds such as pyoverdine may render this element unavailable for *C. elegans* causing its premature death. *P. aeruginosa* may compete with *C. elegans* for iron in a similar manner in which the bacterium competed for iron within the blood of a mammal host. It is also possible that pyoverdine and other pigments may directly or indirectly through activating other enzyme(s) act on *C. elegans*. Independently from the actual mode of action, it appears that to have and express these pigmented compounds may have allowed *P. aeruginosa* to fend off the nematode as predator effectively “training” to survive in the blood of mammals.

Since *exoU*-bearing islands appeared to be associated with the production of pyoverdine, pyocyanin and other pigments, it is possible that the capacity to produce and/or regulate these compounds resides in the accessory genome. For example, the deletion of *tRNA^Pro21-*associated genomic island in PA14 led to a visible reduction of green hue development when grown in King’s B broth in comparison with PA14 wildtype (data not shown). Acquisition of genomic island bearing some of these functions may improve *P. aeruginosa* survivability in presence of *C. elegans* or other yet unidentified conditions which could have had the unintentional consequence to improve survivability in blood.
Appendix A

Diameter measured for growth film of *Acanthamoeba* spp. grazing on *P. aeruginosa* PA14 WT, PA14ΔPAPI-1, PA14ΔPAPI-2, PA14Δ1Δ2 and PAO1

<table>
<thead>
<tr>
<th>Strain</th>
<th>CFU/plate</th>
<th>Diameter of <em>A. castellanii</em> (mm ± st.dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>PA14 WT</td>
<td>$1.58 \times 10^8$</td>
<td>$14.3 \pm 1.1$</td>
</tr>
<tr>
<td>PA14 ΔPAPI-1</td>
<td>$8.20 \times 10^7$</td>
<td>$41.8 \pm 10.5$</td>
</tr>
<tr>
<td>PA14 ΔPAPI-2</td>
<td>$1.51 \times 10^8$</td>
<td>$16.0 \pm 1.8$</td>
</tr>
<tr>
<td>PA14 Δ1Δ2</td>
<td>$1.90 \times 10^8$</td>
<td>$16.0 \pm 1.3$</td>
</tr>
<tr>
<td>PAO1</td>
<td>$1.68 \times 10^8$</td>
<td>$23.5 \pm 1.6$</td>
</tr>
</tbody>
</table>

**Table A-1: Diameter of *A. castellanii* film grazing on *P. aeruginosa* strains**

Table showing the diameter of *A. castellanii* cultures grazing on non-nutrient agar plates layered with *P. aeruginosa* strains. The diameter was measured at day 3, 6, 9 and 12. Amount of bacteria plated (CFU/plate) and amoeba culture diameter (mm) were determined for 10 plates/strain (equivalent to 10 single colonies) and shown as average ± standard deviation.
### Diameter of A. polyphaga (mm ± st.dev.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>CFU/plate</th>
<th>3 days</th>
<th>6 days</th>
<th>9 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14 WT</td>
<td>1.47 × 10^8 ± 5.55 × 10^7</td>
<td>16.3 ± 0.8</td>
<td>31.0 ± 2.1</td>
<td>49.55 ± 3.6</td>
</tr>
<tr>
<td>PA14ΔPAPI-1</td>
<td>2.18 × 10^8 ± 9.04 × 10^7</td>
<td>17.4 ± 1.6</td>
<td>33.9 ± 3.5</td>
<td>54.95 ± 6.5</td>
</tr>
<tr>
<td>PA14ΔPAPI-2</td>
<td>1.34 × 10^8 ± 5.74 × 10^7</td>
<td>18.1 ± 1.4</td>
<td>34.3 ± 3.3</td>
<td>55.80 ± 4.7</td>
</tr>
<tr>
<td>PA14Δ1Δ2</td>
<td>1.21 × 10^8 ± 3.92 × 10^7</td>
<td>17.1 ± 0.6</td>
<td>31.4 ± 1.0</td>
<td>49.65 ± 3.0</td>
</tr>
<tr>
<td>PAO1</td>
<td>2.70 × 10^8 ± 1.14 × 10^8</td>
<td>13.8 ± 1.4</td>
<td>32.3 ± 4.4</td>
<td>60.05 ± 5.9</td>
</tr>
</tbody>
</table>

#### Table A-2: Diameter of A. polyphaga film grazing on P. aeruginosa strains

Table showing the diameter of A. polyphaga cultures grazing on non-nutrient agar plates layered with P. aeruginosa strains. The diameter was measured at day 3, 6, and 9. Amount of bacteria plated (CFU/plate) and amoeba culture diameter (mm) were determined for 10 plates/strain (equivalent to 10 single colonies) and shown as average ± standard deviation.
Appendix B

ORFs associated with genomic islands integrated at \textit{tRNA}^{Pro21}, \textit{tRNA}^{Gly19} and \textit{tRNA}^{Ser11}

Sequenced genomes of \textit{P. aeruginosa} strains identified as “complete” or as “chromosomes with gaps” deposited in the NCBI database were surveyed for presence of genetic elements inserted at \textit{tRNA}^{Pro21}, \textit{tRNA}^{Ser11} and \textit{tRNA}^{Gly19}. Genomic islands or islet were identified as not-conserved genetic elements inserted between universally conserved upstream and downstream flanking regions larger than or smaller than 10 kb, respectively.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Island size (bp)</th>
<th>No. hypothetical ORFs</th>
<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>17,956</td>
<td>10</td>
<td>2</td>
<td>DNA helicase; helicase</td>
</tr>
<tr>
<td>M18</td>
<td>11,465</td>
<td>2</td>
<td>5</td>
<td>Type I restriction-modification enzyme (restriction, methylases and specificity enzymes); ABC transporter; transposase</td>
</tr>
<tr>
<td>PA96</td>
<td>16,667</td>
<td>6</td>
<td>3</td>
<td>Ddel (modification methylase); ATP/GTP-binding protein; MazG (nucleotide pyrophosphohydrolase domain protein)</td>
</tr>
<tr>
<td>PA1</td>
<td>16,699</td>
<td>4</td>
<td>5</td>
<td>MazG (nucleotide pyrophosphohydrolase domain protein); ATP/GTP-binding protein; cytosine-specific methyltransferase protein; FtsK (cell division protein); transposase IS3/IS911</td>
</tr>
<tr>
<td>MTB-1</td>
<td>36,518</td>
<td>11</td>
<td>5</td>
<td>Transcriptional regulator; transposase; truncated transposase; DNA helicase; helicase</td>
</tr>
<tr>
<td>B136-33</td>
<td>24,968</td>
<td>6</td>
<td>6</td>
<td>Integrase; transposase; DNA or RNA helicase; phage integrase; ATP/GTP-binding protein; MazG (nucleotide pyrophosphohydrolase domain protein)</td>
</tr>
<tr>
<td>SCV20265</td>
<td>17,896</td>
<td>6</td>
<td>8</td>
<td>Helicase (Snf2/rad54 family); type III restriction-modification system - methylases and helicase; mobile element protein (∗ 4); phage DNA-binding protein</td>
</tr>
<tr>
<td>DK2</td>
<td>34,652</td>
<td>24</td>
<td>9</td>
<td>ISPpu14 (transposase Orf3); cytoplasmic protein; HNH endonuclease type IV restriction enzyme; XRE family transcriptional regulator; carbon storage regulator; DndE (DNA sulphur modification protein); phage integrase; ATP/GTP-binding protein; MazG (nucleotide pyrophosphohydrolase domain protein)</td>
</tr>
</tbody>
</table>

Table B-1: ORFs annotation for representative \( tRNA^{Pro21} \)-associated genomic islands

ORFs associated with \( tRNA^{Pro21} \) genomic islands found in completely or nearly-completely sequenced \( P. aeruginosa \) genomes. The size of the island in basepairs (bp) is indicated alongside the number of open reading frames (ORFs) recorded as hypothetical protein and the number of ORFs with annotation in NCBI database.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Island size (kb)</th>
<th>No. hypothetical ORFs</th>
<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LESlike4</td>
<td>45,015</td>
<td>22</td>
<td>13</td>
<td>Transposase (× 5); restriction endonuclease; alkaline phosphatase; restriction endonuclease subunit M; protein kinase; integrase; peptidase P60; phage minor tail protein L; transcriptional regulator</td>
</tr>
<tr>
<td>LESlike7</td>
<td>45,024</td>
<td>22</td>
<td>13</td>
<td>Transposase (× 5); restriction endonuclease; alkaline phosphatase; restriction endonuclease subunit M; protein kinase; integrase; peptidase P60; phage minor tail protein L; transcriptional regulator</td>
</tr>
<tr>
<td>LESB58</td>
<td>45,015</td>
<td>17</td>
<td>11</td>
<td>Transposase A (× 2); OrfB (insertion sequence IS407; × 2); restriction endonuclease-like GTPase; transcriptional regulator-like protein; serine/threonine protein kinase; phage integrase (× 2); NlpC/P60 family protein; phage minor tail protein L</td>
</tr>
<tr>
<td>LESlike1</td>
<td>45,016</td>
<td>20</td>
<td>17</td>
<td>Transposase (× 3); transposase IS407 (× 2); GTPase; RadC (DNA repair protein); hydrolase or metal-binding protein; alkaline phosphatase; Der-disrupted (restriction endonuclease subunit M); membrane protein; protein kinase; integrase (× 2); tail protein; peptidase P60; transcriptional regulator</td>
</tr>
<tr>
<td>PAO1</td>
<td>11,843</td>
<td>5</td>
<td>1</td>
<td>Restriction-modification system protein</td>
</tr>
<tr>
<td>PA7</td>
<td>19,504</td>
<td>10</td>
<td>3</td>
<td>Prophage encoded two-component systems; patch repair protein; Ddel (modification methylase)</td>
</tr>
<tr>
<td>NCGM 1900</td>
<td>27,379</td>
<td>6</td>
<td>9</td>
<td>RecQ (ATP-dependent DNA helicase); DprA (DNA protecting protein); N-7 DNA methylase; N-6 DNA methylase; SMC domain-containing protein; UvrD/REP helicase; restriction enzyme type I helicase; transposase (× 2)</td>
</tr>
</tbody>
</table>

Table B-1 (continued): ORFs annotation for representative tRNAPro21-genomic islands
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<th>Strain</th>
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<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSE305</td>
<td>116,041</td>
<td>41</td>
<td>68</td>
<td>Type III secretion system ATPase; phage integrase; deoxyguanosinetriphosphate triphosphohydrolase; resistance-nodulation-cell division (RND) efflux protein; queuine tRNA-ribosyltransferase; glutamate synthase subunit α; radical activating enzyme; ATP-dependent DNA helicase; AMP-binding domain protein; RecQ (ATP-dependent DNA helicase); CobN (cobaltochelatase subunit); HepA (ATP-dependent helicase); selenocysteine-specific elongation factor; dihydrodipicolinate synthase; ATPase central domain-containing protein; methylthioribose-1-phosphate isomerase; chemotaxis transducer; stomatin-like protein; transcriptional factor; VqsM (AraC-type transcriptional regulator); non-ribosomal peptide synthetase; PpiC (peptidyl-prolyl cis-trans isomerase); lipoprotein; transcriptional factor; SuhB (extragenic suppressor protein); two-component response regulator; phage-related endonuclease-like protein; carbon storage regulator; ABC-type amino acid transport/signal; leucine dehydrogenase; short chain alcohol dehydrogenase; transcriptional regulator; putative Fmn oxidoreductase (x 2); hydrolase; phosphate transporter; transport protein; choline transporter; hemolysin; penicillin-binding protein 3; small-conductance mechanosensitive channel; superoxide dismutase; AmpR (transcriptional regulator); transposase (x 3); phenylalanyl-tRNA synthetase subunit α; Mfd (transcription-repair coupling protein); XerC (site-specific tyrosine recombinase); acyl-CoA dehydrogenase; acyl-CoA lyase β chain; porin; acetyl-CoA acetyltransferase; hydrolase; transcriptional regulator (x 2); cyclase family protein short chain dehydrogenase; DhcA (dehydrocarcinine CoA transferase) subunit A and subunit B; acetyl-CoA thioesterase II; major facilitator superfamily (MFS) transporter; muconolactone δ-isomerase; acetyl-CoA hydrolase/transferase; periplasmic spermidine/putrescine-binding protein; 2-Nitropropane dioxygenase; Ant (anti-repressor protein)</td>
</tr>
</tbody>
</table>

Table B-1 (continued): ORFs annotation for representative $tRNA^{Pro21}_P$-associated genomic islands
<table>
<thead>
<tr>
<th>Strain</th>
<th>Island size (bp)</th>
<th>No. hypothetical ORFs</th>
<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LESB58</td>
<td>112,919</td>
<td>46</td>
<td>69</td>
<td>Integrase (× 2); LysR family transcriptional regulator (× 2); transcriptional regulator; D-alanyl-D-alanine endopeptidase; 4-hydroxyphenylpyruvate dioxygenase; AsnC family transcriptional regulator; heavy metal transport/detoxification protein; [2Fe-2S] ferredoxin; ATPase; ArsR family transcriptional regulator (× 2); dihydrolipoamide dehydrogenase; AcrB (multidrug transporter); membrane protein; outer membrane efflux protein; major facilitator transporter; ZitB (zinc transporter); ATPase; transporter - disrupted; multidrug transporter; MerR family transcriptional regulator (× 3); MFS transporter (× 2); TetR family transcriptional regulator; mercuric reductase; GntR family transcriptional regulator (× 2); glutaredoxin; 2-dehydro-3-deoxyphosphooc-tonate aldolase; relaxase; membrane protein; secreted protein; RadC (DNA repair protein); DSBA oxidoreductase; conjugal transfer protein (× 2); RAQPRD family plasmid; DNA-binding protein; cell division protein; DNA polymerase subunit β; ATPase; membrane protein; TraG (conjugal transfer protein); lytic transglycosylase; DEAD/DEAH box helicase; O-methyl transferase family domain-containing protein (× 2); signal peptide protein; transposase; mercuric reductase; mercury transporter (× 2); GTPase; uridylate kinase; ABC transporter substrate-binding protein; ATPase P; sterol desaturase; cation transporter; DNA topoisomerase III; single-stranded DNA-binding protein; integrase; coproporphyrinogen III oxidase; cobyricin acid a,c-diamide synthase; transcriptional regulator</td>
</tr>
<tr>
<td>LESlike7</td>
<td>112,918</td>
<td>46</td>
<td>69</td>
<td>As LESB58</td>
</tr>
</tbody>
</table>

Table B-2: ORFs annotation for representative trNA\textsuperscript{Gly19}-associated genomic islands

ORFs associated with trNA\textsuperscript{Gly19} genomic islands found in completely or nearly-completely sequenced P. aeruginosa genomes. The size of the island in basepairs (bp) is indicated alongside the number of open reading frames (ORFs) recorded as hypothetical protein and the number of ORFs with annotation in NCBI database.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Island size (bp)</th>
<th>No. hypothetical ORFs</th>
<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LES400</td>
<td>112,923</td>
<td>45</td>
<td>69</td>
<td>As LESB58 but without one of the GntR family transcriptional regulator; Integrase (× 2); LysR family transcriptional regulator (× 2); transcriptional regulator; D-alanyl-D-alanine endopeptidase; 4-hydroxyphenylpyruvate dioxygenase; AsnC family transcriptional regulator; heavy metal transport/detoxification protein; glutaredoxin; [2Fe-2S] ferredoxin; transcriptional regulator; ATPase; ArsR family transcriptional regulator (× 2); dihydrolipoamide dehydrogenase; AcrB (multidrug transporter); membrane protein; transporter; NcrB; major facilitator transporter; ZitB (zinc transporter); ATPase; transporter - disrupted; multidrug transporter; MerR family transcriptional regulator (× 3); MFS transporter (× 2); TetR family transcriptional regulator; mercuric reductase; GntR family transcriptional regulator (× 2); glutaredoxin; 2-dehydro-3-deoxyphosphooctonate aldolase; relaxase; membrane protein; integrative-conjugative element protein (× 3); RadC (DNA repair protein); DSBA oxidoreductase; conjugal transfer protein (× 11); TIGR03750 family conjugative transfer region protein; RAQPRD family plasmid; DNA-binding protein; cell division protein; DNA polymerase subunit β; ATPase; membrane protein; TraG (conjugal transfer protein); lytic transglycosylase; DEAD/DEAH box helicase; O-methyl transferase family domain-containing protein (× 2); signal peptide protein; transposase; mercuric reductase; mercury transporter (× 2); GTPase; uridylate kinase; ABC transporter substrate-binding protein; ATPase P; sterol desaturase; cation transporter; DNA topoisomerase III; single-stranded DNA-binding protein; integrase; coproporphyrinogen III oxidase; ParB (integrative conjugative element); cobyricinic acid a,c-diamide synthase; transcriptional regulator; receptor protein-tyrosine kinase</td>
</tr>
<tr>
<td>LESlike1</td>
<td>112,919</td>
<td>29</td>
<td>85</td>
<td></td>
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Table B-2 (continued): ORFs annotation for representative tRNA<sup>Gly19</sup>-associated genomic islands
<table>
<thead>
<tr>
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<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
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<tbody>
<tr>
<td>PA96</td>
<td>36,430</td>
<td>11</td>
<td>12</td>
<td>LysE family efflux protein; major facilitator superfamily protein; glycoside hydrolase family protein; transposase IS4 (× 2); OrfA (IS3-family transposase); OrfB (IS3-family transposase); pathogenesis-related protein; VprG - disrupted (type IV secretion system protein); peptidase M23; ISPsy24-like transposase; phage-related integrase;</td>
</tr>
<tr>
<td>DK2</td>
<td>104,159</td>
<td>62</td>
<td>52</td>
<td>Integrase; integrase catalytic subunit; ATP-binding protein (istb domain-containing protein); Czc (heavy metal RND efflux outer membrane protein); transposase (× 3); transposase IS4 family; transcriptional regulator; copper resistance protein D, C and B; copper-translocating P-type ATPase; copper resistance protein B; IrlR (CopA family copper resistance protein); transcriptional activator protein; CusS (sensor kinase); ArsR (regulatory protein); glyoxalase/bleomycin resistance protein/dioxygenase; protein tyrosine phosphatase; sodium bile acid symporter family protein; arsenate reductase; PadR-like family transcriptional regulator; chromate transporter; N-acetyltransferase GCN5; ParB domain-containing protein nuclease; hydrophobe/amphilile efflux-1 (HAE1) family trans; MexE (membrane fusion protein); RadC (DNA repair protein); VirB4 component (type IV secretory pathway); signal recognition particle GTPase; DNA binding domain, excisionase family protein; AAA ATPase; lytic transglycosylase, catalytic; PilL lipoprotein (type IV pili); reverse transcriptase; helicase; O-methyl transferase family domain-containing protein; O-methyl transferase family protein; transposase Tn3; mercuric reductase; mercuric transporter periplasmic component; mercuric transport protein; Hg(II)-responsive transcriptional regulator; signal peptidase II; heavy metal translocating P-type ATPase; MerR family transcriptional regulator; Co/Zn/Cd efflux system protein; DNA topoisomerase III; single-stranded DNA-binding protein; integrase regulator R protein; transcriptional regulator; Cobiycin acid a,c-diamide synthase;</td>
</tr>
</tbody>
</table>

Table B-2 (continued): ORFs annotation for representative \( tRNA^{Gly19} \)-associated genomic islands
<table>
<thead>
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<th>Strain</th>
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<th>No. hypothetical ORFs</th>
<th>No. annotated ORFs</th>
<th>ORFs annotation in NCBI</th>
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<tr>
<td>PSE305</td>
<td>7,679</td>
<td>4</td>
<td>6</td>
<td>Bacteriophage integrase; inorganic polyphosphatase/ATP-NAD kinase; flavodoxin; Resistance-nodulation-cell division (RND) divalent protein</td>
</tr>
<tr>
<td>NCGM 1984</td>
<td>54,475</td>
<td>34</td>
<td>14</td>
<td>Lytic enzyme; structural protein;  λ gpG-like protein; major tail subunit; phage head-tail adapter protein; capsid protein; peptidase; histone H1; terminase; tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Asn}, transposase; tRNA\textsuperscript{Met}, transposase</td>
</tr>
<tr>
<td>DK2</td>
<td>6,860</td>
<td>6</td>
<td>1</td>
<td>Phage integrase</td>
</tr>
</tbody>
</table>

**Table B-3: ORFs annotation for representative tRNA\textsuperscript{SerI11}-associated islets and genomic island**

ORFs associated with tRNA\textsuperscript{SerI11} genomic islands or islets found in completely or nearly-completely sequenced *P. aeruginosa* genomes. The size of the island in basepairs (bp) is indicated alongside the number of open reading frames (ORFs) recorded as hypothetical protein and the number of ORFs with annotation in NCBI database.
Appendix C

Growth curves for selected wildtype/tRNA-associated genomic island deletion mutant pairs

*P. aeruginosa* strains were grown overnight in Luria broth (LB), diluted in fresh LB medium and aliquoted in six or nine wells of 96-well plate. The 96-well plates were incubated at 37°C while shaking and OD at 600nm was measured every 10 min for 24 h using Varioskan Flash (ThermoScientific). The original raw data (OD values) were provided by Dr Barbara Rieck.

![Growth curves for KR877-related strains](image)

**Figure C-1: Growth curves for KR877-related strains**

Plot showing the OD measured at 600nm (OD$_{600nm}$) over time for KR877 wildtype strain and its derived deletion mutants. At each time point, average OD of six wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

*P. aeruginosa* strains tested:

- = KR877 wildtype
- = KR877 without tRNA$^{Pro21}$ island (KR877ΔPro21, KR1228)
- = KR877 without tRNA$^{Ser11}$ island (KR877ΔSer11; KR1278)
Figure C-2: Growth curves for KR373-related strains

Plot showing the OD measured at 600nm (OD\textsubscript{600nm}) over time for KR373 wildtype strain and its derived deletion mutant. At each time point, average OD of six wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

\textit{P. aeruginosa strains tested:}
- red = KR373 wildtype
- blue = KR373 without \textit{tRNA\textsuperscript{Pro21}} island (KR373ΔPro21, KR1281)
Figure C-3: Growth curves for MID9245-related strains

Plot showing the OD measured at 600nm (OD$_{600\text{nm}}$) over time for MID9245 wildtype strain and its derived deletion mutant. At each time point, average OD of nine wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

*P. aeruginosa* strains tested:
- = MID9245 wildtype
- = MID9245 without $tRNA^{Pro21}$ island (MID9245ΔPro21, KR986)
Figure C-4: Growth curves for PA103-related strains

Plot showing the OD measured at 600nm (OD$_{600nm}$) over time for PA103 wildtype strain and its derived deletion mutant. At each time point, average OD of nine wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

*P. aeruginosa strains tested:*

- **= PA103 wildtype**
- **= PA103 without tRNA$_{Pro21}$ island (PA103ΔPro21, KR977)**
**Figure C-5: Growth curves for KR873-related strains**

Plot showing the OD measured at 600nm (OD$_{600nm}$) over time for KR873 wildtype strain and its derived deletion mutant. At each time point, average OD of six wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

*P. aeruginosa strains tested:*
- Red = KR873 wildtype
- Blue = KR873 without tRNA$^{\text{Ser}11}$ island (KR873ΔSer11, KR1276)
Figure C-6: Growth curves for KR352-related strains

Plot showing the OD measured at 600nm (OD$_{600nm}$) over time for KR352 wildtype strain and its derived deletion mutant. At each time point, average OD of six wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

_P. aeruginosa_ strains tested:

- = KR352 wildtype
Figure C-7: Growth curves for KR822-related strains
Plot showing the OD measured at 600nm (OD$_{600nm}$) over time for KR822 wildtype strain and its derived deletion mutant. At each time point, average OD of six wells ± the standard deviation is shown. For each strain, the solid line represents the average and the dotted lines represent the standard deviation.

*P. aeruginosa* strains tested:
Appendix D

*C. elegans* N2 hightthroughput assay score for *tRNA*-associated genomic island deletion mutants and their isogenic wildtype strains

<table>
<thead>
<tr>
<th>Wildtype strain/ Deletion mutant</th>
<th>Wildtype HTA score</th>
<th>ΔPro21 HTA score</th>
<th>Difference Mutant – WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>K6/KR504</td>
<td>0.1</td>
<td>0.4</td>
<td>+0.3</td>
</tr>
<tr>
<td>LES431/KR969</td>
<td>2.8</td>
<td>2.3</td>
<td>-0.5</td>
</tr>
<tr>
<td>MID9245/KR976</td>
<td>5.0</td>
<td>4.0</td>
<td>-1.0</td>
</tr>
<tr>
<td>PA103/KR977</td>
<td>5.0</td>
<td>6.0</td>
<td>+1.0</td>
</tr>
<tr>
<td>PA14/KR1034</td>
<td>0.1</td>
<td>1.9</td>
<td>+1.8</td>
</tr>
<tr>
<td>PA14/KR1035</td>
<td>0.1</td>
<td>1.5</td>
<td>+1.4</td>
</tr>
<tr>
<td>PA2192/KR1229</td>
<td>1.8</td>
<td>1.3</td>
<td>-0.5</td>
</tr>
<tr>
<td>RW41/KR974</td>
<td>6.0</td>
<td>5.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>KR222/KR1248</td>
<td>6.0</td>
<td>5.5</td>
<td>-0.5</td>
</tr>
<tr>
<td>KR224/KR1165</td>
<td>1.0</td>
<td>0.6</td>
<td>-0.4</td>
</tr>
<tr>
<td>KR373/KR1281</td>
<td>2.6</td>
<td>2.6</td>
<td>0.0</td>
</tr>
<tr>
<td>KR378/KR1114</td>
<td>0.5</td>
<td>0.9</td>
<td>+0.4</td>
</tr>
<tr>
<td>KR378/KR1235</td>
<td>0.3</td>
<td>0.4</td>
<td>-0.1</td>
</tr>
<tr>
<td>KR822/KR1164</td>
<td>0.3</td>
<td>0.4</td>
<td>+0.1</td>
</tr>
<tr>
<td>KR836/KR1022</td>
<td>0.0</td>
<td>0.3</td>
<td>-0.3</td>
</tr>
<tr>
<td>KR842/KR1163</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>KR877/KR1228</td>
<td>2.8</td>
<td>4.0</td>
<td>+1.2</td>
</tr>
</tbody>
</table>

**Table D-1: tRNA<sup>Pro21</sup>-GI deletion mutants vs. wildtype**

Hightthroughput assay (HTA) score assigned to *P. aeruginosa* wildtype strains and isogenic mutants that underwent deletion of *tRNA<sup>Pro21</sup>* genomic island through homologous recombination. The last column reports the score difference for each pair calculated by subtracting the score of the wildtype from the score of the mutant (HTA mutant – HTA wildtype); a positive difference was indicative of mutant strain less virulent than wildtype.
### Table D-2: \( tRNA^{\text{Gly}19}\)-GI deletion mutants vs. wildtype

Highthroughput assay (HTA) score assigned to \( P. \ aeruginosa \) wildtype strains and isogenic mutants that underwent deletion of \( tRNA^{\text{Gly}19} \) genomic island through homologous recombination. The last column reports the score difference for each pair calculated by subtracting the score of the wildtype from the score of the mutant (HTA mutant – HTA wildtype); a positive difference was indicative of mutant strain less virulent than wildtype.

<table>
<thead>
<tr>
<th>Wildtype strain/ Deletion mutant</th>
<th>Wildtype HTA score</th>
<th>( \Delta \text{Gly19} ) HTA score</th>
<th>Difference Mutant – WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>LES431/KR1243</td>
<td>1.6</td>
<td>3.4</td>
<td>+1.8</td>
</tr>
<tr>
<td>KR352/KR1236</td>
<td>3.3</td>
<td>2.5</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

### Table D-3: \( tRNA^{\text{Ser}11}\)-GI deletion mutants vs. wildtype

Highthroughput assay (HTA) score assigned to \( P. \ aeruginosa \) wildtype strains and isogenic mutants that underwent deletion of \( tRNA^{\text{Ser}11} \) genomic island through homologous recombination. The last column reports the score difference for each pair calculated by subtracting the score of the wildtype from the score of the mutant (HTA mutant – HTA wildtype); a positive difference was indicative of mutant strain less virulent than wildtype.

<table>
<thead>
<tr>
<th>Wildtype strain/ Deletion mutant</th>
<th>Wildtype HTA score</th>
<th>( \Delta \text{Ser11} ) HTA score</th>
<th>Difference Mutant – WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR352/KR1274</td>
<td>3.3</td>
<td>2.9</td>
<td>-0.4</td>
</tr>
<tr>
<td>KR873/KR1276</td>
<td>2.3</td>
<td>4.5</td>
<td>+2.2</td>
</tr>
<tr>
<td>KR877/KR1278</td>
<td>2.9</td>
<td>4.6</td>
<td>+1.7</td>
</tr>
</tbody>
</table>
Appendix E

Contingency tables and $\chi^2$ tests

Analysis of categorical variables was performed using the CrossTab option in SPSS.

<table>
<thead>
<tr>
<th>Pigment production by <em>P. aeruginosa</em> strains</th>
<th>Isolation source</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Sputum</td>
</tr>
<tr>
<td>Count</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Expected Count</td>
<td>21.0</td>
<td>23.0</td>
</tr>
<tr>
<td>% within pigmentation by <em>P. aeruginosa</em> strain</td>
<td>31.8%</td>
<td>68.2%</td>
</tr>
<tr>
<td>% within <em>P. aeruginosa</em> isolation source</td>
<td>25.5%</td>
<td>50.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>12.2%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>-1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presence of pigment either in King’s broth or on NGM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Expected Count</td>
<td>22.0</td>
<td>24.0</td>
</tr>
<tr>
<td>% within pigmentation by <em>P. aeruginosa</em> strain</td>
<td>58.7%</td>
<td>41.3%</td>
</tr>
<tr>
<td>% within <em>P. aeruginosa</em> isolation source</td>
<td>49.1%</td>
<td>31.7%</td>
</tr>
<tr>
<td>% of Total</td>
<td>23.5%</td>
<td>16.5%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>1.1</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presence of pigment both in King’s broth and on NGM</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Expected Count</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>% within pigmentation by <em>P. aeruginosa</em> strain</td>
<td>56.0%</td>
<td>44.0%</td>
</tr>
<tr>
<td>% within <em>P. aeruginosa</em> isolation source</td>
<td>25.5%</td>
<td>18.3%</td>
</tr>
<tr>
<td>% of Total</td>
<td>12.2%</td>
<td>9.6%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>0.6</td>
<td>-0.6</td>
</tr>
</tbody>
</table>

| Total | | |
| Count | 55               | 60    | 115   |
| Expected Count | 55.0               | 60.0  | 115.0 |
| % within pigmentation by *P. aeruginosa* strain      | 47.8%            | 52.2% | 100.0%|
| % within *P. aeruginosa* isolation source            | 100.0%           | 100.0%| 100.0%|
| % of Total                                           | 47.8%            | 52.2% | 100.0%|
| Standard Residual                                    | 0.6              | -0.6  |       |

Table E-1: Production of pigment during HTA vs. source of isolation of *P. aeruginosa* clinical isolates

The production of pigment in King’s B broth and on NGM for *P. aeruginosa* clinical isolates was recorded during highthroughput assay and tabulated against the source of isolation of the strains. $\chi^2$ test $P$-value below 0.05 indicates that this distribution is significantly different from what expected by chance. Standard residual values indicate over-representation of pigment-negative strains among sputum-derived isolates and under-representation among blood-derived strains.

Pearson $\chi^2$ test= 7.366; degree of freedom= 2; $P$-value (exact, 2-sided)= 0.027
Cramer’s $V$= 0.253 (intermediate effect); $P$-value (exact)= 0.027
### Table E-2: Production of pigment vs. presence of exoU in *P. aeruginosa* clinical isolates

The production of pigment in King’s B broth and on NGM for *P. aeruginosa* clinical isolates was recorded during high-throughput assay and tabulated against the presence of exoU assessed by PCR assay. \( \chi^2 \) test \( P \)-value below 0.05 indicates that this distribution is significantly different from what expected by chance. Standard residual values indicate that strains producing pigments in both media are over-represented among exoU-positive strains.

Pearson \( \chi^2 \) test = 8.994; degree of freedom = 2; \( P \)-value (exact, 2-sided) = 0.012

Cramer’s \( V \) = 0.289 (intermediate effect); \( P \)-value (exact) = 0.012

<table>
<thead>
<tr>
<th>Pigment production by <em>P. aeruginosa</em> strains</th>
<th>Presence of exoU</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Count</strong></td>
<td>32</td>
<td>7</td>
</tr>
<tr>
<td><strong>Expected Count</strong></td>
<td>30.0</td>
<td>9.0</td>
</tr>
<tr>
<td>% within pigment production by <em>P. aeruginosa</em> strain</td>
<td>82.1%</td>
<td>17.9%</td>
</tr>
<tr>
<td>% within presence of exoU</td>
<td>38.6%</td>
<td>28.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>29.6%</td>
<td>6.5%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>0.4</td>
<td>-0.7</td>
</tr>
<tr>
<td><strong>Count</strong></td>
<td>38</td>
<td>7</td>
</tr>
<tr>
<td><strong>Expected Count</strong></td>
<td>34.6</td>
<td>10.4</td>
</tr>
<tr>
<td>% within pigment production by <em>P. aeruginosa</em> strain</td>
<td>84.4%</td>
<td>15.6%</td>
</tr>
<tr>
<td>% within presence of exoU</td>
<td>45.8%</td>
<td>28.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>35.2%</td>
<td>6.5%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>0.6</td>
<td>-1.1</td>
</tr>
<tr>
<td><strong>Count</strong></td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td><strong>Expected Count</strong></td>
<td>18.4</td>
<td>5.6</td>
</tr>
<tr>
<td>% within pigment production by <em>P. aeruginosa</em> strain</td>
<td>54.2%</td>
<td>45.8%</td>
</tr>
<tr>
<td>% within presence of exoU</td>
<td>15.7%</td>
<td>44.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>12.0%</td>
<td>10.2%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>-1.3</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Count</strong></td>
<td>83</td>
<td>25</td>
</tr>
<tr>
<td><strong>Expected Count</strong></td>
<td>83.0</td>
<td>25.0</td>
</tr>
<tr>
<td>% within pigment production by <em>P. aeruginosa</em> strain</td>
<td>76.9%</td>
<td>23.1%</td>
</tr>
<tr>
<td>% within Presence of exoU</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>76.9%</td>
<td>23.1%</td>
</tr>
</tbody>
</table>
Table E-3: Source of isolation vs. presence of exoU and/or exoS in *P. aeruginosa* clinical isolates

The origin of *P. aeruginosa* clinical isolates from blood-culture or sputum-culture was tabulated against the presence of exoU and exoS assessed by PCR assay. $\chi^2$ test P-value above 0.05 indicates that this distribution is not significantly different from what expected by chance.

Pearson $\chi^2$ test = 3.285; degree of freedom = 3; P-value (exact, 2-sided) = 0.369
<table>
<thead>
<tr>
<th>P. aeruginosa isolation source</th>
<th>Presence of exoY</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>8</td>
<td>61</td>
</tr>
<tr>
<td>Expected Count</td>
<td>7.4</td>
<td>61.6</td>
</tr>
<tr>
<td>% within P. aeruginosa isolation source</td>
<td>11.6%</td>
<td>88.4%</td>
</tr>
<tr>
<td>% within presence of exoY gene</td>
<td>57.1%</td>
<td>52.1%</td>
</tr>
<tr>
<td>% of Total</td>
<td>6.1%</td>
<td>46.6%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>0.2</td>
<td>-0.1</td>
</tr>
<tr>
<td>Sputum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>6</td>
<td>56</td>
</tr>
<tr>
<td>Expected Count</td>
<td>6.6</td>
<td>55.4</td>
</tr>
<tr>
<td>% within P. aeruginosa isolation source</td>
<td>9.7%</td>
<td>90.3%</td>
</tr>
<tr>
<td>% within presence of exoY gene</td>
<td>42.9%</td>
<td>57.1%</td>
</tr>
<tr>
<td>% of Total</td>
<td>4.6%</td>
<td>42.7%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>-0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>117</td>
</tr>
<tr>
<td>Expected Count</td>
<td>14.0</td>
<td>117.0</td>
</tr>
<tr>
<td>% within P. aeruginosa isolation source</td>
<td>10.7%</td>
<td>89.3%</td>
</tr>
<tr>
<td>% within presence of exoY gene</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>% of Total</td>
<td>10.7%</td>
<td>89.3%</td>
</tr>
</tbody>
</table>

Table E-4: Source of isolation vs. presence of exoY in P. aeruginosa clinical isolates

The origin of P. aeruginosa clinical isolates from blood-culture or sputum-culture was tabulated against the presence of exoY assessed by PCR assay. $\chi^2$ test $P$-value above 0.05 indicates that this distribution is not significantly different from what expected by chance.

Pearson $\chi^2$ test= 0.126; degree of freedom= 1; $P$-value (exact, 2-sided)= 0.783
P. aeruginosa strain colour when grown in King’s broth

<table>
<thead>
<tr>
<th>Pigment-negative Count</th>
<th>P. aeruginosa strain colour when grown on NGM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pigment-negative</td>
<td>Pigment-positive</td>
</tr>
<tr>
<td>Expected Count</td>
<td>35.6</td>
<td>51.4</td>
</tr>
<tr>
<td>% within P. aeruginosa strain colour when grown in King’s broth</td>
<td>50.6%</td>
<td>49.4%</td>
</tr>
<tr>
<td>% within P. aeruginosa strain colour when grown on NGM</td>
<td>93.6%</td>
<td>63.2%</td>
</tr>
<tr>
<td>% of Total</td>
<td>38.3%</td>
<td>37.4%</td>
</tr>
<tr>
<td>Standard Residual</td>
<td>1.4</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

| Pigment-positive Count | 3 | 25 | 28 |
| Expected Count         | 11.4 | 16.6 | 28.0 |
| % within P. aeruginosa strain colour when grown in King’s broth | 10.7% | 89.3% | 100.0% |
| % within P. aeruginosa strain colour when grown on NGM | 6.4% | 36.8% | 24.3% |
| % of Total             | 2.6% | 21.7% | 24.3% |
| Standard Residual      | -2.5 | 2.1 | |

| Total Count | 47 | 68 | 115 |
| Expected Count | 47.0 | 68.0 | 115.0 |
| % within P. aeruginosa strain colour when grown in King’s broth | 40.9% | 59.1% | 100.0% |
| % within P. aeruginosa strain colour when grown on NGM | 100.0% | 100.0% | 100.0% |
| % of Total | 40.9% | 59.1% | 100.0% |

Table E-5: Production of pigment in King’s broth vs. production of pigment on NGM for P. aeruginosa clinical isolates

The production of pigment for P. aeruginosa clinical isolates was recorded when grown in King’s B broth and tabulated against the presence of pigment when grown on NGM during high throughput assay. $\chi^2$ test P-value below 0.05 indicates that this distribution is significantly different from what expected by chance. Standard residual values indicate that strains producing pigment in King’s broth were over-represented among strains producing pigment on NGM medium and under-represented among strains that did not produce pigment on NGM.

Pearson $\chi^2$ test=13.927; degree of freedom= 1; P-value (exact, 2-sided)< 0.001

Phi= 0.348 (intermediate effect); P-value< 0.001
Table E-6: Production of pigment vs. presence of *exoY* in *P. aeruginosa* clinical isolates

The production of pigment in King’s broth and on NGM for *P. aeruginosa* clinical isolates during high-throughput assay and tabulated against the presence of *exoY* assessed by PCR assay. $\chi^2$ test $P$-value above 0.05 indicates that this distribution is not significantly different from what expected by chance.

$\chi^2$ test $= 3.946$; degree of freedom $= 2$; $P$-value (exact, 2-sided) $= 0.128$
Appendix F

To elucidate *A. castellanii* film grazing on *P. aeruginosa*

Expansion of *A. castellanii* film is facilitated when *P. aeruginosa* PA14 was plated at higher density

The classic non-nutrient agar assay is done by adjusting the bacterial strains OD$_{600nm}$ to 0.1. In order to establish whether bacterial density may affect the ability of *A. castellanii* to expand on bacterial-layered plates, cultures from PA14 or PAO1 single colonies were adjusted to OD$_{600nm}$= 0.1 and compared to conditions in which bacterial cell were more spares (serially diluted) or more crowded (concentrated).
Figure F-1: *A. castellanii* film expansion on *P. aeruginosa* plated at different densities

Plot showing *A. castellanii* film expansion on *P. aeruginosa* PAO1 and PA14 strains layered on NNA plates at different densities. Three colonies from each strain were plated on TSA and OD$_{600}$ = 0.1 prepared. OD = 0.01 and OD = 0.001 were prepared by serially diluting the original bacterial suspension. OD = 1 was prepared by collecting the bacteria by centrifugation and resuspending the pellet in 1/10th of the original volume. Bacterial suspensions were prepared for each colony and spread on NNA plates.

Dilution or concentration of PAO1 did not seem to affect the *A. castellanii* film expansion while concentration but not dilution of PA14 affected *A. castellanii* film expansion. In fact, at OD = 1, the difference between expansion on PAO1 and PA14 was minimized.
Expansion of *A. castellanii* film is bacterial growth conditions-dependent and strain-dependent.

**Figure F-2: *A. castellanii* film expansion on *P. aeruginosa* grown on different media**

Plot showing *A. castellanii* film expansion on *P. aeruginosa* PAO1 and PA14 strains grown on different media previous of layering on NNA plates. Three colonies from each strains were chosen and plated on TSA, LA or M9 (minimal medium) agar. Bacterial suspension was prepared for each colony from each growth condition as explained in Material and Methods and layered on NNA plates.

Expansion on PAO1 previously grown on M9 appeared to be hindered compared to expansion on PAO1 grown on LA or TSA. The difference was greater when PA14 was used. In fact, the expansion of *A. castellanii* on PA14 grown on LA was slower than on PA14 grown on TSA while the amoebas were unable to expand on PA14 previously grown on M9.
Expansion of *A. castellanii* film is not affected by type three secretion system effectors.

**Figure F-3: A. castellanii expansion on PA14 mutants lacking T3SS effector genes**

Plot showing *A. castellanii* film expansion on *P. aeruginosa* PA14 WT and derived mutant strains lacking *exoU*, *exoT* or *exoY* and all three effector genes. Three colonies for each strain were chosen and plated on NNA plates.

Expansion of *A. castellanii* on PA14 mutants lacking T3SS effector genes was not different from the expansion on PA14 wildtype.
Appendix G

To elucidate *A. polyphaga* film grazing on *P. aeruginosa* strains

Expansion of *A. polyphaga* film is not affected by plating *P. aeruginosa* strains at different densities

The classic non-nutrient agar assay is done by adjusting the bacterial strains OD$_{600nm}$ to 0.1. In order to establish whether bacterial density may affect the ability of *A. polyphaga* to expand on bacterial-layered plates, cultures from PA14 or PAO1 single colonies were adjusted to OD$_{600nm}= 0.1$ and compared to conditions in which bacterial cell were more spares (serially diluted) or more crowded (concentrated).
Figure G-1: *A. polyphaga* film expansion on *P. aeruginosa* plated at different densities

Plot showing *A. polyphaga* film expansion on *P. aeruginosa* PAO1 and PA14 strains layered on NNA plates at different densities. Three colonies from each strain were plated on TSA and OD$_{600}$ = 0.1 prepared. OD = 0.01 and OD = 0.001 were prepared by serially diluting the original bacterial suspension. OD = 1 was prepared by collecting the bacteria by centrifugation and resuspending the pellet in 1/10$^{th}$ of the original volume. Bacterial suspensions were prepared for each colony and spread on NNA plates.

Dilution or concentration of either PAO1 or PA14 did not seem to affect the *A. polyphaga* film expansion. The difference between PAO1 and PA14 was also minimized.
Expansion of *A. polyphaga* film is independent from bacterial growth conditions.

**Figure G-2: A. polyphaga film expansion on *P. aeruginosa* grown on different media**

Plot showing *A. polyphaga* film expansion on *P. aeruginosa* PAO1 and PA14 strains grown on different media previous of layering on NNA plates. Three colonies from each strains were chosen and plated on TSA, LA or M9 (minimal medium) agar. Bacterial suspension was prepared for each colony from each growth condition as explained in Material and Methods and layered on NNA plates.

No difference between bacterial growth conditions was detected.
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