Phenotypic variation in *Photorhabdus temperata* K122

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For the degree of Doctor of Philosophy

National University of Ireland, Maynooth

Department of Biology

2011

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Acknowledgements

This has been an epic trip. Along the way, I have had the privilege of meeting, consulting, working and socialising with many astounding people who have contributed to the development of not only this thesis, but also myself as a person.

The journey began in NUIM and I'd firstly like to thank Dr. Barbara Dowds under whose direction the journey began, as well as the members of the Molecular genetics laboratory at that time – Dr. Antonia Volgyi and Dr. Declan Roche. Moving to the University of Bath, I would sincerely like to thank Dr. David Clarke and Dr. Susan Joyce, both of whom have provided guidance, advice and encouragement following Barbara's retirement and without whom none of this would have been possible. The members of the Clarke lab at that time also deserve a huge thank you – Jane, Rob, Helen, Yahui and Lionel – it was nice to be able to enjoy a 'quiet drink' with you all! Thanks also to Rob Rees for the Celtic camaraderie and a special mention for my co-commuter Liz who helped me keep it real! Back in NUIM, I'd like to thank Prof. Ann Burnell who has always had a kind word and facilitated me wherever possible. I'd also like to thank Prof. Kay Ohlendieck for enabling the completion of this work. To Gemma and the rest of the technical staff, much appreciation and thanks for making my time working there enjoyable and memorable!

My family also deserve thanks for their continued support – My parents and my brother Seán who have helped me through thick and thin and dealt with the best and the worst of me. Erina, you've been amazing with the patience you've shown and the support you've given me, I couldn't have done it without you. Finally, I'd like to thank my son Darragh for showing me how to climb mountains again.

Declaration

This thesis has not been submitted, in whole or part, to this or any other University for any degree and is, except where otherwise stated, the original work of the author.

Signed ____________________

Hilton D. McWeeney.

List of abbreviations

AA	Amino acid
Amp	Ampicillin
AMP	Anti microbial peptides
ATCC	American Tissue Culture Collection
AQ	Anthraquinone
Bp	Base pairs
^0C	Degrees Celsius
CFU	Colony forming units
Cm	Chloramphenicol
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
C-terminus	Carboxy-terminus
dH_2O	Distilled Water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
Fig.	Figure
g	Gram
g	G-force (Relative centrifugal field)
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IJ	Infective juvenile

Km	Kanamycin
kb	Kilobase
kDa	Kilo Dalton
L	Litre
LB	Luria Bertani media
LPS	Lipopolysaccharide
M	Molar
mA	Milliamps
MCA	McConkey agar
mg	Milligrams
min.	Minute
ml	Millilitre
mM	Millimolar
MOPS	3-(n-Morpholino)propanesulphonic acid
mRNA	Messenger RNA
MW	Molecular weight
µg	Microgram
µl	Microlitre
µM	Micromolar
NBTA	Nutrient agar supplemented with TTC and bromothymol blue
NCBI	National Center for Biotechnology Information
ng	Nanogram
N-terminus	Amino-terminus
OD	Optical Density
Ω	Resistance (Ohms)
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	$-\log_{10}[H^+]$
rDNA	Recombinant DNA
RFLP	Restriction fragment polymorphism
Rif	Rifampicin
Rif^R	Rifampicin resistant
RLU	Relative light unit
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
spp.	Species
SSC	Sodium chloride sodium citrate
ST	3-5-dihydroxy-4-isopropylstilbene
Subsp.	Subspecies
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
TEMED	N, N, N', N'-tetramethyl ethylene diamine
Tn	Transposon
Tris	Tris(hydroxymethyl)aminomethane
TTC	Triphenyltetrazolium chloride
U	Unit
UV	Ultraviolet
V	Volts
v/v	Volume/volume
w/v	Weight/volume

Abstract

Photorhabdus spp. are Gram negative enterobacteria which have a life cycle involving mutualism with a nematode partner and pathogenicity towards insects. *Photorhabdus* reside as intestinal symbionts of the infective juvenile (IJ) stage of entomopathogenic nematodes of the *Heterorhabditis* family. The bacteria and its nematode partner together form an insect killing (entomopathogenic) complex that kills the soil dwelling larval stages of a wide variety of insect species. *Photorhabdus* spp. have the ability to exist in either of two phenotypically different forms. The bacterial form isolated from the nematode is referred to as the primary phenotype variant, whilst the phenotypically different form is referred to as secondary phenotype variant. This secondary variant is isolated after prolonged stationary phase growth of the primary variant. The secondary variant does not support mutualism with the nematode partner and does not produce pigment, bioluminescence, extracellular enzymes, antibiotics and displays a different colony morphology to the primary variant. Study of the phenotypic switch undergone by *Photorhabdus* has identified two signalling pathways which play a role in the regulation of phenotypic variation - the LysR-type transcriptional regulator HexA and the 'adaptation to stationary phase' AstR-AstS two component signal transduction system. A putative DNA binding protein, Ner, has also been shown to influence the phenotypic switch when overexpressed. This study has confirmed the role of HexA in the regulation of symbiosis, virulence and phenotypic variation in *P. temperata* K122 and furthermore, has identified a role for HexA in regulating the small RNA global regulator *csrB*. Other genes, including *hipB* (part of a toxin/antitoxin system) and *sdiA* a LuxR type regulator have also been identified as having potential roles in phenotypic variation. A broad range of other genes that influence phenotypic variation either directly or indirectly have also been identified, providing further insight into the reasons for the occurrence of phenotypic variation in *P. temperata* K122.

Chapter 1

1. Phenotypic variation in *Photorhabdus temperata* K122

1.1 General Introduction

Photorhabdus spp. are Gram negative bacteria of the family *Enterobacteriaceae* found predominantly in a symbiotic association with nematodes of the *Heterorhabditis* family (Forst & Clarke 2002). One group of *Photorhabdus* strains have been isolated from human wounds (Farmer *et al.,* 1989). The bacteria and nematode together form an insect killing (entomopathogenic) complex which infects and kills the soil dwelling larval stages of a wide variety of insect species. The closely related genus, *Xenorhabdus*, which is symbiotic with the *Steinernema* family of nematodes, also forms similar insect-killing complexes (Dowds and Peters 2002).

1.1.1 Taxonomy

Originally, *Photorhabdus* spp. isolates were classified as *Xenorhabdus luminescens* (Thomas and Poinar 1979). These isolates were separated from other *Xenorhabdus* species on the basis of fatty acid patterns (Janse and Smits, 1990). This distinctness was confirmed and a new genus was then described – *Photorhabdus* – incorporating these previously identified bacteria on the basis of phenotypic characteristics and DNA relatedness (Boemare *et al*., 1993).

Within this genus a single species, *Photorhabdus luminescens* was initially defined, however DNA-DNA hybridisation (Akhurst *et al*., 1996; Farmer *et al*., 1989), 16S rDNA sequencing (Liu *et al*., 1997; Rainey *et al*., 1995 and Szállás *et al*., 1997) and PCR ribotyping (Brunel *et al*., 1997) have shown the *Photorhabdus* genus to be a heterogeneous group of organisms. Restriction fragment length polymorphism analysis (RFLP) of PCR-amplified 16S rDNA genes on 92 *Photorhabdus* isolates, from a variety of locations worldwide, differentiated twelve 16S RFLP types (Fischer-

Le Saux *et al.*, 1998). Most recently, a polyphasic approach to classification has been applied to these 16S rDNA RFLP groups. Incorporating DNA relatedness along with ΔT_m measurements (difference between the T_m of homologous and heterologous duplexes), morphological, biochemical and physiological phenotypic tests and complete 16S rRNA gene sequencing, the polyphasic approach is the most reliable method for distinguishing species. This approach proposed three subspecies of *P. luminescens*: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov. and *P. luminescens* subsp. *laumondii* subsp. nov.
Two new species were also proposed, *Photorhabdus asymbiotica* (isolated from human wounds) and *Photorhabdus temperata* – incorporating the subspecies *P. temperata* subsp. *temperata* subsp. nov. (Fischer-Le Saux *et al*., 1999).

Photorhabdus and *Xenorhabdus* have been assigned to the gamma group of Proteobacteria (Purple Bacteria) (Fischer-Le Saux *et al.,* 1999). Whether this was part of the family *Enterobacteriaceae* or as their own unconnected family had been questioned as a result of their comparative fatty acid patterns (Rainey *et al.*, 1995), and as a result of their inability to reduce nitrate (Janse and Smits, 1990). Other unique features not found in other *Enterobacteria* include *Xenorhabdus* being catalase negative and *Photorhabdus'* bioluminescence and pigment production. 16S rRNA sequences have however shown that both *Xenorhabdus* and *Photorhabdus* branch well within the *Enterobacteriaceae*, with *Photorhabdus* seeming to have evolved subsequent to the radiation of *Xenorhabdus* (Forst *et al*., 1997, Marokhazi *et al*., 2003).

The genera *Photorhabdus* and *Xenorhabdus* differ significantly despite having similar life cycles and being closely related in the same phylum. As mentioned, both form symbiotic relationships with separate families of nematodes and both differ in certain biochemical traits. Allied to this, many traits that are superficially shared by both bacteria and appear similar at phenotypic level (e.g. antibiotic, protease, lipase and pigment production) have been found to be quite distinct at the molecular level (Forst and Nealson, 1996) indicating independent acquisition/development and that, potentially, parallel evolution has occurred within these lineages. An example of such a difference is seen during insect infection, where the bacteria must survive the insect immune response, including exposure to bacterial membrane targeting antimicrobial

peptides (AMPs) produced by the insect humoral response (Nappi & Ottaviani, 2000). *Photorhabdus* appears to counteract the activity of such AMPs by modification of its lipopolysaccharide, whist the mechanism employed by *Xenorhabdus* appears to be one of suppression of AMP expression (Goodrich-Blair & Clarke, 2007). The different mechanisms employed by the two related species to achieve similar outcomes provides a useful tool for the comparative analysis of gene function and may also provide insight into the evolution of mutualism and pathogenicity.

1.1.2 Life Cycle

Photorhabdus spp. reside as intestinal symbionts of the infective juvenile (IJ) stage of entomopathogenic nematodes of the *Heterorhabditis* family (Fig. 1.1). Starting and ending with the free-living, non-feeding, soil-dwelling IJ form of the nematode , the cycle is described as consisting of three stages (I-III) according to development of the bacteria and nematode (Forst & Clarke, 2002). In stage I, the free-living IJs seek out and physically invade insect hosts. Once the IJs enter the digestive tract of the larval stage of a suitable insect they enter the haemocoel (the insect blood system). Entrance to the haemocoel can occur via the intestine, respiratory spiracles, anus or by direct penetration through the insect cuticle (Akhurst and Dunphy, 1993; Poinar, 1990). When in the haemocoel, the bacteria are released from the nematode gut into the haemolymph (insect blood) in response to an unidentified compound found in the haemolymph (Ciche and Ensign, 2003). The entire complement of intestinal bacterial symbionts is released by the nematode which has been hypothesized to occur to ensure selection of fit, virulent symbiotic bacteria (Ciche *et al*., 2008). Once released, they replicate rapidly (Daborn *et al*, 2001). Bacteria and nematode together rapidly kill the insect larva (Akhurst and Dunphy, 1993) although the bacteria alone have been shown to be cause insect death both by artificial introduction into the insect body (injection) (Bowen & Ensign, 1998; Götz *et al.,* 1981) or by simulated natural uptake (ingestion) (Rajagopal *et al.*, 2006).

Stage II begins as the bacteria grow to stationary phase within the insect cadaver, producing enzymes and toxins which result in the bioconversion of the insect cadaver

and the nematodes develop and begin to sexually reproduce, using the bacteria as a food source. The IJs recover to a hermaphrodite stage in response to haemolymph food signals (Strauch & Ehlers, 1998; Han & Ehlers 2000) and lay eggs, from which males and females arise and begin to reproduce sexually, resulting in the development of adult hermaphrodite nematodes which undergo *endotokia matricida* (intrauterine egg hatching and use of the mother as a food source) leading to Stage III – the development of bacterial symbiont carrying IJs (Ciche *et al*., 2008) which then leave the insect cadaver in search of a new host. *Endotokia matricida* is utilised by *H. bacteriophora* nematodes for symbiont transmission. The bacteria infect the adult hermaphrodite and invade specific cells in the intestinal tract – rectal gland cells (RGCs). As the IJs develop, the RGCs of the maternal hermaphrodite rupture, releasing *Photorhabdus* into the body cavity of the nematode, where each developing IJ is colonized by a single bacterial cell. The bacteria the replicate inside the gut lumen of the IJ to give a clonal symbiotic bacterial population (Ciche *et al*., 2008).

Stationary phase *Photorhabdus* secrete many extracellular products including degradative enzymes that break down the insect cadaver, enabling its use as a food source by the developing nematodes. *Photorhabdus* also secrete various antimicrobial compounds into the insect cadaver to prevent competition from non-symbiotic bacteria and colonisation of the insect cadaver by saprophytic soil organisms (Eleftherianos, 2009). Studies indicate nematode reproduction is optimal when the symbiotic bacteria dominate the microbial flora. This suggests a role for the bacteria as a food source and also as a provider of essential nutrients that are required for nematode development (Akhurst and Dunphy, 1993), indeed the nematode cannot develop/reproduce in the absence of *Photorhabdus* (Akhurst *et al*., 1996; Ehlers *et al*., 1998; Ehlers *et al*., 1990; Gerritsen & Smits 1993).

Heterorhabditis nematode life cycle.

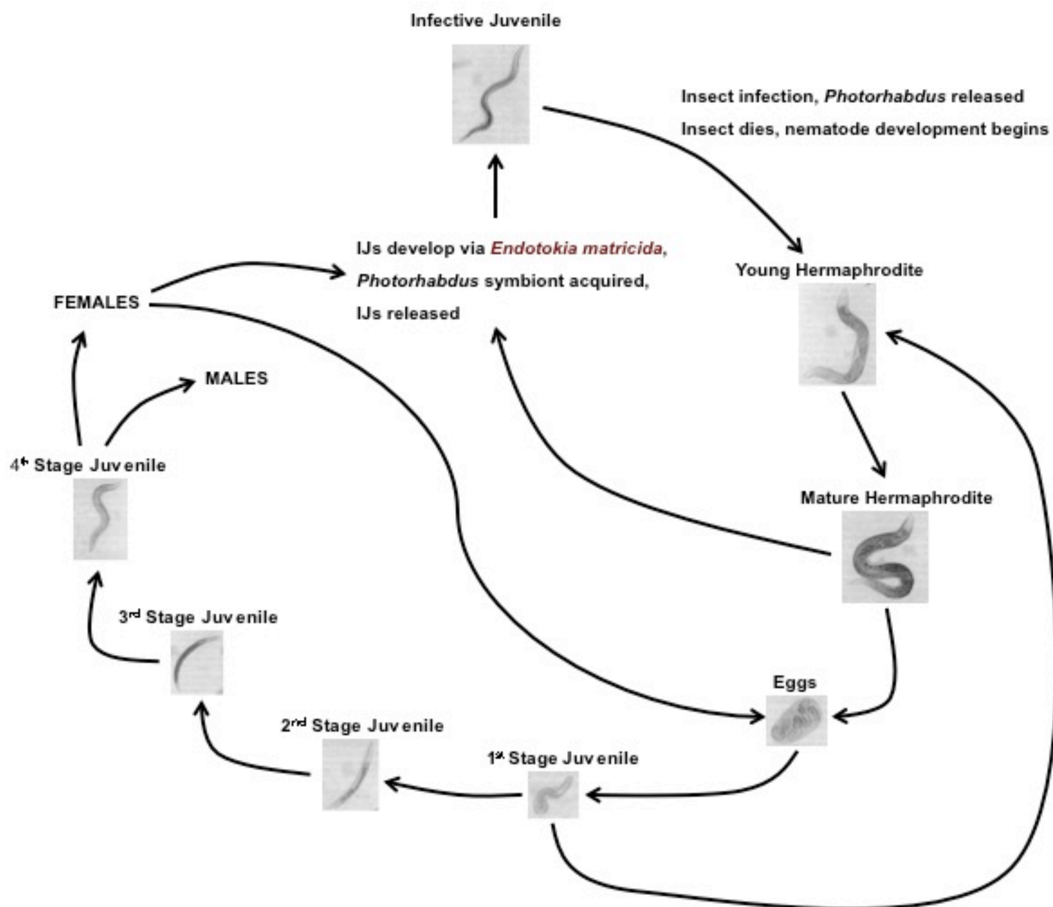


Fig. 1.1, Development of the *Heterorhabditis* nematode in conjunction with *Photorhabdus*. Infective Juveniles (IJs) develop exclusively from intrauterine egg-laying (*Endotokia matricida*) (Ciche *et al.,* 2008). During nematode development, *Photorhabdus* and insect breakdown products are used as food source.

1.1.3 Symbiosis and Pathogenicity – Linked or Distinct?

Interactions of a pathogenic nature invariably cause damage or death of the host organism whilst symbiotic interactions are generally considered to be beneficial. Despite the widely varying outcomes of these organism-host interactions, symbiosis and pathogenesis share similar molecular mechanisms, with both facilitating colonisation of a particular host niche (Hentschel *et al*., 2000).

Pathogenicity can generally be described as the ability to cause disease and the genes responsible are termed "virulence factors" (Hentschel *et al,*. 2000). As a result of their action, colonisation factor expression, the release of toxins, and the manipulation/usurping of host cell functions results in host cell damage or death.

The term "symbiosis factor" (Hentschel *et al.,* 2000) has been utilised to describe features of a bacterium that lead to a beneficial interaction with a host organism. Metabolites and enzymes can be classified under this heading. Several symbiosis factors can also be involved or described as pathogenicity factors, e. g. invasion, colonisation and host modulation factors are all required for the onset of a successful symbiosis. In some cases, toxins have also been described as symbiosis factors where there is an overall net benefit after the organism suffers initial damage (Hentschel and Steinart 2001) (Fig. 1.2).

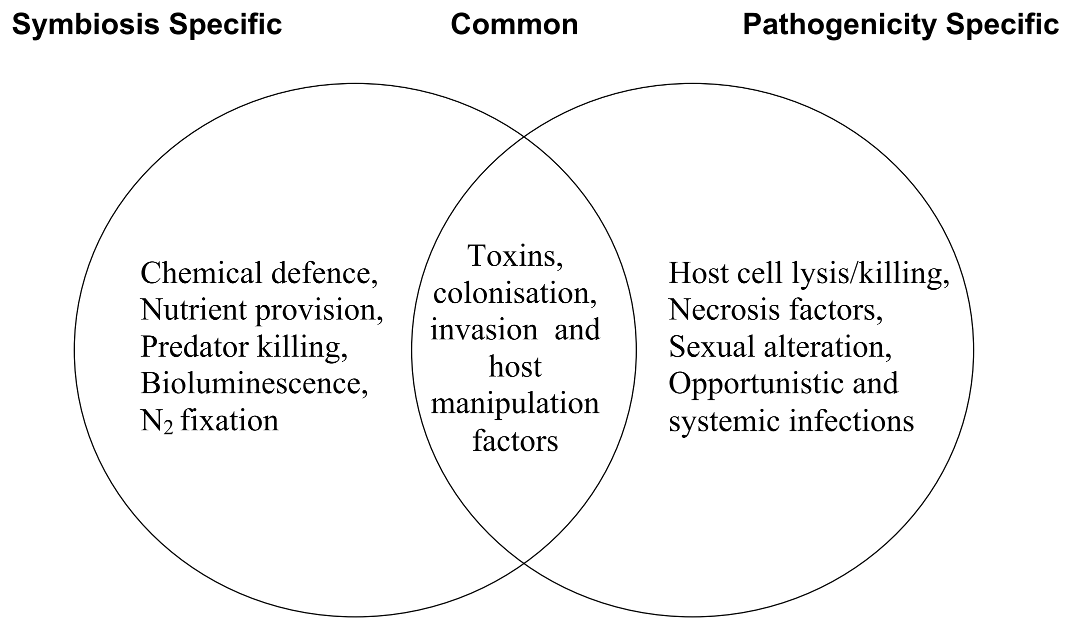


Fig. 1.2 – Examples of the overlap between symbiosis and pathogenicity, adapted from Hentschel *et al*., 2000.

1.1.4 Mutualism/Mutualistic Specificity

Individual *Heterorhabditis* nematode species are specifically associated with only one species of *Photorhabdus*, though the bacteria may interact with more than one species of nematode. It has been suggested that the specificity of the nematode-bacterial interaction is determined by the bacteria's ability to colonize the intestine of the IJ (Akhurst and Boemare, 1990). Nutrient provision by the bacterium has also been implicated (Akhurst and Dunphy, 1993). It is also likely that the bacteria are adapted to withstand the onslaught of the nematode immune system, which remains largely unexplored, but has been somewhat characterised in the non-pathogenic closely related nematode *Caenorhabditis elegans* (Irazoqui *et al*., 2010). Bacterial contamination by other species rarely occurs in live nematodes and is usually limited to the intracuticular spaces of the nematode sheath. Conversely, IJ recovery can be initiated by direct inoculation onto agar plates with high densities of *Photorhabdus* indicating that the nematode responds to an element produced by the bacteria – this has been termed the 'food signal' (Strauch & Ehlers 1998). Part of this food signal may involve the antibiotic 3-5-dihydroxy-4-isopropylstilbene (ST) which has shown to be a necessary requirement for IJ to hermaphrodite recovery *in vitro* (Joyce *et al*., 2008)

Other *Photorhabdus* genes that have been identified as having a role in/being required for the maintenance of a successful mutualistic relationship include a recently identified novel fimbrial locus, *mad* (maternal adhesion defective) which, though not required for insect pathogenicity, is necessary for *Photorhabdus* to develop mutualistically with the nematode - in the absence of Mad fimbriae, *Photorhabdus* fail to adhere to the intestine of the maternal nematode and bacterial transmission to the IJs does not occur (Somvanshi *et al*., 2010). The *mad* locus has also been implicated in the determination of host specificity (Somvanshi *et al*., 2010). A mutation in a gene encoding a phosphopantetheinyl transferase – *ngrA*, involved in the manufacture of siderophores, antibiotics and other bioactive molecules, has also been shown to be unable to support nematode growth and development (Ciche *at al*., 2001). Inactivation of either of the genes *cipA* or *cipB*, encoding the stationary phase crystal protein inclusion bodies of *Photorhabdus* also result in an inability of the

bacteria to support nematode growth and development (Bintrim & Ensign, 1998; Bowen & Ensign, 2001). A requirement for *Photorhabdus* iron uptake has also been demonstrated as a necessary component in establishment of successful mutualism, with a mutation of the *Photorhabdus exbD* gene, resulting in a defect in bacterial iron uptake, being unable to support growth and development of the nematode (Watson *et al*., 2005).

Practically all *Photorhabdus* (and its close relative *Xenorhabdus*) isolates studied to date have been isolated from nematodes of soil origin. *Photorhabdus* can be cultured independently *in vitro* under laboratory conditions on standard media, however axenic *Heterorhabditis* nematodes, where *Photorhabdus* is absent, are unable to complete their life cycle either *in vivo* or *in vitro* (Han and Ehlers, 2000). Given that *Photorhabdus* has been attributed a 0.0004% survival rate after 7 days in soil (Bleakley and Chen 1999), and that free-living forms of the bacteria have yet to be isolated from soil or water sources, evidence suggests the bacteria-nematode interaction *in vivo* may be obligate in the environment (Akhurst, 1993; Kaya and Gaugler, 1993). *Photorhabdus* spp. enable *Heterorhabditis* nematode growth and development whilst the nematodes facilitate *Photorhabdus* growth and survival in the soil environment as well as transportation to a food source and assisting in physical evasion of the insect immune system during entry into the haemolymph.

1.1.5 Pathogenicity

Cumulative evidence indicates the primary virulence events effected by *Photorhabdus* that lead to the death of the insect host involve multiple factors including the secretion of insecticidal toxins and other extracellular products, the release of LPS molecules from the bacterial envelope and the anti-hemocytic properties of the cell surface (Ensign *et al*., 1990; Dunphy & Webster, 1991; Clarke & Dowds, 1995; Dunphy, 1995).

After release of *Photorhabdus* from the nematode into the insect haemocoel, the bacteria encounter the insect immune system which consists of both a cellular and

humoral response (Kanost *et al*., 2004). With the cellular response, *Photorhabdus* encounters phagocytic hemocytes of the insect immune system. These hemocytes try to engulf or encapsulate the bacterial invaders. Whilst avoiding the hemocytes, *Photorhabdus* must also escape the insect peptide-mediated immune response (the humoral response), which acts via the release of several anti-bacterial peptides. (ffrench-Constant *et al.,* 2003). The insect immune responses are a result of direct recognition of foreign (non-self) surface characteristics. Once a 'non-self' particle or organism has been recognised, hemocytes will either phagocytose and destroy the foreign body or encapsulate and isolate it (nodulation) for later destruction (Lavine and Strand 2001). Whilst the absolute specifics of this avoidance of the immune system have yet to be elucidated, some possible mechanisms of avoiding the cellular response have been observed, including re-emergence after encapsulation by hemocytes (Dowds and Peters 2002) and repression of phagocytosis (Van Sambeek and Wiesner 1999; Silva *et al.,* 2002). In support of this, the Tc and Mcf toxins of *Photorhabdus* have been shown to induce apoptosis in insect immune cells (Waterfield *et al*., 2001; Daborn *et al*., 2002, Au *et al*., 2004; Dowling *et al*., 2004; Waterfield *et al*., 2005) indicating an ability to resist nodulation. A type III secretion system (TTSS) also appears to pay a part in insect immune evasion by *Photorhabdus*, with a TTSS producing the LopT effector protein, shown to inhibit phagocytosis (Brugirard-Ricaud *et al*., 2004 & 2005). The ST antibiotic (3-5-dihydroxy-4-isopropylstilbene) has been shown to also have a function in suppression of insect host defenses during *Photorhabdus* infection where it inhibits phenoloxidase activity – a requirement for successful nodulation to occur (Eleftherianos *et al*., 2007).

As mentioned, both aspects of the insect immune response must be circumnavigated by *Photorhabdus* before a successful colonisation and infection can be established. Evidence for the efficacy of the insect humoral response to bacterial infection is provided by the observation by Eleftherianos *et al* (2006) that *Photorhabdus* cannot infect insects with existing high levels of antimicrobial peptides (AMPs) stimulated by pre-immunization with *E. coli*. Clarke (2008) suggests that upon encountering the humoral response, *Photorhabdus* adapts through modification of the outer membrane modulated by the PhoPQ two-component pathway (Derzelle *et al*., 2004b). PhoPQ controls the expression of the *pbgPE* operon, mutatations in which result in attenuated

virulence and sensitivity to the polymixin B antimicrobial peptide (Bennett & Clarke 2005).

Upon successful evasion of the immune system, *Photorhabdus* must adhere to a surface within the host to establish a successful infection (Dowds & Peters 2002). Genes similar to those of the *Yersinia* spp. attachment/invasion locus have been identified in *Photorhabdus* and implicated in this process (ffrench-Constant *et al.,* 2000b). Meslet-Cladiere *et al* (2004) identified a phase variable mannose resistant fimbrial locus (*mrf*) that is expressed by *P. temperata* K122 during insect infection which may also play a role in successful establishment of infection. Whilst motility has been shown not be an absolute requirement for either pathogenicity or symbiosis, it has been shown to confer a competitive advantage during insect colonisation (Easom & Clarke 2008). Once an infection has been established, the bacteria then destroy the insect midgut (Silva *et al.,* 2002). This prevents the insect from feeding and has also been implicated in a loss of body turgor (ffrench-Constant *et al.,* 2003).

Genetic and biochemical studies of *Photorhabdus* have revealed several factors that may play a role in pathogenicity. Many of these factors include toxins, proteases and other similar types of molecule to those listed above. With genomic sequence data increasingly available, the application of rapid virulence annotation (RVA) techniques has lead to the preliminary identification of many *Photorhabdus* genes/loci with potential roles in virulence (Waterfield *et al*., 2008).

1.1.6 Toxins

Analysis of the *Photorhabdus luminescens* genome has resulted in the identification of more putative toxin genes than had been found in any other bacterium sequenced at the time (Duchaud *et al.,* 2003). Insecticidal toxicity in *Photorhabdus* has been shown to be associated with high molecular weight protein complexes secreted directly from the bacterium (Bowen *et al.,* 1998). Three groups of toxins have been well characterized in *Photorhabdus*, with a possible fourth class identified as *Photorhabdus* Virulence Cassettes (PVC) (Yang *et al.,* 2006). It has been suggested

the production of several toxin types by *Photorhabdus* strains may have developed to broaden the possible insect target range (Waterfield *et al*., 2004).

The first class of *Photorhabdus* toxins, the toxin complexes (*tc* toxins) can be orally active (Bowen *et al.,* 1998). First purified from the *Photorhabdus* W14 strain, these toxins are high molecular weight complexes encoded by loci of two distinct genomic organisations in *Photorhabdus* (ffrench-Constant & Bowen 2000) alongside other putative virulence factors (Waterfield *et al.,* 2001). Four toxin complexes, Tca, Tcb, Tcc and Tcd were isolated by column and high-performance liquid chromatography and the genetic loci, *tca, tcb, tcc,* and *tcd* respectively, that encode them were cloned using antibodies against the four complexes (Bowen *et al.,* 1998). The *tc* genes display high levels of similarity to each other. The large peptides produced by the four loci are cleaved by proteases to produce the various subunits that make up the active toxins (Bowen *et al.,* 2000). Homologs of the *tc* loci have been found in other bacteria including *Xenorhabdus nematophila*, *Serratia entomophila* (insect host) and *Yersinia pestis* (insect vector) (Waterfield *et al.,* 2001). The activity of these toxins appears to be specifically located on the midgut epithelium of the insect, resulting in its disintegration. This activity occurs irrespective of mode of toxin introduction to the insect, be it via injection or ingestion (Blackburn *et al.,* 1998).

The second class of *Photorhabdus* toxin, the "makes caterpillars floppy" (Mcf) toxins are members of a family of potent high molecular mass toxins that encode differing effector domains at their N-termini and are active upon injection. Mcf1 and Mcf2 display limited homology to the cytotoxin B of *Clostridium difficile* (Waterfield *et al.,* 2003). Mcf1 is a high molecular weight toxin associated with persistence in the host and insect death. The predicted structure of Mcf1 includes an N-terminal pro-apoptotic BH3 domain. Upon exposure to Mcf1, changes in cell morphology of hemocytes and midgut epithelial cells occur as a result of toxin triggered apoptosis (programmed cell death) (Daborn *et al.,* 2002). The second Mcf toxin (Mcf2) shows N-terminal similarity to the HrmA avirulence protein from the plant pathogen *Pseudomonas syringae* (Waterfield *et al.,* 2003). Recognition of HrmA triggers localised plant cell death thereby limiting bacterial infection although the site and mode of action of Mcf2 remain unclear.

The third class, the *Photorhabdus* insect related proteins (PirAB) are binary toxins with both oral and injectable activities in some insects (Waterfield *et al.,* 2005). Displaying limited similarity to δ-endotoxins from *Bacillus thuringiensis* (Duchaud *et al.,* 2003) their mechanisms of action and of secretion from the bacterial cell remain unclear (Waterfield *et al.,* 2005). PirAB toxins also display sequence similarity to a developmentally regulated protein from the *Leptinotarsa decemlineata* beetle. Initially suggested to have juvenile-hormone esterase (JHE) activity (Duchaud *et al.,* 2003) implying a mode of action via disruption of the insect developmental process, it has subsequently been shown that PirAB toxins lack such JHE activity and the mode of toxin action is yet to be elucidated (Waterfield *et al.,* 2005).

The most recently identified fourth class of toxin, the *Photorhabdus* virulence cassettes (PVCs) are injectably active, functional homologs of the anti-feeding genes of the free-living entomopathogen *Serratia entomophila*. Whilst similar in structure to the R-type pyocin bacteriocins (Hurst *et al.,* 2004), they display no antibacterial activity and instead are active against insect hemocytes, causing actin cytoskeleton condensation (Yang *et al.,* 2006). Sequence analysis revealed a transposon flanked variable effector sequences and a phage like structure (Yang *et al.,* 2006).

Given the vast array of putative toxin genes on the *Photorhabdus* genome, it is likely many other toxins remain uncharacterised. Such putative toxins include the Txp40 insecticidal toxin protein identified in the similarly nematode symbiotic insect pathogenic bacterium *Xenorhabdus nematophila* (Brown *et al.,* 2006). Txp40 is a novel insect lethal secreted protein identified in *X. nematophila* (Brown *et al.,* 2004). Histopathological studies indicate this toxin is also primarily active against the midgut epithelium of the insect. Sequence analysis of further *Xenorhabdus* and *Photorhabdus* strains revealed this to be a ubiquitous toxin with a high degree of conservation (Brown *et al.,* 2006).

The range and variety of toxin proteins at the disposal of *Photorhabdus* imply that some level of redundancy occurs, however it is also an indication of the important roles played by the toxin products in symbiosis and pathogenesis through initiation of colonisation of the insect midgut epithelium.

1.2 Variation

Photorhabdus spp. have the ability to exist in either of two phenotypically different forms. The bacterial form isolated from the nematode is referred to as the primary phenotype variant, whilst the phenotypically different form is referred to as the secondary phenotype variant. This secondary variant is isolated after prolonged stationary phase growth of the primary variant (Akhurst 1980; Boemare and Akhurst 1988). Secondary variant *Photorhabdus* has never been isolated from wild caught nematodes (Forst & Clarke 2002). Whilst the two phenotypic variants produce toxin to the same degree and are equally pathogenic towards insects, they differ in a broad range of characteristics, including biochemical properties as well as in colonial and cellular morphology (Fig. 1.3).

The primary variant of *Photorhabdus* is characterised by its ability to produce several extracellular proteins including antimicrobial agents, lipases, phospholipases and proteases. Other primary specific factors include pigment and bioluminescence production. Primary type cells are also motile, have distinct colony morphology, dye adsorption patterns and develop large intracellular crystal protein inclusions (Forst *et al.,* 1997). In general, these properties are specific to the primary variant and are necessary for the symbiotic interaction between the bacteria and its nematode partner. These traits allow successful symbiosis with the nematode, enabling normal nematode growth and development to occur and thus have been termed 'Symbiosis Factors' (Joyce & Clarke 2003). In contrast, the secondary variant is lacking or has severely reduced levels of these symbiosis factors (Boemare & Akhurst 1988).

Despite, as previously mentioned, being equally virulent to insects, the secondary variant lacks the ability to form a successful symbiosis with the nematode (Akhurst 1980). Although one case of *Heterorhabditis* nematode proliferation with secondary form bacteria has been reported (Gerritsen & Smit 1993), this resulted in low infective juvenile (IJ) yield compared to wild type. Typically, the nematode cannot retain the secondary form and nematode development is curtailed (Hans & Ehlers 2001).

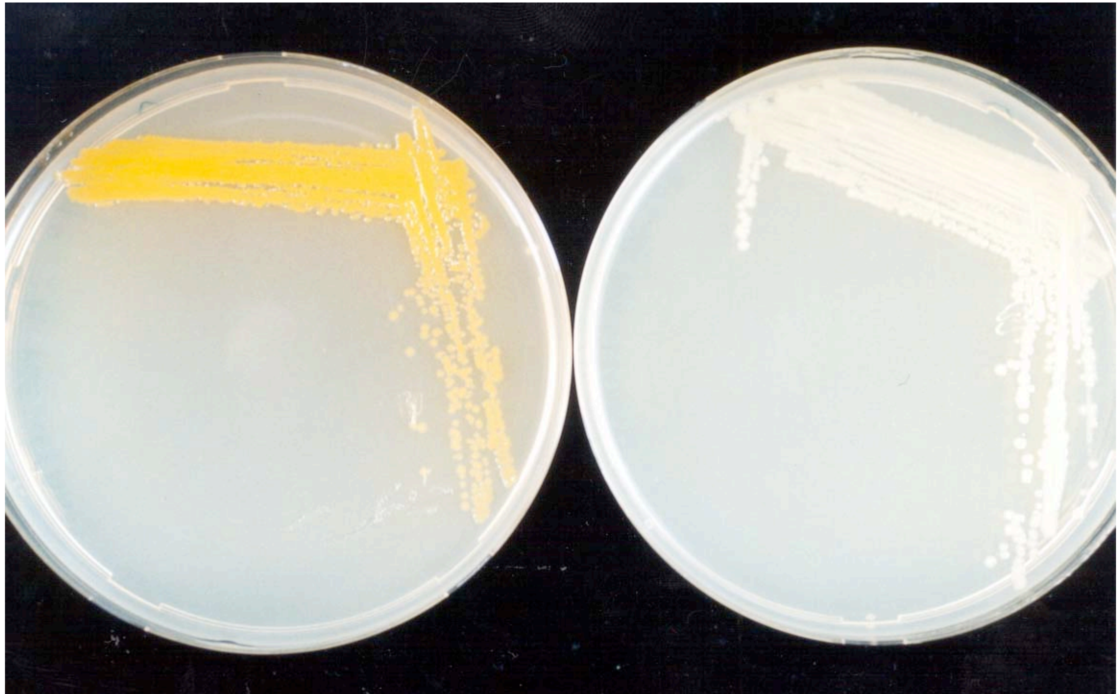


Fig. 1.3 Photograph illustrating pigment difference between primary phase (orange colonies) and secondary phase (white colonies) of *P. temperata* K122.

The secondary variant displays higher levels of cellular metabolism, respiration (Boemare *et al.,* 1997; Smigielski *et al.,* 1994) and also recovers from starvation more quickly than their primary counterparts (Bleakley & Nealson 1988; Boemare *et al.,* 1997). From this, it has been hypothesized that the secondary cell is adapted as a free-living, soil-dwelling form (Boemare *et al.,* 1997) and that the switch from primary to secondary phenotypic variant, is a response to environmental conditions that are unfavourable for nematode association (ffrench-Constant *et al.,* 2003). Phenotypic variation has been described in other bacteria, including the Gram positive organism *Staphylococcus aureus* which has been shown to undergo a reversible phenotypic switch to a resistant small colony variant (SCV) when exposed to the gentamicin antibiotic (Massey *et al*., 2001). Such SCVs are believed to have defective electron transport systems, reduced range of carbohydrate utilization, lack pigmentation and fail to produce multiple virulence factors (von Eiff *et al*., 1997).

Proteomic comparisons of the primary and secondary phenotypic variants of *Photorhabdus* have further highlighted the differences between the two types of cell. In all, 32 membrane proteins and 54 cytoplasmic proteins were observed to have altered levels of synthesis in the secondary variant. These proteins cover a broad range of bacterial cell functions including energy production and conversion, transport, biosynthesis, transcription and translation as well as proteins involved in adaptation and stress responses (Turlin *et al.,* 2006).

1.2.1 What constitutes phenotypic/phase variation?

Bacterial division occurs as a result of binary fission, a replication mechanism that does not involve the exchange of genetic material as occurs in sexual organisms. As a result of this, disease causing, bacterial strains are often populations derived from a single source and are therefore said to be clonal (Rubin 1987). Improving molecular and cultural techniques mean that the complexity involved in survival and establishment/maintenance of an infection in the face of host immune defences and varying environmental conditions is becoming more lucid (Ehrlich *et al.,* 2005)

Whilst a bacterial population may appear to be clonal, it is necessary that significant bacterial adaptation and diversification occurs within the population on order to ensure survival and success of these asexually reproducing organisms in the face of hostile and volatile environments (Deitsch *et al.,* 1997).

Such diversification in a population – termed 'bacterial plurality' (Ehrlich *et al.,* 2005) – can result from both phenotypic and genotypic variation. Given the requirement for mechanisms to avoid host immune responses, it is no surprise that phenotypic variation is a phenomenon that has been observed and studied mainly in bacterial pathogens (Van der Woude & Baumler 2004). Variation in phenotype occurs as a result of both altered gene expression from genotypically identical cells, as well as from the expression of different sets of genes possessed by genotypically distinct cells (Ehrlich *et al.,* 2005).

Switching between different phenotypes of individuals in a clonal population has been termed phase (or phenotypic) variation. In general, the term phase variation refers to a reversible switch between an "all-or-none" (on/off) expressing phase that results in a variation of the expression levels of one or more proteins between individual cells of a clonal population (Van der Woude & Baumler 2004). Frequently, genetic changes are the driving force behind phase variation, for example through genomic inversions, strand –slippage mechanisms or DNA modifications (Smits *et al*., 2008). Phase variation is distinguishable from genetic noise (unimodal variation in the expression of a gene due to random fluctuations in the rate and synthesis of the gene product (Ozbudak *et al*., 2002)) and from classical gene regulation by virtue of there being a genetic or epigenetic mechanism that allows such variability to be heritable (Van der Woude & Baumler 2004). Genetic noise can however lead to another non-unimodal type of variation resulting in defined subpopulations, referred to as 'bistability' (Dubnau & Losick 2006). Bistable switches are however epigenetic and as such are not mediated by genetic changes, providing a distinction from phase variation derived phenotypic changes (Dubnau & Losick 2006). Such non-genetically diverse phenotypically heterogenous populations as arise from bistability allow for a 'bet-hedging' strategy whereby each phenotypic variant may develop into one of several specialized types (Cooper & Kaplan 1982; Seger & Brockmann, 1987). This bet-hedging is useful in maximising an organism's fitness where environmental

uncertainty exists (Donaldson-Mataschi *et al*., 2008). Persister cells are an example of the benefits of maintaining a phenotypically heterogenous population. First reported during studies on the mechanism of penicillin action on staphylococcal infections (Bigger 1944), persister cells are a subpopulation of cells that survive antibiotic exposure yet do not have dedicated resistance mechanisms to the antibiotic in question. Persister cells have subsequently been shown to occur in bacterial biofilms (Brooun *et al*., 2000; Spoering & Lewis, 2001) where persisters survive antimicrobial exposure and repopulate the biofilm (Lewis 2001). It has been suggested that the problem of biofilm resistance to most therapeutic antimicrobials is as a result of persisters (Lewis 2007). Two types of persiter have been described – Type I persisters are generated in stationary phase whilst Type II persisters arise continuously during growth (Balaban *et al*., 2004).
The observation of the occurrence of *Photorhabdus* secondary variant cells during reproduction in insects (Hurlbert, 1994) could suggest that the secondary variant is a sub-population of *Photorhabdus* enabling it to respond to environmental fluctuations and adversity.

1.2.2 Phenotypic switch associated phenotypes

Due to their ease of observation in the laboratory, colony morphology and opacity changes are the most readily identifiable phenotypic-switch associated characteristics. Such changes include mucoid/non-mucoid, ruffled/smooth, dry/moist and opaque/translucent colony types. Such changes have been attributed to variation in a range of surface-exposed proteins of the capsule and of cell wall composition (Van der Woude & Baumler 2004). Changes in dye adsorption (indicated by colour change on specific media) are also attributable to phase variation of proteins that interact with the relevant dye.
In general, any change in colony morphology, opacity or colour can be indicative of a change in expression (phase variation) of one or more proteins.

1.2.3 Molecular Mechanisms of Phase Variation

There is no correlation between a phase-varying phenotype and its regulatory mechanism (Van der Woude & Baumler 2004), with multiple mechanisms affecting similar variations of phenotype. Genetic regulation occurs where the change in phase expression (between "on" and "off") occurs as a result of a DNA sequence change at a specific locus. There are several mechanisms to effect such DNA sequence change, including, as previously mentioned, genomic inversions and strand –slippage mechanisms however, such DNA rearrangement resulting in phenotypic variation has been termed a "shufflon" irrespective of the mechanism involved (Komano, 1999). A classic example of phase variation by DNA inversion occurs in the expression of the type I fimbriae of *E.coli* (Abraham *et al*., 1985) whilst strand slippage resulting in frame mutations has been shown to result in phase variation of virulence factors in *Bordetella pertussis* (Stibitz *et al*., 1989). DNA methylation can also play a role in phase variation, for example the methylation pattern of the DNA can affect the binding of a transcriptional regulator of the *pap* pilus operon in *E. coli* (Van der Woude *et al*., 1996).

It is important to note however, as previously mentioned, that whilst phase variation can affect phenotype, a variation in phenotype need not be as a result of changes in sequence of/modifications of the DNA (Smits *et al*., 2008). Variations in growth stage of cells in a population can result in the expression of different phenotypes and cell cycle-related variation has an important role in coordinated expression of genes involved in replication and cell division (Holtzendorff *et al*., 2006; Avery 2006).

1.2.3.1 Phase variation in *Photorhabdus*

Phase variable factors characterised to date in *Photorhabdus* spp. include the two previously mentioned *mrf* and *mad* fimbrial loci.

mrf

The *mrf* fimbrial locus that is expressed by *P. temperata* K122 during insect infection has been shown to undergo differential expression between phenotypic variants. Controlled by an inversion mechanism, the primary variant population contains cells with the *mrf* locus in both the ON and OFF orientations. It is interesting to note that in secondary variant populations the *mrf* locus is locked in the ON position (Meslet-Cladiere *et al*., 2004). The locus also appears to be regulated temporally, with low levels of *mrf* expression occurring in early exponentially growing cells, switching to elevated expression levels during late exponential phase, suggesting a role for the Mrf pili in the latter stages of infection (Meslet-Cladiere *et al*., 2004).

mad

The *mad* gene locus, implicated in symbiosis and host specificity, encodes a fimbria regulated by an ON/OFF invertible promoter switch (Somvanshi *et al*., 2010). *P. luminescens* TTO1 cells associated with maternal nematode intestines, where the bacteria reside as part of a biofilm, have the promoter switch in the ON orientation, as opposed to the OFF orientation which is observed in most other cells (Somvanshi *et al*., 2010).

1.2.3.2 Phenotypic Variation of *Photorhabdus*

Aside from the phase variable loci mentioned above, *Photorhabdus* undergoes a unidirectional coordinated phenotypic switch, which results in the formation of the previously described secondary variant. Whilst the overall mechanism controlling phenotypic variation in *Photorhabdus* remains unidentified, the wide range of varying phenotypes suggests a genetically complex system involving a large number of genes in a major cascade that is tightly controlled and regulated. The unidirectional nature of the phenotypic switch and the fact that it has been documented as occurring under stress conditions (Krasomil-Osterfeld 1995; Boemare *et al.,* 1997; ffrench-Constant *et al.,* 2003) suggest that it is an extreme reaction to ensure bacterial survival.

1.2.3.3 Phenotypes differentially expressed in the phenotypic variants of *Photorhabdus*

Extracellular enzymes

Two of the major extracellular enzymes have been well characterised (lipase & protease) and their regulation during phenotypic variation examined. Wang & Dowds (1993) found that both the lipase and protease enzymes are made/produced in the secondary variant *P. temperata* K122 cell in an inactive form. SDS treatment (which linearises proteins, and may remove the hydrophobic leader sequence of the proteins, thereby rendering them active) restored the activities of both these extracellular enzymes, indicating regulation at the post-translational level. The production of active lipase and protease in the primary variant suggests that phenotypic variation results in the loss of post-translational activation/secretion of these exoenzymes.

Luminescence

For bioluminescence to occur in *Photorhabdus*, expression of the *lux* genes is required. Despite poor bioluminescence of the secondary variant, the *lux* genes are transcribed equally in both phenotypes of *P. temperata* K122 (Wang & Dowds 1992). Addition of rifampicin (an inhibitor of RNA synthesis) to *P. luminescens* Hm secondary variant cells results in a similar bioluminescent profile to that of rifampicin exposed primary variants cells (Hosseini & Nealson, 1995). Taken together, these

data are indicative of post-transcriptional regulation of bioluminescence and furthermore suggests that there may be post-transcriptional regulation of phenotypic variation occurring, potentially in response to an unstable RNA. The biological role for bioluminescence in *Photorhabdus* has yet to be determined.

Fimbriae/Pili

The previously mentioned phase variable *mrf* fimbrial locus of *P. temperata* K122 that is expressed during insect infection undergoes differential expression between phenotypic variants via an inversion event that may be driven by the activity of a nearby putative recombinase gene *mrfI*. Whilst the primary variant locus may be in the ON or OFF orientations, the locus in secondary variant populations is locked in the ON position (Meslet-Cladiere *et al*., 2004).

Crystalline proteins

Encoded by the *cipA* and *cipB* genes, the development of the two kinds of crystalline inclusion bodies found in *Photorhabdus* is specific to the primary variant, where they amount to 55-60% of total protein in *P. luminescens* stationary phase cells (Bintrim & Ensign, 1998). Bintrim and Ensign (1998) have shown that inactivation of either of the *cipA* or *cipB* genes, encoding the stationary phase crystal protein inclusion bodies of *Photorhabdus* NC1 was pleiotropic and resulted in the formation of a bacterial cell that was secondary-like for many traits including loss of exoenzyme production, bioluminescence and pigment production. Mutants inactivated for activity of both genes were not isolated and such double mutants may be non-viable. Whilst the structures of CipB (11.3 KDa large rectangular body) and CipA (11.6KDa smaller bipyramidal body) are known, their exact function is not. They display some homology to each other but not to any other proteins (Bowen & Ensign 2001). Proposed functions include food storage (Bowen & Ensign 2001) or use as a food source (Forst & Nealson 1996; You *et al*., 2006). Expression of the *Photorhabdus* Cip proteins in *E. coli* enables successful development of the *Xenorhabdus nematophila* symbiont nematode *Steinernema carpocapsae* in liquid cultures, confirming a role in nematode development, however, *Heterorhabditis bacteriophora* nematode development was not supported by the Cip expressing *E.coli* strains indicating a requirement for other *Photorhabdus* specific factors (You *et al.,* 2006).

Glycocalyx

Studies using transmission electron microscopy have highlighted differences in the surface capsular material between phenotypic variants of *Photorhabdus* (Brehélin *et al*., 1993). The thickness of the glycocalyx layer of primary variant cells was found to be over twice that of the secondary variant (85nm as opposed to 40nm). It has been hypothesized that the greater thickness and any chemical difference in the glycocalyx layer may contribute to the increased ability of the primary variant cells to adhere to the intestinal cells of the nematode (Forst *et al*., 1997).

Antibiotics

The main antibiotic compounds produced by *Photorhabdus* are of the hydroxystilbene class of molecules (Paul *et al*., 1981), with polyketide-synthase-like genes also having been identified on the genome (ffrench-Constant *et al*., 2000). The aforementioned 3-5-dihydroxy-4-isopropylstilbene (ST) small antibiotic molecule which has been shown to be a required signal for IJ to hermaphrodite recovery (Joyce *et al*., 2008) was also identified as having a function in suppression of insect host defenses during *Photorhabdus* infection (Eleftherianos *et al*., 2007) is consistent with the inability of the antibiotic deficient *Photorhabdus* secondary variant to undergo successful mutualism with the *Heterorhabditis* nematode. A role for *Photorhabdus* produced antibiotic compounds in the suppression of potential competitor microorganisms has also been proposed (Hu & Webster, 2000).

Pigment

Photorhabdus cultures are characterised by the production of pigments that can vary yellow to brick red/green depending on the strain and/or species, e.g. *P. temperata* K122 produces bright yellow colonies on LB agar and green insect cadavers whilst *P. luminescens* TTO1 produces orange colonies and brick red insect cadavers. In all cases where it has been studied, the pigment has been shown to be an anthraquinone (AQ) – a polyketide compound that has been shown to have some antimicrobial activity (Richardson *et al.,* 1988, Li *et al.,* 1995). The biochemical pathway for the synthesis of AQ has been described by Brachmann *et al.* (2007) and this study represents the first characterisation of AQ production in Gram negative bacteria and only the second type II polyketide synthase identified in Gram negative species. However the role of AQ in symbiosis/pathogenicity is as yet unclear.

Proteome analysis

As previously mentioned, proteomic analysis via the comparison of cellular, extra-cellular and membrane-associated protein extracts from primary and secondary variant cultures has led to the identification of stationary phase differences in the proteomes of the respective phenotypic variants (Turlin *et al.,* 2006). Differences occurred between the variants in all of the extracts analysed, with proteins involved in energy production and conversion, transport, biosynthesis, transcription and translation, as well as proteins involved in adaptation and stress responses all displaying differential expression between variants. Interestingly, comparison of the extra-cellular extracts indicated that all proteins found in the primary supernatants were lacking or severely reduced in those of the secondary variant.

1.2.3.4 Control/Regulation of Phenotypic Variation in *Photorhabdus*

Whilst the phenotypic differences between primary and secondary cells are readily identifiable, the nature of the phenotypic switch, and the reasons for its occurrence have yet to be elucidated. Despite this lack of clarity however, current evidence tends to support the hypothesis of a complex regulatory cascade of interacting genes as a control mechanism for phenotypic variation in *Photorhabdus.* Transcriptional analysis of a *Photorhabdus luminescens* TTO1 derived colonial and phenotypic variant displaying delayed pathogenicity (VAR*) (Lanois *et al*., 2011) lends weight to this suggestion. The main difference between VAR* and wild-type TTO1 is the presence in VAR* of a single-block 275kb duplication. 148 genes were transcriptionally affected, with 55 genes shown to have reduced expression in the VAR* mutant when compared to the wild-type strain, with the majority of thee changes occurring in the stationary phase as a result of both transcriptional repression and an absence of transcriptional induction (Lanois *et al*., 2011). The transcription of 15 regulators/potential regulators was affected, with 10 transcribed to higher levels in the VAR* mutant, including a putative LuxR transcriptional regulator and 3 Ner-like regulatory proteins. The observed transcriptional regulation of regulators and of genes coding for some wild-type traits, suggests the occurrence of mixed (transcriptional and post-transcriptional) regulation, indicating that phenotypic variation in *Photorhabdus* involves a complex networks of regulators (Lanois *et al*., 2011).

HexA

Joyce and Clarke (2003) isolated a gene in *P. temperata* K122 with a role in phenotypic variation. This gene displays homology with a member of the LysR type transcriptional regulator (LTTR) family – *hexA* - found in the plant pathogen *Erwinia carotovora*. In *P. temperata* K112, *hexA* is required to maintain the secondary variant. Disruption of *hexA* in the secondary variant results in the derepression of the primary-specific phenotypes, enabling the *hexA* mutant to support nematode growth and development. The *hexA* mutant also results in attenuation of virulence towards larvae of the Greater Wax Moth *Galleria mellonella*, suggesting temporal regulation of pathogenicity and symbiosis in *Photorhabdus*, with *hexA* an important mediator of the process. The conservation of the HexA protein across the enterobacteria (Fig 1.4)

indicates it is a part of the enteric backbone. HexA in *Erwinia carotovora* has been identified as having a role in the regulation of motility and virulence factors (Harris *et al*., 1998) whilst in *E.coli*, the HexA homologue LrhA represses the transcription of genes involved in the formation of type I fimbriae, motility, chemotaxis and is required for biofilm formation (Blumer *et al*., 2005; Lehnen *et al.,* 2002). LrhA has also been shown to reduce the expression of the stationary phase sigma factor RpoS (Gibson & Silhavy, 1999; Peterson *et al*., 2006). LrhA in *Xenorhabdus nematophila* is required for virulence, motility, toxin expression and lipase activity (Richards *et al*., 2008). In *X. nematophila* however, LrhA has been identified as positive regulator of gene function as opposed to the HexA/LrhA in the other bacteria (Harris *et al*., 1998; Lehnen *et al.,* 2002; Joyce & Clarke 2003; Richards *et al*., 2008). HexA of *Photorhabdus* appears to have broader effects than its *Xenorhabdus* counterpart where there is no requirement for LrhA in the regulation of RpoS (Richards *et al*., 2008).

The LysR family of genes is the largest known family of autoregulatory transcriptional regulators (LTTRs). In general, each LysR responds to a different coinducer to activate the divergent transcription of either linked target genes, or unlinked regulons encoding extremely diverse functions (Schell 1993).
The structure of the majority of LTTRs (Fig. 1.5) can be described as consisting of 3 domains:

1) DNA binding domain incorporating a helix-turn-helix (HTH) motif, usually located within residues 1-65
2) Coinducer Recognition and/or Response Domain located around residues 100-173 and 196-206.
3) DNA-binding and Coinducer Response Domain, sited around residues 227-253.

(Henikoff *et al.,* 1988; Henikoff *et al.,* 1990)

Whilst the coinducer and target genes of HexA in *Photorhabdus* remain as yet undetermined, a model of action/interactions has been proposed by Joyce *et al.* (2006) (Fig 1.6) whereby HexA has a role in phenotypic variation and the regulation of mutualism, pathogenicity (Joyce & Clarke 2003), with the AstRS (adaptation to stationary phase) two component pathway (see below) temporally regulating

phenotypic variation, potentially in response to stress (Derzelle *et al*., 2004a), and a link between AstRS and HexA proposed by Joyce *et al*. (2006).

AstR/AstS two-component pathway

The AstRS two component pathway has been shown to affect the timing of *Photorhabdus luminescens* phenotypic variation (Derzelle *et al.,* 2004a). Mutation of *astR*, the gene encoding the regulator protein results in a reduction in the competitive advantage of primary variant cells during stationary phase conditions, affects antibiotic synthesis and also induces an earlier switch to the secondary variant. Another effect of the *astR* mutation was the derepression of *flhDC* mRNA during exponential phase, resulting in early onset of swarming behaviour. A role for AstRS in the oxygen dependent regulation of motility during phenotypic variation has also been suggested (Joyce *et al*., 2006). Additionally, Joyce *et al* (2006) speculate on a potential link between the AstRS pathway and that regulated by HexA (Fig. 1.6). The *astR* mutant strain displayed altered stationary phase synthesis levels of 17 proteins with roles in electron-transport, energy metabolism, iron acquisition and stress responses. The molecular chaperonin GroEL, responsible for the folding, repair and degradation of proteins is also affected, seeing a five-fold increase occurring in the mutant strain (Derzelle *et al.,* 2004a). GroEL is produced in response to various environmental stresses, including heat shock and salt stress (Yura *et al*., 1993; Meury and Kochiyama, 1991; Kilstrup *et al*., 1997) and is generally involved in the maturation of newly synthesized proteins, as well as the refolding or degradation of denatured proteins (Georgopoulus and Welch, 1993; Hartl *et al*., 1994). The increase of GroEL expression in the *Photorhabdus* AstRS mutant strain gives a further indication that the phenotypic switch observed in *Photorhabdus* may occur as part of an overall response to environmental stress.

```
P.luminescens      MINANRPIMNLDLDLLRTFVAVADLNTFAAAAAA----VCRTQSAVSQQMQRLEQLVGRE 56
P.asymbiotica      MINANRPIMNLDLDLLRTFVAVADLNTFAAAAAA----VCRTQSAVSQQMQRLEQLVGRE 56
P.temperata        MINANRPIMNLDLDLLRTFVAVADLNTFAAAAAA----VCRTQSAVSQQMQRLEQLVGRE 56
X.nematophila      MINANRQIINLDLDLLRTFVAVADLNTFAAAAAA----VSRTQSAVSQQMQRLEHLVGRE 56
Y.Pestis           MTNANRPIINLDLDLLRTFVAVADLNTFAAAAAAAAAAVCRTQSAVSQQMQRLEQLVGKE 60
E.carotovora       MTSANRPVLNLDLDLLRTFVAVADLNTFAAAATA----VNRTQSAVSQQMQRLEQLIGKE 56
E.coli             MISANRPIINLDLDLLRTFVAVADLNTFAAAAAA----VCRTQSAVSQQMQRLEQLVGKE 56
                   * .*** ::***********************:*    * **************:*:*:*

P.luminescens      LFARHGRNKLLTEHGLQLLGYARQILRANDDASASLTYSDAEGELRIGASDDTVDTLLPF 116
P.asymbiotica      LFARHGRNKLLTEHGLQLLGYARQILRANDDASASLTYSDAEGELRIGASDDTVDTLLPF 116
P.temperata        LFARHGRNKLLTEHGLQLLGYARQILRANDDASASLTYSDAEGELRIGASDDTVDTLLPF 116
X.nematophila      LFARHGRNKLLTEHGLQLLGYARQILRANDDATASLTYNDADGELRIGVSDDAVDTLLPF 116
Y.Pestis           LFARHGRNKLLTEHGLQLLGYARKILRFNDEACTSLMYSNMEGSLIIGASDDTADTLLPF 120
E.carotovora       LFARHGRNKLLTEHGIQFLGYARKILQFNDEACISLMYSDIQGTLTIGASDDTADTILPY 116
E.coli             LFARHGRNKLLTEHGIQLLGYARKILRFNDEACSSLMFSNLQGVLTIGASDESADTILPF 116
                   ***************:*:*****:**: **:*  ** :.: :* * **.**::.**:**:

P.luminescens      LLNRIASVYPRMAIDVRIKRTQFIESMLDNHEIDLALTTAKISHHPRTVLRSTPVLWHCA 176
P.asymbiotica      LLNRIASVYPRMAIDVRIKRTQFIESMLDNHEIDLALTTAKISHHPRTVLRSTPVLWHCA 176
P.temperata        LLNRIASVYPRMAIDVRIKRTQFIESMLDSHEIDLALTTAKISHHPRTVLRSTPVLWHCA 176
X.nematophila      LLNRIASVYPRVAVDVRIKRAQFIESMLDNHEIDLALTTAKINQHPKTILRSSPVLWHCA 176
Y.Pestis           LLNRVATLYPRLAIDVRVKRSPFIADMLSSGEVDLAITTAKVDSHPHVILRTSPTLWYCS 180
E.carotovora       ILHRVTSVFPKLSVNVSVKRSAEMMEMLNQGKIDLVITTMNGVVFPHVLLRSSPTLWYCA 176
E.coli             LLNRVSSVYPKLALDVRVKRNAYMAEMLESQEVDLMVTTHRPSAFKALNLRTSPTHWYCA 176
                   :*:*:::::*:::::* :**   : .**.. ::** :** .    .    **::*. *:*:

P.luminescens      PDFQLQVNEPVPLVVMDETNPFRQLALDTLDEVGVSWRIAYEAASLSAVRTAVNAEVGIT 236
P.asymbiotica      PDFQLQPNEPVPLVVMDETNPFRQLALDTLDEVGVSWRIAYEAASLSAVRTAVNAEVGIT 236
P.temperata        PDFQLQPNEPVPLVVMDETNPFRQLALDTLDEAGVSWRIAYEAASLSAVRTAVNAEVGIT 236
X.nematophila      PDFQLQANEPVPLVVMDETNPFRQIALETLDMAGISWRIAYEAASLSAVRAAVNAEVGIT 236
Y.Pestis           VDYQFQPGEPVPLVVMDEPSLYREMAIEHLTQAGVPWRIAYVASSLSAIRAAVRAGLGVT 240
E.carotovora       ADYQFRSQEPVPLVVLDEPSPFRTLATQQLTAAGIPWRIAYVASTLSAVRAAVKAGMGIT 236
E.coli             AEYVLQKGEPIPLVLLDDPSPFRDMVLATLNKADIPWRLAYVASTLPAVRAAVKAGLGVT 236
                    :: ::  **:***::*:.. :* :.   *  ..:.**:** *::*.*:*:**.* :*:*

P.luminescens      ARPLEMQNADLRILGESEGLPRLPETQFSLYRHSNEQNESVLTVFSAIENKKTPYTITSV 296
P.asymbiotica      ARPLEMQNADLRILGESEGLPRLPETQFSLYRHSNEQNESVLTVFSAIENKKTPYTISSV 296
P.temperata        ARPLEMQNADLRILGESEGLPRLPETQFSLYSHSNEQNESVLTVFSAIENKKTPYTISGV 296
X.nematophila      ARPLEMHNADLRILGESEGLPRLPEIQFFLYRNTNEQNESVLTVFDAIENKKTPYTVTPI 296
Y.Pestis           ARPIEMMSPDLRVLGETEGLPGLPETRYVLCKDKQCDNELALAIFSALQN-SYQHTMSSE 299
E.carotovora       VRSVEMMSPELRVLGEEEGLPRLPETRYFLCQNPNQENELATAIFNVIESGK-PSHITPV 295
E.coli             ARPVEMMSPDLRVLSGVDGLPPLPDTEYLLCYDPSSNNELAQVIYQAMESYHNPWQYSPI 296
                   .*.:** ..:**:*.  :*** **: .: *  . . :** . .::..::.       :

P.luminescens      SEE-LPDDDDIIDE-------------------- 309
P.asymbiotica      SDE-PSDDEDIIEE-------------------- 309
P.temperata        SEEPLSDDAHIIDE-------------------- 310
X.nematophila      EVDESDETDESIDTIGPIKSTDSVDETSAENIDE 330
Y.Pestis           SSLILDSDYLTGDED------------------- 314
E.carotovora       SMLANSTNEKLSSDPSLKDAI------------- 316
E.coli             SAPEGDDSLLIERDIE------------------ 312
```

Fig. 1.4 ClustalW2 (Larkin *et al.*, 2007; Goujon *et al.*, 2010) protein alignment of HexA/LrhA from *P.luminescens* TTO1, *P. asymbiotica* ATCC 43949, *P. temperata* K122, *Xenorhabdus nematophila*, *Yersinia pestis*, *Erwinia carotovora* subsp. carotovora and *Escherichia coli* O157:H7.

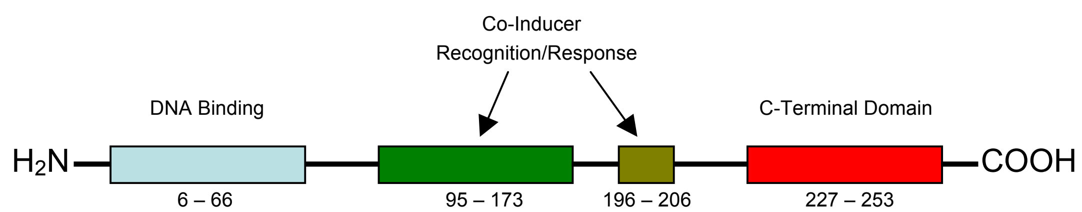


Fig.1.5 Model For Domain Organization Of An LTTR (Adapted from Schell 1993)

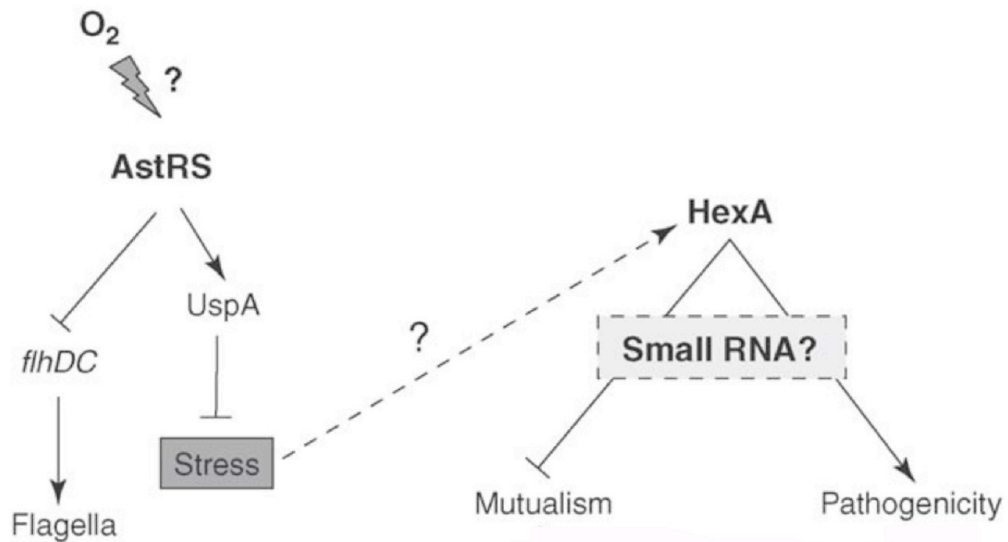


Fig. 1.6 Possible regulatory interactions of pathways governing phenotypic variation in *Photorhabdus* (From Joyce *et al*., 2006). HexA has a role in phenotypic variation and the regulation of mutualism, pathogenicity (Joyce & Clarke 2003). AstRS temporally regulates phenotypic variation, potentially in response to stress (Derzelle *et al*., 2004a), with a link between AstRS and HexA proposed by Joyce *et al*., (2006).

ner

O'Neill *et al* (2002) have demonstrated that the expression of a gene (*ner*), encoding a putative DNA-binding protein, from a multi-copy plasmid during stationary phase results in a primary to secondary phenotypic switch in *P. temperata* K122, suggesting a role for *ner* as a repressor of stationary phase primary variant specific gene expression. Ner has been shown to regulate transposition and repressor synthesis of phage Mu (Goosen & Van Den Putte 1986) and homologues have been identified in *E.coli* and other gram negative species (Choi *et al*., 1989; Autexier and Du Bow 1992; Tinsley & Nassif, 1996). The *E. coli ner* homologue *nlp(stsB)* has been implicated in the positive regulation of metabolism of two sugars via increasing activity of the *malQ, malP* and l*acZ* genes encoding maltose and lactose utilising enzymes (Choi *et al*., 1989), however its overall function in the bacterial cell remains unclear.

H-NS

A potential role for the histone-like DNA binding protein and global regulator H-NS has also been proposed, with increased levels of the protein detectable in the secondary variant. *hns* expression is growth dependent in *Photorhabdus* primary variant cells (Turlin *et al.,* 2006) and in *E. coli,* where it is highly expressed during stationary phase. The H-NS protein has been shown to be involved in the regulation of genes involved in the *E. coli* response to osmolarity and oxygen starvation (Atlung & Ingmer 1997). Upregulation of H-NS in the secondary variant would therefore seem consistent with the hypothesis that the phenotypic switch in *Photorhabdus* occurs in response to stress conditions (Boemare *et al.,* 1997; ffrench-Constant *et al.,* 2003). H-NS, a repressor of gene expression, may also be playing a negative regulatory role in the secondary variant, resulting in lower expression or lack of synthesis of numerous proteins in the secondary cell (Turlin *et al.,* 2006). Such a role for H-NS may arise in response to stressful environmental conditions resulting in a more hardy variant adapted for survival in the absence of a nematode host.

1.3 Summary

The enterobacterium, *Photorhabdus temperata*, has a symbiotic relationship with nematodes of the *Heterorhabditis* family. After prolonged *in vitro* growth, stable phenotypic variants that do not facilitate normal nematode development appear in the population. Such cells are termed secondary variants (distinct from primary variants - those that do support nematode development). Phenotypic variants can be distinguished by a variety of different phenotypes, for example, pigment production, dye adsorption, antibiotic production, protease production and bioluminescence. In general, primary variants are positive for these characteristics whilst secondary variants show diminished or lack of production of these characters. With phenotypic variation in *Photorhabdus* occurring after prolonged growth under stressful conditions, the unidirectional nature of the phenotypic switch may be due to the lack of the presence of an as yet unidentified environmental signal. The emerging evidence points to an increasingly complex series of events and interactions leading to the primary to secondary phenotypic switch, with a master regulator having yet to be identified. In spite of the complex nature of interactions observed to date with involvement in phenotypic variation, the limited circumstances in which phenotypic switching occurs are indicative of a tightly controlled, regulated process.

1.4 Aims and Objectives

The aim of this project is to further our understanding of the mechanism of phenotypic switching in *P. temperata* by investigating the genetic factors involved in controlling phenotypic variation. It has been hypothesized that phenotypic switching occurs as part of a gene cascade, regulated by one major controller gene (Forst *et al.*, 1997, Dowds, 1997). This project hopes to investigate this hypothesis, and examine the molecular mechanism controlling these factors in *P. temperata*, with the goal of identifying the gene(s) involved – thereby enabling manipulation of the phenotypic switch and facilitating further study of the tripartite bacteria-nematode-insect host interaction.

Chapter 2

2. Material and Methods

2.1 Bacterial strains, plasmids and growth conditions

The strains used in this study are listed in Table 2.1. *Photorhabdus* strains were grown at 28°C whilst *E. coli* and *M. luteus* strains were cultivated at 37°C unless otherwise stated. Liquid cultures were incubated with agitation at 25*g*. Strains were maintained as 20% (v/v) glycerol stocks stored at -80^{0}C.
Plasmids used in this study are listed in Table 2.2.

2.2 Media, chemicals and enzymes

Luria-Bertani (LB) medium (1% (w/v) peptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride in water) (Merck) was used for liquid culture where stated. For solid media, agar (Merck) to a concentration of 15g/L was added. Where required, antibiotics used at the following final concentrations: Rifampicin 100μg/ml, Kanamycin 50μg/ml, Ampicillin 100μg/ml, Gentamycin 30μg/ml and Chloramphenicol 20μg. Bacterial cell densities were determined by measuring the OD_{600nm} of cultures.
Unless otherwise stated, all other chemicals were obtained from the Sigma-Aldrich.

Strain	Genotype/Description	Source/Reference
Photorhabdus temperata K122 Rif^R Primary	Spontaneous Rif^R mutant of wild isolate from Ireland	Laboratory stock
Photorhabdus temperata K122 Rif^R Secondary	Spontaneous Rif^R mutant of secondary isolated after prolonged stationary phase culture of primary cells	Laboratory stock
Photorhabdus luminescens TT01		Laboratory Stock
Sm11, 13, 15		This study
Pm1-127		This study
Escherichia coli XL1-blue	F' *proAB* $lacI^qZ\triangle M15$ Tn*10*(Tet^r)	Promega Corporation
Escherichia coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, △(lac-proAB)/F' [*traD*36, $proAB^+$,$lacI^qZ\triangle M15$]	Stratagene Corporation
Escherichia coli S-171 λ-*pir*	*pro res* mod^+ RP4-2 Tet::Mu-Km::Tn*7*	Laboratory stock
Escherichia coli EC100	F^- *mcrA Δ(mrr-hsdRMS-mcrBC), Φ80dlacZΔM15 ΔlacX74 recA1, endA1 araD139 Δ(ara, leu)7697, galU galK* λ^- *rpsL* (Str^R) *nupG*	Epicentre Biotechnologies
Escherichia coli DH5α	*rec*A1, *end*A1, ϕ080d*lac*Z∇M15	Laboratory stock
Micrococcus luteus	ATCC 4698	Laboratory stock

Table 2.1 Strains used in this study

Plasmid	Description	Source/Reference
pHP45Ω-Km	Source of Km^r interposon	Fellay *et al.,* 1987
pBAD33	$P_{BAD,}$ *araC,* Cm^r	Guzman *et al.,* 1995
pBAD24	$P_{BAD,}$ *araC,* Amp^r	Guzman *et al.,* 1995
pUTkm2	Amp^r, mini-Tn5 Km^r	Herrero *et al.,* 1990
pJQ200sk	Gtm^R, *Sst*I/MCS/*Kpn*I, *traJ*, *ori*T, *sacB*	Quandt & Hynes, 1993
pTRC99a	Amp^r, Tet^r	Laboratory stock
pBR322	*ori* colE1, Amp^r, Tet^r	Laboratory stock
pIM5	Partial *E. coli csrB* clone (+1 to +337) in pT218U	Baker *et al.,* 2002
pHMW011	pJQ200sk with Km^r interposon from pHP45Ω-Km	This study
pHMW014	pJQ200sk with Km^r interposon from pHP45Ω-Km	This study
pBAD24hexA	pBAD24 carrying *hexA*	Laboratory stock

Table 2.2, Plasmids used in this study

2.3 Nematode stocks

Nematodes used were *Heterorhabditis downsei* K122 (Stock *et al*., 2002), the cognate partner of *P. temperata* K122. Nematode cultures were maintained via passage through *Galleria mellonella* larvae.

2.4 Phenotypic tests

The tests listed in Table 2.3 were applied to *Photorhabdus* wild type and mutant strains to distinguish phenotypic variants. For clarity, the expected phenotype of the primary and secondary variants for each test are also listed. The procedure for each test is given below. In each case, all strains for testing were grown overnight in liquid media as described above, then adjusted to an OD_{600nm} of 1.0, of which 5µl of the resultant culture solution was pipetted onto the relevant test media, allowed to dry and then incubated for 48h at 30^0C. Control strains that represented stable primary and secondary phenotypic variants were included in all experiments.

2.4.1 Luminescence

Bacteria were grown to stationary phase (in liquid and on solid culture media) and bioluminescence was assayed using a Fujifilm Intelligent Darkbox II. Plate cultures were observed after exposure for ~5 minutes. Liquid cultures were monitored in duplicate over time in 100µl aliquots of Luria broth in black 96 well plates using a TECAN Genios microplate reader to record the level of light production at that particular time.

2.4.2 Pigment Production

Pigment production was observed following 48 hours incubation on LB agar at 30^0C. Primary phenotypic variant colonies have an orange/yellow appearance whilst secondary variant colonies appear cream/white, with pigmentation deepening with age for each culture type.

2.4.3 Colony Morphology/Consistency

Stationary phase colonies on LB agar were observed and then manipulated with a sterile wire loop. Primary colonies are sticky and glutinous (mucoid), while secondary variants detach easily from the plate surface (non-mucoid).

2.4.4 Lipase Assay

Liquid bacterial cultures were normalized to OD_{600nm}=1 as detailed above, then inoculated as 5µl drops onto Tween 80 agar (10g peptone, 5g NaCl, 15g agar per litre, made up to 989 ml with dH_2O, to which, 10ml autoclaved Tween 80 (Sigma) and 1ml 1M $CaCl_2$ was added after autoclaving) (Bleakley and Nealson, 1988). Between 5-7 days incubation, a halo of precipitated Tween 80 breakdown products became visible around lipase producing colonies.

2.4.5 Antibiotic Assay

Strains from liquid overnight cultures adjusted to OD_{600nm} = 1 were inoculated as 5µl drops onto 9cm LB agar plates and incubated for 48 hours at 30^0C. The plates were then overlaid with warm (45^0C) soft agar (0.6% (w/v) LB agar) to which 1ml per 100ml of an overnight culture of *Micrococcus luteus* had been added. Once the soft agar had set, the plates were incubated at 37^0C for a further 48 hours (*Photorhabdus* does not grow at this temperature). Clear zones where *M. luteus* growth is inhibited become visible around antibiotic producing *Photorhabdus* colonies.

2.4.6 Dye adsorption

2.4.6.1 Colour on NBTA medium

NBTA medium was prepared as follows: Nutrient agar (Oxoid) with 0.1mM $MgCl_2$ and 20mg/l triphenyltetrazolium chloride (TTC) was autoclaved and 1

ml/L filter sterilized bromothymol blue stock solution (25mg/ml in ethanol) added after cooling to 55^0C. Dye uptake was measured after incubation at 30^0C for 48 hours. Primary variant colonies appear green, with secondary variant colonies appearing blue/red. The difference arises as the primary variants adsorb bromothymol blue and reduce TTC whilst the secondary variants reduce TTC but do not adsorb the bromothymol blue constituent of the media. These changes are indicative of membrane differences between the phenotypic variants.

2.4.6.2 Colour on MacConkey (MCA) medium

MacConkey No.3 medium (Oxoid) was made up as per manufacturer's directions and normalized liquid overnight cultures were inoculated as previously described above. Colonies were observed and scored following 48 hours incubation at 30^0C. The primary phenotypic variant produced red colonies due the adsorption of the neutral red dye component of MCA and secondary phenotypic variant colonies appear white since these variants lack the dye adsorption abilities of the primary variant.

2.4.6.3 Clearing on Congo Red medium

After autoclaving and cooling to 55^0C, LB agar was supplemented with 0.01%(w/v) Congo Red before pouring. Cultures were normalized as before and plates were inoculated with normalized cultures as previously described. Results were recorded after 48 hours incubation at 30^0C. As a result of dye uptake, a zone of clearing is observed surrounding primary phenotypic variant colonies. This zone of clearing is absent around secondary phenotypic variants.

2.4.7 Haemolysin Assay

Blood agar plates were prepared by addition of 15ml/l defibrinated horse blood (E&O Laboratories) to autoclaved and cooled LB agar at 55^0C. A zone of haemolysis can be observed around haemolysin producing primary phenotypic

variant colonies after incubation at 30^{0}C for 5-7 days. Secondary phenotypic variant colonies do not produce haemolysin.

2.4.8 Nematode Production (Mutualism assays)

Assays designed to observe mutualism with the nematode were employed to examine the ability of the nematodes to recover from the infective juvenile (IJ) stage, to reproduce and re-enter the IJ stage in the presence of the bacteria being tested. Fat rich media was prepared by adding 10ml lipid agar (10g corn syrup, 5g yeast extract, 5ml cod liver oil, 23g nutrient agar, 0.01M $MgCl_2$ per litre in 4.5cm petri dishes) and inoculating with 50 µl of an overnight culture of the relevant *Photorhabdus* strain spread as a "Z". These plates were allowed to incubate at 30^{0}C for 48 hours to give a confluent growth of culture before addition of surface sterilized nematodes to each plate. Antibiotics were not added to the plates.

K122 Nematodes (laboratory stock) were surface sterilized by pelleting in 1.5ml tubes for 10 seconds at 15,700*g* and resuspending in 800µl 0.9% (w/v) NaCl, before adding 100µl 12% (v/v) bleach (NaOCl), mixing by inversion and pelleting by centrifugation as before. Nematodes were then washed 3 times in 0.9% (w/v) NaCl by resuspending and pelleting as above. Nematode numbers were estimated by counting the numbers in 5x2 µl spots under 10x magnification using a binocular microscope (Cambridge Instruments). An average of 50 nematodes were added to each plate (the same number to each plate within each replication of the experiment). After the nematodes were added the plates were wrapped in parafilm and incubated at 25^{0}C. Nematode production was measured by monitoring the development of IJ nematodes on the plates under a binocular microscope. On completion of the assay, IJ nematodes were recovered by washing of the petri dish lid with sterile PBS.

Phenotypic Test	Expected Phenotype Primary	Secondary
Luminescence	+++	-
Pigmentation (on LB)	Orange/Yellow	Cream/White
Colony consistency	mucoid	non-mucoid
Lipase activity	+++	-
Protease activity	+++	-
Haemolysin activity	+++	-
Antibiotic activity	+++	-
Crystal production	yes	no
Colour on LBTA	green	red
Congo Red clearing	+++	-
Nematode production	yes	no
Virulence (*Galleria*)	virulent	virulent

Table 2.3 Phenotypic tests carried out on *Photorhabdus temperata* K122 and the observed phenotype of the primary and secondary variants for each trait.

2.4.9 Virulence assay

To establish the insect killing ability of *Photorhabdus*, 1ml of the relevant overnight culture was centrifuged at 15,700*g* for 1 minute and washed twice in sterile 1X phosphate buffered saline (PBS). To ensure equivalence between inocula, the OD_{600nm} was taken and each strain adjusted to an $OD_{600nm} = 1$, corresponding to approximately $2x10^8$ cells/ml (Clarke, 1993). The suspension was further diluted to allow an injection concentration of 10 cell/µl. Using a Hamilton syringe, 10µl were injected directly into the haemocoel of each of 10 *Galleria mellonella* larvae, which were then incubated at 25^0C. Control insects were injected with 10µl sterile PBS. The insects were monitored for insect death. Virulence was measured by comparing the LT_{50} (the time taken for 50% of the larvae to die) between variants.

2.4.10 Growth Curves

Liquid overnight cultures were prepared for each of the strains to be analysed. Each culture was diluted in 100 ml fresh liquid LB to a final OD_{600nm} of 0.05 and subsequently incubated at 30^0C with agitation at 25*g*. Samples were removed at regular intervals for OD_{600nm} measurement.

2.5 Basic molecular techniques

2.5.1 Genomic DNA Extraction

Except where otherwise indicated, DNA was extracted using a method adapted from the miniprep isolation method of Ausubel *et al.* (1998). 3 ml cells were pelleted by centrifugation (15,300*g*, 5 minutes) and resuspended in 560 µl TE buffer (10 mM Tris, 1 mM EDTA, pH8.0). 60 µl 10% (w/v) SDS and 10 µl 20 mg/ml Proteinase K was added and the sample mixed and incubated for one hour at 37^0C. 100 µl 5M NaCl was added and the sample mixed thoroughly by vortexing. 80 µl CTAB/NaCl solution (10% (w/v) hexadecyltrimethyl

ammonium bromide in 0.7 M NaCl) was added and the sample was mixed thoroughly and incubated for 10 minutes at 65^{0}C. 700 μl chloroform/isoamyl alcohol (24:1) was added and the sample mixed and centrifuged at 2,300*g* for 5 minutes. The aqueous supernatant was removed, extracted with an equal volume of phenol/chloroform/isoamyl alcohol 25:24:1 and centrifuged at 2650*g* for 5 minutes. The supernatant was removed and 0.6 volumes isopropanol added to precipitate the DNA. The sample was centrifuged at 15,300*g* for 30 minutes at 4^{0}C. The pellet was then washed in 300 μl 70% (v/v) ethanol and centrifuged at 15,700*g* for 15 minutes at 4°C. The pellet was resuspended in 100 μl sterile distilled water (SDW).

The concentration and purity of the DNA was determined on a spectrophotometer as described by Sambrook *et al.* (1989).

2.5.2 Plasmid DNA Extraction

Plasmid DNA was prepared using the Wizard® Plus SV miniprep DNA purification system (Promega) for small volumes and the QIAGEN Plasmid midi kit (Qiagen) for large volumes. Kits were used as per manufacturers instructions.

2.5.3 Electrophoresis of DNA

DNA was electrophoresed on 0.7% or 0.1% (w/v) agarose (depending on fragment size) in 1x TAE (1 l of 50x = 242 g Tris, 57.1 ml acetic acid, 100 ml 0.5 M EDTA pH 8.0) or 1xTBE (1 l of 5x = 54 g Tris, 27.5 g boric acid, 20 ml 0.5 M EDTA pH 8.0) buffer (Sambrook *et al*., 1989) 10 mg/ml ethidium bromide stock was added to gels to give a final concentration of 0.5 mg/ml. The loading buffer used was 6x Blue/Orange Loading Dye (Promega Corporation), used at 1x concentration. The marker used was 1kb DNA ladder (Promega Corporation). Gels were electrophoresed at 50-100 V until the dye had reached the required distance from the wells, then visualised by UV light and photographed.

2.5.4 RNA Extraction

The mucoid nature of *Photorhabdus* bacterial colonies results in difficulties in dispersion in liquid cultures (Boemare & Akhurst, 1988). An adapted protocol was used for RNA extraction. Buffers used in RNA work were treated to remove RNase by adding diethyl pyrocarbonate (DEPC) to 0.01%, leaving overnight with stirring at room temperature and autoclaving to degrade DEPC.

5ml of the relevant culture grown to OD_{600nm}= 0.5 were mixed with 2ml of STOP Reaction solution (5% phenol pH 4.3, 95% Ethanol) in a 15ml Falcon tube and incubated on ice for 15 minutes. After centrifugation (2,320*g*, 5 minutes, 4^0C) the cell pellet was flash frozen on dry ice with ethanol and stored at -80^0C until required.
The pellet was resuspended in 1.4ml of freshly made, pre-warmed (65^0C) Lysis Buffer (0.1M Sucrose, 10mM Sodium acetate pH 4.2, 1.5% SDS, 30mM EDTA). 700µl of this suspension was pipetted into each of 2 x 2ml eppendorf tubes, which were then incubated for 10 minutes at 65^0C. To each of these, 700µl pre-warmed acidic phenol (pH 4.3) were added and mixed well by vortexing, followed by incubation for a further 3 minutes at 65^0C. After cooling on ice for 3 minutes, the phases were separated by centrifugation (15300*g*, 5 minutes, 4^0C). This phenol extraction was repeated on the samples a further 4 times. If required, 100µl DEPC treated water was added to aid with cleaning and recovery.

Once these extraction steps were completed, 700µl acidic phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature were added. The samples were vortexed for 20 seconds and centrifuged (15,300*g*, 5 minutes, 4^0C). 700µl room temperature chloroform:isoamyl alcohol (24:1) were then added, followed by further centrifugation (15,300*g*, 5 minutes, 4^0C).
The aqueous phase was separated and 0.1X volumes (~35µl) 3M Sodium acetate (pH 5.2) added. After mixing, 2.5X volumes of absolute ethanol were added. The samples were left at -80^0C overnight to allow RNA to precipitate. Following this, the RNA was pelleted by centrifugation (15,300*g*, 30 minutes,

4^0C). The supernatant was removed and the pellets allowed to air-dry for 15 minutes before resuspension in 50μl DEPC-treated water. The RNA was then checked for quantity and quality by spectrophotometer (OD_{260nm}) and by loading samples on a 1.2% non-denaturing TAE agarose gel respectively.

Where required, DNaseI treatment was carried out by addition of 5μl DNaseI buffer (10X), 5μl DNaseI (1U/μl) to a 50μl reaction containing 50-100μg RNA with incubation at 37^0C for 30 minutes. 650μl DEPC-treated water was then added and an acidic phenol extraction as described above was carried out.

2.5.5 Electrophoresis of RNA

All buffers and equipment were sterilized as for RNA extraction. RNA electrophoresis was performed using 1.8% (w/v) agarose gels containing 1x MOPS buffer (10X = 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA) (Sambrook *et al*., 1989), and 2% (v/v) formaldehyde (from a 37% (v/v) stock, added after the gel had been melted and cooled to 55^0C), using 1X MOPS as running buffer. RNA samples (5-10μg) were mixed with 2X RNA loading buffer (1.5x MOPS, 3.3% (v/v) formaldehyde, 60% (v/v) formamide, 16% (v/v) 6X Blue/Orange Loading Dye (Promega)), heated at 65^0C for 10 minutes and immediately cooled on ice. 15 to 20 μl each sample were loaded and gels were run at 90 V for 2-3 hours until the rapid blue front had run 2/3 the length of the gel. The 1X MOPS running buffer was circulated regularly to prevent a pH gradient occurring across the gel.

2.5.6 Northern Blot Analysis

The extracted RNA was transferred from the agarose gel, to Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech), by capillary transfer as described by Sambrook *et al*., 1989, using 20X SSC buffer (3M Sodium chloride, 0.3M Sodium citrate, pH 7.0) overnight. The total RNA was fixed to the membrane by applying 1300 KJoules to crosslink it. Before application of the probes, the membrane was allowed to prehybridize with 20ml pre-heated Emilson-Kurland

buffer solution (900mM NaCl, 90mM Tris-HCL pH7.5, 9mM EDTA, 1% SDS, 1mg/ml polyvinylpyrrolidine, 1mg/ml Ficoll, 200μg/ml salmon sperm DNA) for 2 hours at 65^0C in a Hybaid oven.

The probes to be used - 5S RNA probe (5'-ACTACCATCGCCGCTACGGC-3') as a loading control, and *E. coli csrB* (prepared by gel purification of an *Eco*RI and *Bam*HI excision from plasmid pIM5) were prepared using the random labelling technique as follows:

5SRNA: 1μl 5S RNA probe was added to 15μl SDW, boiled for 5 minutes and placed on ice. To this was added 2μl kinase buffer, 1μl T4 kinase and 1μl α^{32}P dATP and the resultant reaction mix was incubated at 30^0C for 30 minutes, followed by incubation at 65^0C for 20 min.

csrB: This probe was radiolabelled in the same way as detailed above however 3μl of probe and 11μl SDW were used.

After the probes had been prepared, they were each applied to a separate pre-equilibrated (TE buffer) sephadex column and 6 x 200μl aliquots of TE buffer were applied to remove unincorporated radioactivity. In each case, Fractions 3 and 4 were retained as this contained the cleaned, radiolabelled probe, ready for use in hybridization.

After pre-hybridisation in Emilson-Kurland buffer was complete, the relevant probe was syringe-filtered into the cylinder and incubated at 65^0C for 1 hour. The temperature was then reduced to 30^0C over 5 hours. The membrane was then washed twice in 6X SSC at room temperature and then washed twice for 30 minutes at 56^0C with 3X SSC containing 0.5% SDS. The membrane was then covered in cling wrap and exposed to film overnight. This process was repeated for the second probe. Between applications of the two probes, the membrane was stripped by placing into a lidded, plastic microwavable container and washing in stripping buffer (Boiled H_20, 0.5% SDS) for 20 minutes with microwaving on the maximum Defrost setting.

2.5.7 Western blotting and analysis

Overnight cultures of the strains to be analysed were diluted to OD_{600nm} = 0.4 then pelleted by centrifugation and resuspended in sterile PBS and Sample buffer (2X stock 125mM Tris pH6.8, 10β-mercaptoethanol, 4% SDS, 20% glycerol, 0.05% Bromophenol Blue). 12.5% Gels were run on a Mini-Protean 3 electrophoresis cell (BioRad) and prepared as follows:
Resolving Gel: 2.5ml Buffer A (0.75 Tris pH8.8, 0.2% SDS), 1.56ml 40% Acrylamide solution, 0.25ml bis-Acrylamide, 0.69ml H_20, 25µl Ammonium persulphate, 7µl TEMED. (TEMED is added last).
Stacking Gel: 1.0ml Buffer B (0.25Tris pH6.8, 0.2%SDS), 0.25ml 40% Acrylamide, 0.6ml H_20, 0.14 2% bis-Acrylamide, 12.5µl Ammonium persulphate, 3.5µl TEMED.

The running buffer used was 25mM Tris, 250mM Glycine (pH8.3), 0.1% SDS. Gels were run at 120V until entry into the resolving gel and then at 150V until complete. Transfer was carried out as per the Mini-Protean directions using transfer buffer (20mM Tris, 150mM glycine, 0.02% SDS, 20% Methanol) Transfer was carried out using 80V at 4^0C for 1 hour 10 minutes.
The membrane was blocked for 1 hour by incubation at 4^0C in PBS 5% (w/v) milk powder (Marvel) with agitation. Primary antibody (anti-*hexA*) was applied in PBS 5% milk overnight at 4^0C with agitation (2µl Antibody in 10ml milk). The membrane was rinsed with PBS, 3 times for 15 minutes. Secondary antibody was applied in PBS 5% milk for 1.5 hours (10µl Anti-rabbit HRP in 30ml milk), followed by a further 3 x 15 minute PBS washes and detection was carried out using the chemiluminescent components of the ECL system (Amersham Pharmacia).

2.5.8 Southern blotting and analysis

Genomic DNA from phenotypic mutant strains was restricted with the *Eco*RV restriction enzyme. The reaction was RNase treated to remove any potential interference from RNA present in the sample. The resultant restricted DNA was electrophoresed on 1% agarose TAE gel, along with molecular weight marker and the excised pUTkm kanamycin cassette as a control. The gel was placed into denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 minutes at room temperature with gentle agitation on a rocker plate. The solution was discarded and a further volume was added and the gel was incubated for a further 15 minutes. The denaturing solution was discarded and replaced with Neutralisation solution (1M Tris pH7.4, 1.5M NaCl) and left for 30 min. at room temperature with gentle agitation as before. This solution was replaced with a further aliquot of Neutralising solution and left for a further incubation period of 15 minutes. The treated gels were then subjected to overnight capillary transfer to nitrocellulose membrane using 20X SSC as described in section 2.4.6. After transfer, the membrane was exposed to UV at 1200 Joules in a Crosslinker to fix the DNA to the membrane. The membrane was placed in a hybridization cylinder and pre-hybridised at 60^0C for 1 hour in filter-sterilised 6X SSC, 5X Denhardt's (50X stock = 1% ficoll, 1% polyvinylpyrrolidine, 1% bovine serum albumin, in distilled H_20, filter-sterilised, diluted to 5X in 6X SSC), 0.5% SDS, 100μg/ml salmon sperm DNA. A radiolabelled probe was prepared via PCR amplification of the kanamycin cassette of plasmid pUTkm2 using primers HB015 (5'- GTTATGAGCCATATTCAACG – 3') and HB016 (5'-TCGAGCATCAAATGAAACTGC -3'). The probe was radiolabelled using the random labelling technique as described in section 2.4.6. The probe was then hybridised to the membrane overnight for 14 hours and then membrane was washed at 55^0C with 2X SSC. This solution was discarded and a second wash was carried out with 0.2X SSC, 0.5% SDS a 60^0C for 20 minutes. The membranes were then exposed to film.

2.5.9 Restriction Digests

Restriction digests were carried out using restriction enzymes and buffers supplied by New England Biolabs. The conditions used were as recommended by the manufacturer. If appropriate, digests were inactivated by heating to 65°C for 15 minutes. If the products were to be used in ligations, reactions were cleaned by extracting twice with 1 volume phenol/chloroform 1:1 and precipitated in 3 volumes ethanol, 0.1 volumes 3M Sodium acetate pH 5.3 with centrifuging at 15,700*g* at 4^{0}C for 30 minutes, then washing by addition of 150 μl 70% (v/v) ethanol and centrifugation at 15,700*g* at 4^{0}C for 15 minutes, before resuspension in an appropriate volume of SDW.

2.5.10 Blunt-ending of Restriction Products

Where required, blunt-ending was carried out by incubation of fragment with Klenow polymerase, along with dNTPs as described in the manufacturer's protocol (Promega Corporation), before cleaning as for restriction digests.

2.5.11 Ligations

Cut vectors, or inserts excised from plasmids, were cleaned by phenol/chloroform extraction as described for restriction digestion. If the insert/vector needed to be separated from fragment(s) generated by restriction digestion, the insert/vector mix was run on a 0.7% agarose gel in TAE buffer and the desired band purified from the gel using the QIAquick Gel Extraction Kit (Qiagen) as per the manufacturer's instructions.

Vector DNA was dephosphorylated using shrimp alkaline phosphatase (SAP) (Roche Corporation). 1U SAP was added directly to inactivated restriction digests or to cleaned DNA (1-5μg DNA), in SAP buffer. The samples were incubated for 10 minutes at 37°C or, if the fragments were blunt-ended, for 60 minutes at 37°C and the SAP was then inactivated by heating to 65°C for 15

minutes. If necessary, the DNA was concentrated by precipitating in ethanol and resuspension in sterile distilled water.

Ligations were carried out using the ratios (vector:insert) of 1:5, 1:3, 1:1 and 3:1. Ligations using cut vector and cut dephosphorylated vector only were also performed as controls. Ligation reactions were performed in 10μl volumes using 1U T4 DNA ligase (Promega) per reaction, with an appropriate amount of T4 ligase buffer. Reactions were incubated at 16^0C overnight, cleaned by extracting with phenol:chloroform (1:1) and precipitated with ethanol as described for restriction digestions, before resuspension in sterile distilled water. Ligations were transformed into cells, with uncut vector used as a control.

2.5.12 Transposon mutagenesis

Transposon mutagenesis of the *Photorhabdus temperata* K122 strains was carried out using the *E. coli* S17-1/pUTkm conjugable suicide mini-Tn*5* delivery system as detailed below.

2.5.13 Conjugation of *Photorhabdus temperata* K122

500μl of the relevant overnight *Photorhabdus* culture was inoculated into 25ml $LBRif_{100}$ and incubated at 30^0C with shaking at 25*g* until log phase growth (OD_{600nm} = 0.5) was achieved. Concurrently, 500μl of an overnight culture of the kanamycin, ampicillin resistant *E.coli* S17-1/pUTkm strain was inoculated into 25ml $LBAmp_{100}Km_{50}$ and incubated a 37^0C with shaking at 25*g* until log phase (OD_{600nm} = 0.5) was achieved. When both cultures were ready, 1ml of the *E.coli* culture and 4ml of the *Photorhabdus* culture were pelleted in individual eppendorf 1.5ml tubes by centrifugation (5900*g*, 2 minutes). The cultures were washed with 200μl LB and repelleted by centrifugation as before. The *Photorhabdus* pellets were resuspended together in 200μl LB by pipetting. The *E. coli* culture was also resuspended in 100μl LB by gentle pipetting. Both cultures were mixed in one eppendorf tube (total volume ~300μl) and the resultant solution was pipetted onto a dry LB plate and left overnight (16-21

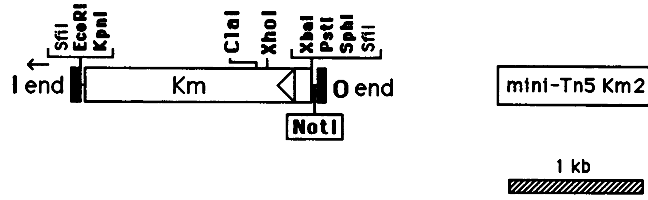
hours) at room temperature. The dried suspension was subsequently washed off with 3 x 500μl LB and mixed by gentle pipetting. This solution was then diluted 2X with LB and 100μl aliquots were spread onto $LBRif_{100}Km_{50}$ agar plates. These were incubated in the dark at 30^0C for 3 days to allow growth of exconjugants. Pigmentation was assessed after the third day.

A control conjugation was carried out using plasmids pHMW011 and pHMW014, which consisted of the kanamycin cassette in both orientations, which had been excised from pHPΩ45km via restriction with *Bam*HI and blunt end ligated into conjugable plasmid pJQ200sk. The conjugation was performed as above.

2.5.14 Cloning and sequencing of transposon insertion sites

The mini-Tn*5* transposon system (Fig. 2.1) is an ideal tool for the identification of a site of insertion/gene disruption as it has unique I and O ends (DeLorenzo *et al.,* 1990). Complementary to either end are the primers P6 (5'-CCTAGGCGGCCAGATCTGAT-3') and P7 (5'-GCACTTGTGTATAAGAGTCAG-3') which allow sequencing of the genomic region directly before and after the site of transposon insertion (Hensel *et al.,* 1995). To provide sufficient quantities of DNA for sequencing and further analysis, Eco*RV* restricted genomic DNA was ligated into the similarly restricted, dephosphorylated pBR322 plasmid vector. This vector was then electroporated into EC100 electrocompetent *E. coli* cells (see section 2.4.18 below) and the resultant transformants selected for ampicillin and kanamycin resistance, thereby indicating the presence of the correct transposon disrupted genomic DNA fragment in the vector. Such colonies were grown up overnight in selective media for plasmid purification. The plasmid DNA was rerestricted with EcoRV and run on agarose gels to give an indication of insert size. Plasmid DNA was then sent for sequencing with the primers P6 and P7 listed above, as well as with primers pBR-F (5'- GTCATCCTCGGCACCGTCACCCTGG – 3')and pBR-R (5'- CCAAAGCGGTCGGACAGTGCTCCGAG – 3') which are complimentary to the restricted ends of the pBR322 vector.

Fig. 2.1 Structure of mini-Tn*5* Km2 element.



2.5.15 Electroporation of *Photorhabdus temperata* K122

Electrocompetent cells of *Photorhabdus* cultures were prepared as follows: 200ml LB was inoculated with 2ml of an overnight starter culture and the culture incubated at 30^{0}C with shaking at 25*g* until early exponential phase was achieved (OD_{600nm} = 0.2). The cultures were chilled on ice for 90 minutes and centrifuged at 1700*g* for 10 minutes at 4^{0}C. The cells were then resuspended in 200 ml ice-cold HEPES-Sucrose buffer (1 mM HEPES pH 7.0, 5% Sucrose) and centrifuged as before. Cells were resuspended in 100ml ice-cold HEPES-Sucrose buffer, centrifuged, resuspended in 20ml ice-cold HEPES-sucrose buffer, centrifuged, and finally resuspended in 2ml ice-cold HEPES buffer and stored on ice. Competent cells had to be freshly prepared as storage at -80^{0}C is not possible, unlike electrocompetent *E. coli* cells.

For electroporation, 50µl cells and 1-5 µl clean DNA (~100ng) all chilled on ice, were mixed and placed in a pre-chilled electroporation cuvette with a 2mm gap, taking care to avoid the formation of bubbles, eliminating any by tapping of the cuvette. The cells were electroporated at 2.1kV, 100Ω, 25µFD. A time constant of ~ 4.1 indicated efficient electroporation. 1ml of pre-warmed LB (30^{0}C) was gently added to the cells and the resuspended cells were incubated in 15ml Falcon tubes at 30^{0}C with gentle shaking (15*g*) for 3 hours. Various volumes of cells (10µl cells, 100µl cells) were pipetted and spread onto selective agar plates. The remaining cells pelleted and resuspended in 100 µl LB before spreading onto the selective media. The plates were then incubated at 30^{0}C for 2-3 days.

2.5.16 Electroporation of *E. coli*

Where required, *E. coli* competent cells were prepared by inoculating 500ml of LB with 1ml from an overnight culture and incubating to 37^{0}C with shaking at 25*g* to an OD_{600nm} of 0.5-0.7 (mid exponential phase). Cultures were centrifuged at 1700*g* for 20 minutes at 4°C. The cells were then resuspended in 500 ml ice-

cold SDW and centrifuged as before. Cells were resuspended in 500 ml ice-cold SDW, centrifuged, resuspended in 250ml ice-cold SDW, centrifuged, and resuspended in 8ml sterile, ice-cold 15% (v/v) glycerol. The cells were centrifuged again and finally resuspended in 800μl sterile, ice-cold 15% (v/v) glycerol, and frozen at –80°C in 100 μl aliquots until required.

For electroporation, 40 μl cells and 1-5 μl DNA (~100ng) were mixed and placed in an ice-cold electroporation cuvette with a 2mm gap and electroporated at 2.5kV, 200Ω, 25μFD. A time constant of ~ 4.5 indicated efficient electroporation. 1ml of LB was added to the cells and the cells were incubated in 15ml Falcon tubes with shaking at 15*g* for 1 hour. The cells were then placed on selective agar as per *Photorhabdus* above. The plates were incubated at 37^{0}C for 24-48 hours.

Commercially produced EC100 high efficiency electrocompetent cells were used when cloning the transposon disrupted *Eco*RV genomic DNA fragments to sequence the region of mini-Tn*5* transposon insertion. The cells were thawed on ice prior to use and 50μl of cells were used per reaction. The electroporation conditions used were as detailed above.

2.5.17 DNA and Protein Sequence Homology Searching

Homology searching was carried out using the National Center for Biotechnology Information (NCBI) databases from the NCBI website (http://www.ncbi.nlm.nih.gov/) using the BLAST search engine (Altschul *et al.,* 1997).

Protein alignments were carried out using the EMBL-EBI ClustalW2 web interface (http://www.ebi.ac.uk/Tools.msa/clustalw2/) (Larkin *et al*., 2007; Goujon *et al*., 2010).

The Photolist web server (http://genolist.pasteur.fr/PhotoList/) was used to identify homologies/compare sequences with *Photorhabdus luminescens* TTO1.

Analysis of the *P. temperata* K122 genome contigs was carried out using the *Photorhabdus* K122 BLAST server (http://tre-biolinux.ex.ac.uk/blast/K122.html).

Contigs were annotated using the Artemis genome browser and annotation tool (http://www.sanger.ac.uk/resources/software/artemis/) (Rutherford *et al.,* 2000).

Chapter 3

3. Secondary to primary phenotypic variation

3.1 Introduction

Two distinct phenotypic variants of *Photorhabdus* have been described – termed the primary and the secondary variants, they differ as a result of changes in physiology and in exoprotein production between the two genetically identical forms.

The primary variant is characterised by the production of what have been termed 'symbiosis factors' which include bioluminescence, pigment, antimicrobial agents, lipases, phospholipases and proteases. The primary variant is also motile, has a distinct colony morphology, dye adsorption pattern and develops large intracellular protein inclusions (Forst *et al.,* 1997). On the other hand, the secondary variant is lacking, or has severely reduced production of these symbiosis factors (Boemare & Akhurt 1988). In the absence of these factors, the mutualistic symbiosis between the bacteria and its associated nematode breaks down.

The factors identified to date which have been shown to play a role in phenotypic variation include:

- Inactivation of the *cipA/cipB* genes, which encode the crystal proteins of *Photorhabdus* NC1, creating a secondary variant like cell (Bintrim & Ensign, 1998)
- Multicopy expression of *ner*, a gene encoding a putative DNA-binding protein which causes a primary to secondary variant phenotypic switch (O'Neill *et al.,* 2002)
- Inactivation of the *hexA* gene in secondary variant cells, thereby restoring production of the primary specific phenotypes (Joyce & Clarke 2003)
- The AstR/AstS two-component signal transduction system involved in adaptation to stationary phase and in delaying onset of primary to secondary phenotypic variation (Derzelle *et al.*, 2004)

Taken together, these observations suggest the presence of a complex regulatory cascade of gene interactions involved in control of phenotypic variation in *Photorhabdus*. To gain further insight into such regulation, this study initially examined phenotypic switching in the direction of the less frequently observed secondary to primary variation.

3.2 Identification of candidate genes involved in secondary to primary phenotypic switching

Early investigations observed phenotypic switching in *P. temperata* in the direction of primary to secondary variant only. In order to investigate whether a switch can occur in the opposite direction (secondary to primary variant) Tn*5* mutagenesis was performed using the *P. temperata* K122 secondary (2^0) variant. Mutants were then screened for colonies exhibiting primary variant phenotypic characteristics. An initial screen for the production of pigment was used. Rifampicin resistant *P. temperata* K122/2^0 was conjugated with *E. coli* S17*λpir* carrying the pUTkm2 suicide plasmid containing a transposable (mini-Tn*5*) kanamycin resistance cassette (see 2.4.15 Materials and Methods). Exconjugants were initially screened for the production of pigment (via cell colour change) and 15 such pigment producing colonies were isolated from a screen of 28,000 mutants. These 15 mutants were then subjected to further phenotypic analyses to confirm that a secondary to primary switch had taken place (Fig. 3.1 & Table 3.1). The mutants were termed secondary mutated (Sm) 1-15.

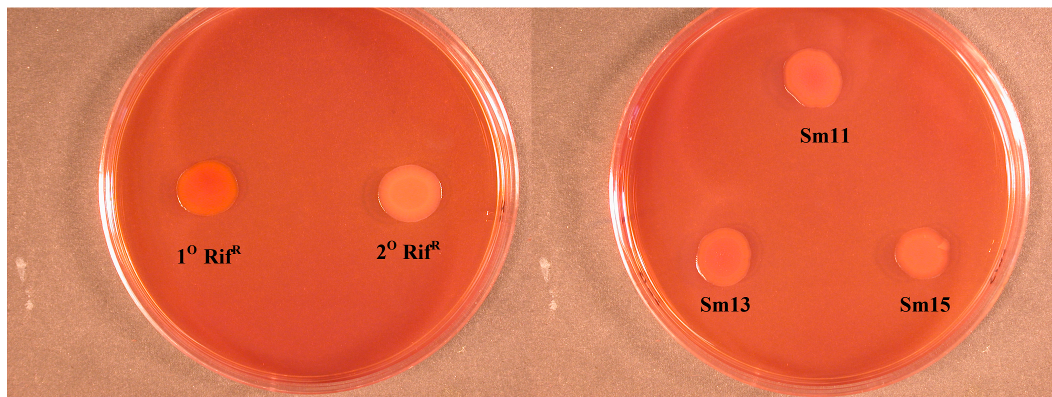
The phenotypic tests included assays for dye adsorption (NBTA; Congo Red & MacConkey Agars), extracellular enzyme production (haemolysing & lipase activity on Blood agar and Lipase agar respectively), pigment production and bioluminescence. From the data obtained, the Sm mutant strains can be separated into 3 broad classes:

1) Completely switched, stable mutants (Sm11, Sm13 & Sm15)
2) Completely switched, unstable mutants (Sm1-8, Sm12 & Sm14)
3) Partially switched, unstable mutants (Sm9 & Sm10)

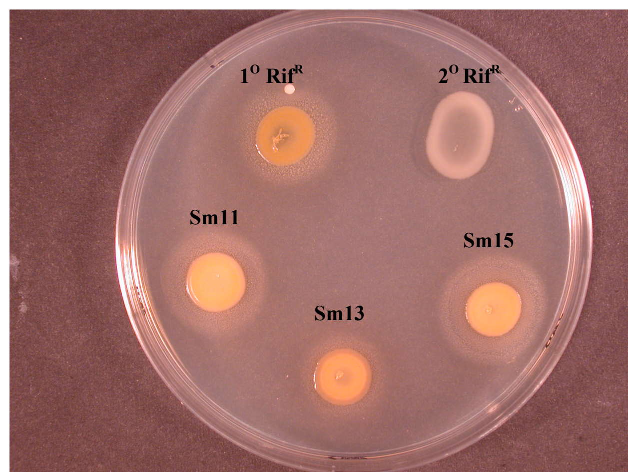
Figure 3.1 Phenotypic tests on the Sm mutants

The mutants were spotted onto different agar plates and compared to the *P. temperata* 1^0 and 2^0 Rif^R variants. Examples depicted are A) MacConkey agar; B) Lipase production agar; C) Antibiotic production (see Materials and Methods).

A)



B)



C)

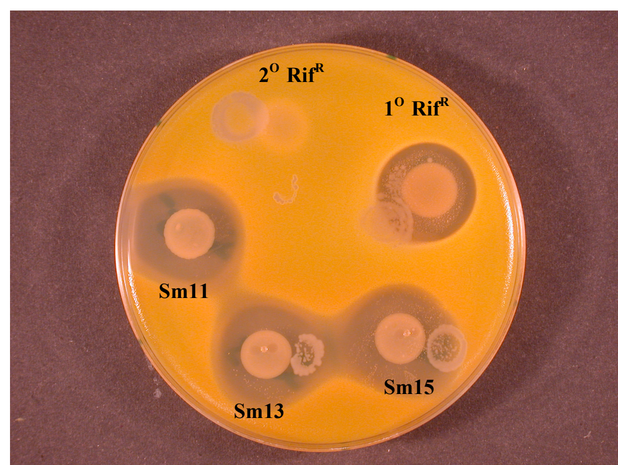


Table 3.1 Phenotypic characterization of Secondary Mutants.

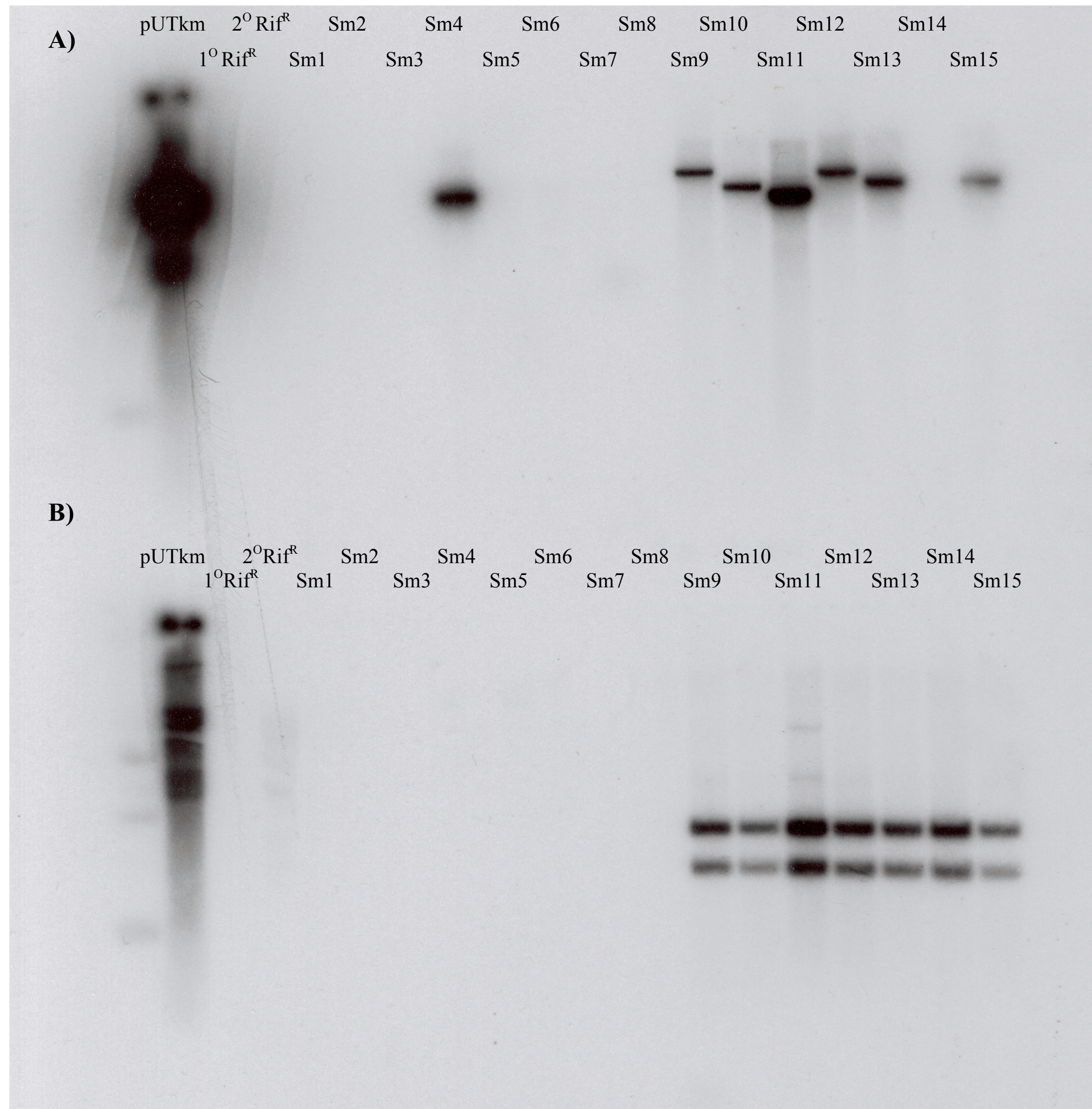
	Colour on LB[a]	Bioluminescence[b]	Colour on NBTA[c]	Colour on Congo Red[d]	Colour on MCA[e]	Activity on Blood Agar[f]	Antibiosis[g]	Lipase Activity[h]	Stability[i]
K122 1° Rif^{R}	Orange	Yes	Green	Red/Orange	Red	Haemolysis	++	++	**Yes**
K122 2° Rif^{R}	White	No	Blue	White	White	No Haemolysis	-	-	**Yes**
Sm1	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm2	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm3	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm4	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm5	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm6	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm7	Orange	Yes	Green	Red/Orange	Red	Haemolysis	++	++	No
Sm8	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	No
Sm9	Yellow/Cream	Yes	Blue	White	White	No Haemolysis	-	++	No
Sm10	Yellow/Cream	No	Blue	White	White	No Haemolysis	-	++	No
Sm11	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	**Yes**
Sm12	Orange	No	Blue	Red/Orange	White	No Haemolysis	+++	++	No
Sm13	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	**Yes**
Sm14	Orange	Yes	Blue	White	White	No Haemolysis	++++	++	No
Sm15	Orange	Yes	Green	Red/Orange	Red	Haemolysis	+++	++	**Yes**

(a) Pigment observed after 48 hours growth on LB agar. **(b)** Bioluminescence measured on a Fujifilm Intelligent Dark Box II **(c)** Colour on NBTA- Nutrient agar supplemented with bromothymol blue (BTB) and triphenyltetrazolium chloride (TTC). Phase I colonies adsorb BTB, obscuring the red colour produced by the reduction of TTC to formazan. Phase II colonies do not. **(d)** Congo Red Agar- LB agar with 0.01% Congo Red, a planar hydrophobic compound that binds lipids and lipoproteins. Differences in Congo Red staining between strains has been interpreted to indicate differences in cell wall components, which in turn may lead to changes in virulence and drug susceptibility **(e)** Colour on MacConkey Agar (MCA) – Phase I colonies are red due to adsorption of the neutral red dye in the medium. Phase II colonies remain off-white **(f)** Activity phase on Blood Agar - Phase I strains have a partial (α) or total (β) haemolysis resulting in a green or clear halo respectively. Phase II cells give no halo or a partial (α) halo dependent on the result obtained from the Phase I test strain. **(g)** Antibiosis – 2 day old *Photorhabdus* cultures that have been spot inoculated onto LB agar plates are overlayed with a soft agar containing an inoculum of 24hr old *Micrococcus luteus*. After incubation a clear zone of inhibition around the *Photorhabdus* colony indicates antibiotic production. Phase I colonies produce much higher quantities of agar diffusible antibiotics than Phase II **(h)** Lipase activity – Cultures are grown on a calcium agar incorporating Tweens (fatty acids combined with polyoxyethylenesorbitan). The fatty acids form a white precipitate indicative of lipolysis around colonies producing lipases. **(i)** Stability – refers to the reculturability of the strain without the occurrence of variations in phenotype

Class 2 and 3 mutants were prone to complete phenotypic reversion upon re-culturing on solid media, or after exposure to the freeze-thaw process associated with the maintenance of frozen bacterial culture stocks. Southern analysis of the Eco*RV* restricted genomic DNA of the 15 mutants (Fig. 3.2) using the 1.8kb mini-Tn*5* as a probe, revealed a single transposon insertion event in mutants Sm9, 10, 11, 12, 13, 14 and 15. No intact Tn*5* insertion was detected in the other mutants (Sm1-8) by the probe, in agreement with the unstable nature of these mutants. These strains were omitted from further study.

In conclusion, transposon mutagenesis and analysis of >20,000 *P. temperata* K122/2^0 clones resulted in the isolation of 3 stable secondary to primary phenotypic variants (Sm11, 13 & 15). In addition, 4 mutants displayed partially switched phenotypes (Sm9, 10, 12 &14). This chapter focuses on Sm11, 13 & 15 as these mutants were subjected to further investigation with the aim of identifying a 'master' switch or regulator for phenotypic variation in *Photorhabdus temperata* K122.

Figure 3.2 Southern analysis of Secondary mutants.



A) Eco*RV* restriction of genomic DNA. B) Hind*III* restriction of genomic DNA to confirm integrity of tn*5* insert. Hind*III* cuts within the transposon, so two bands should be detected.

3.3 Growth and bioluminescence

The phenomenon of bioluminescence normally occurs only in the primary variant as high cell densities are achieved on entry into stationary phase, indicating some form of temporal or cell-density dependent regulation (Frackman *et al.,* 1990; Daborn *et al.,* 2001). Bioluminescence in other bacteria is controlled through quorum sensing pathways which, with the exception of *luxS*, are absent in *Photorhabdus* species.

Growth of the Sm mutants generated in this study was similar to the wild type primary and parent secondary variants (Fig 3.3) however, bioluminescence was not restored to wild-type primary variant levels and appears to drop to lower levels in stationary phase compared to the primary variant, but is nonetheless, greater than those of the parental secondary variant (Fig. 3.4). Unlike the BMM215 *hexA* mutant generated by Joyce and Clarke (2003) however, where it was reported that bioluminescence was constitutive and independent of cell growth phase, mutants Sm13 and Sm15 display a bioluminescent profile more akin to that of the primary variant, with the major increase in luminescence beginning upon entry into stationary phase, building to a peak and tapering off during late stationary phase.

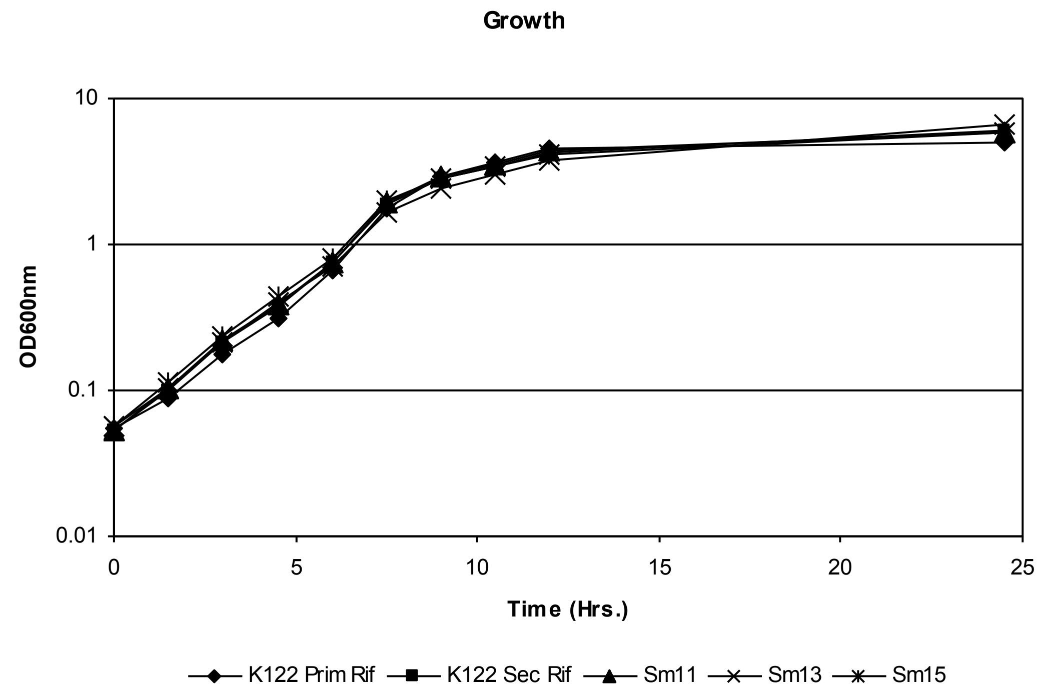


Figure 3.3. Growth curve of *P. temperata* K122 1° and 2° Rif^{R} variants with mutants Sm11, 13 & 15 compared to both phases of wild type strain. Cultures were grown overnight and inoculated (1:100) into fresh LB broth. Growth of the bacteria at $28^{0}C$ was measured by observing the OD_{600nm} of the culture over time.

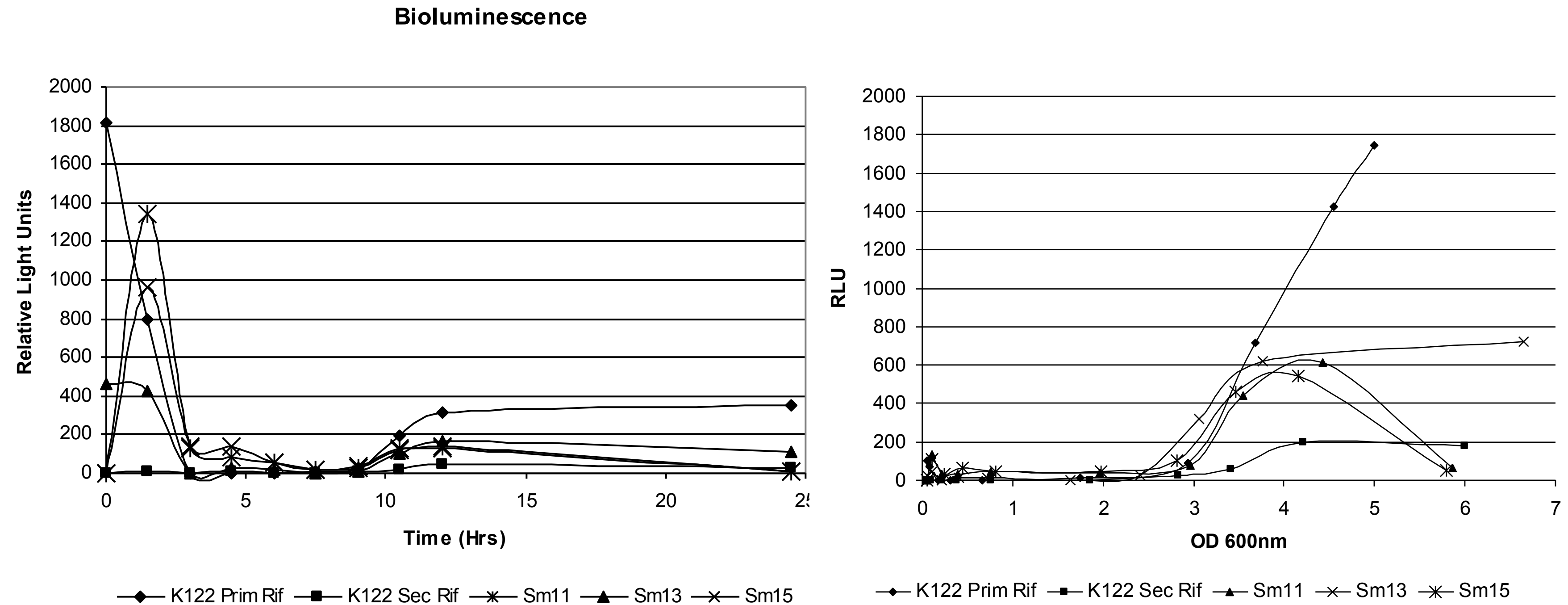


Figure 3.4, Bioluminescence of mutant strains compared to wild type parent Rif^R strains, shown as Relative light Units vs. Time (Left) and Relative light units vs. OD600nm (Right)

Bioluminescence data comparing primary like mutants to wild type strains. Relative Light Units (RLU) are a function of bioluminescence units per OD_{600nm} of cells, where an $OD_{600nm} = 1$, corresponding to approximately $2x10^8$ cells/ml (Clarke, 1993). The initial luminescence seen is as a result of inoculating test cultures from stationary phase overnights. Levels of luminescence of test cultures are expected to be higher overnight (12-20hrs – readings not taken) returning to lower levels when final reading is taken the following day.

3.4 Identification and sequencing of sites of transposon insertion

The mini-Tn*5* transposon system is an ideal tool for the identification of a site of insertion/gene disruption as it has unique I and O ends (Fig. 2.1 Materials and Methods) (DeLorenzo *et al.,* 1990). Complementary to either end are the primers P6 (5'-CCTAGGCGGCCAGATCTGAT-3') and P7 (5'-GCACTTGTGTATAAGAGTCAG-3') which allow sequencing of the genomic region directly before and after the site of transposon insertion (Hensel *et al.,* 1995). Further more, the transposable element lacks an Eco*RV* restriction site. To provide sufficient quantities of DNA for sequencing and further analysis, Eco*RV* restricted genomic DNA was ligated into the pBR322 plasmid vector, thereby allowing cloning of the region of the transposon along with the flanking genomic DNA fragments. These vectors were then electroporated into *E. coli* EC100 electrocompetent cells and the resultant transformants selected for kanamycin resistance, thereby indicating the presence of the correct transposon disrupted genomic DNA EcoRV fragment in the vector.

Sm11 returned mainly sequence of the pBR322 vector, with only short genomic sequence fragments available for analysis indicating that the site of insertion was probably flanked by Eco*RV* restriction sites. The identification of this mutant will be discussed further in Section 3.9. Sequencing of the other two mutants however revealed that the transposon region and the flanking 3' and 5' DNA consisted of inserts into the same gene – *hexA* – encoding a member of the LysR type transcriptional regulator (LTTR) family. In Joyce & Clarke's mutant BMM215 (Joyce & Clarke 2003), the gene has been disrupted at codon 35 whilst here, Sm13 and Sm15 have been disrupted in the regions of codon 205 and codon 242 respectively (Fig 3.5). The insertion in BMM215 disrupts the *hexA* gene in the part which encodes the N-terminus helix-turn-helix DNA binding domain whilst, in mutants Sm13 and Sm15 *hexA* is disrupted in the region coding for the C-terminal domain (Fig 1.5). This difference may explain the reported differences in the bioluminescent profiles between BMM215 and the Sm mutants as a result of the production of some truncated form of HexA in Sm13 and Sm15 that retains some limited functionality.

Figure 3.5 Alignment of sequences obtained from mutant strains with that of the *Photorhabdus temperata*

```
Sm13P7     GAATAACAAT GATA.AATGN A..ATCGTCC GATAATGAAT CTCGAT..CT CGATNTGTTA
Sm15P7     TNNAACCAAT NNTTCAATGC CCAATCGTCN GCTCATGAAT ATNGAATCCC CCNAATGNCN
hexA       ........AT GATA.AATGC A.AATCGTCC GATAATGAAT CTCGAT..CT CGATCTGCTA
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     60

Sm13P7     AGAACTTTTG GTTGCTGTTG CTGATTTAAA T.ACGTTCGC AGCA.GCAGC AGCAGCAGTC
Sm15P7     ACNACTNNTG TNNGNTGTTG NTGATTTAAA TAACGTTNGC AGCCAGCAGC AGCAGCAGTC
hexA       AGAACTTTTG TT.GCTGTTG CTGATTTAAA T.ACGTTCGC AGCA.GCAGC AGCAGCAGTC
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     120

Sm13P7     .TGCAGAACG CAA.TCGCTT GTAAGTCAGC AAATGCAACG T.TTGGAGCA GTTGGTGGGC
Sm15P7     TTGCAGAACG CCACTCGNCT GTAAGTCAGC AAATGCAACG TTTTGGAGCA NTTGTTGGGC
hexA       .TGCAGAACG CAA.TCGGCT GTAAGTCAGC AAATGCAACG T.TTGGAGCA GTTGGTGGGC
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     180

Sm13P7     AGAGAGTTGT TTGCCCGTCA CGGCNGTAAT AAGCTTCTTA CAGAGCATGG TCTTCAACTT
Sm15P7     AGNGANTTGT TTGCCNGTNA CGGCGGTAAT AAGCTTNTTA CAGAGCATGG TCNTCAACTT
hexA       AGAGAGTTGT TTGCCCGTCA CGGCCGTAAT AAGCTTCTTA CAGAGCATGG TCTTCAACTT
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     240

Sm13P7     CTTGGCTATG CAAGACAGAT CCTGCGCGCT .AATGATGAT GCCAGTGCAT CATTAACCTA
Sm15P7     CTTGGCTATG CAAGACAGAT CTTGCGAGCN TAATGATGNT GCCAGTGCNT CATTAACCTA
hexA       CTTGGCTATG CAAGACAGAT CCTGCGCGCT .AATGATGAT GCCAGTGCAT CATTAACCTA
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     300

Sm13P7     TAGCGATGCA GAAGGCGAGT TAAGAATCGG TGCATCC.GA TGATACTGTG GATACACTGC
Sm15P7     TAGCGATGCA GAAGGCGAGT TAAGAATCGG TGCATCCCGA TGATACTNTG GATACACTGC
hexA       TAGCGATGCA GAAGGCGAGT TAAGAATCGG TGCATCC.GA TGATACTGTG GATACACTGC
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     360

Sm13P7     TCCCTTTTTT GTTAAATCGC ATCGCTTCGG TTTATCCGCG GATGGCAATA GATGTTCGTA
Sm15P7     TCCCTTNTTT GCTAAATCGC ATCGNTTCGG TTTATCCGCG GATGGCAATA GATGTTNGTA
hexA       TCCCTTTTTT GTTAAATCGC ATCGCTTCGG TTTATCCGCG GATGGCAATA GATGTTCGTA
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     420

Sm13P7     TAAAGCGAAC GCAATTTATT GAAAGTATGC TGGATAGCCA TGAGATCGAT TTGGCGTTAA
Sm15P7     TAAAGCGAAC GCAATTTCTN GAAACATGCT TGGNTANCCA TGAGATCGAN TTGGCGNTAA
hexA       TAAAGCGAAC GCAATTTATT GAAAGTATGC TGGATAGCCA TGAGATCGAT TTGGCGTTAA
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     480

Sm13P7     CAACGGCCAA GATCAGTCAT CATCCACGGA CTGTTCTGCG CTCGACACCG GTATTATGGC
Sm15P7     CAACGGCCAA GATCAGTCCN CATCCACGGA CTGTNATGCG CTCGACACCG GTATTATCGC
hexA       CAACGGCCAA GATCAGTCAT CATCCACGGA CTGTTCTGCG CTCGACACCG GTATTATGGC
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     540

Sm13P7     ATTGCGCTCC TGATTTTCAG CTACAGCCAA ACGAACCTGT GCCGCTGGTT GTGATGGATG
Sm15P7     ATTGCGCTCA TGATTTTCAG CCACAGCCAA ACGAACCTNT GCCGCTGGTT GTGATGGATG
hexA       ATTGCGCTCC TGATTTTCAG CTACAGCCAA ACGAACCTGT GCCGCTGGTT GTGATGGATG
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... .......... .......... ..........     600

Sm13P7     AAACAAACCC ATTCCGTCAG CTGGCGCTGG ANACGNGG.. .....AAAGG GGAAATGA..
Sm15P7     AAACAAACCC ATTCCGTCAG CTGGCGCTGG ATACGCTTGG ATGAGGCTGG GGTATCGTGG
hexA       AAACAAACCC ATTCCGTCAG CTGGCGCTGG ATACGCT.GG ATGAGGCTGG GGTATCGTGG
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     .......... .......... .......... TTTNAATN.C CATTTGCTAA GGTCCCGTGG     660

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     CGTATTGCTT ATGAGGCTGC TTCTCTGTCG GCTGTTCGCA CAGGCAGTGA ATGCAGAAGT
hexA       CGTATTGCTT ATGAGGCTGC TTCTCTGTCG GCTGTTCGCA CG.GCAGTGA ATGCAGAAGT
Sm15P6     .......... .......... .......... .......... .......... ..........
Sm13P6     CGTATTGCTT ATGAGGCTGC TTCTCTGTCG GCTGTTCGCA CG.GCANTGA ATGCAGAAGT     720

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     CGGTATTACT GCCCGTCCTT TCGGNNTGCA ACAG...... ...CTNANC. ..........
hexA       CGGTATTACT GCCCGTCCTT TGGAAATGCA AAAT...... GCTGATCTGC GTATTTTAGG
Sm15P6     .......... .......... .GTGTGNGNC TGANATNTGG CCNGATCTGC GTATTTTATG
Sm13P6     CGGTATTACT GCCCGTCCTT TGGAAATGCA AAAT...... GCTGATCTGC GTATTTTAGG     780

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     .......... .......... .......... .......... .......... ..........
hexA       CGAGAGTGAA GGGCTTCCTC GTTTGCCTGA AACCCAGTTC TCTCTATATA GCCATAGTAA
Sm15P6     CGAGAGTGAA NGGCTTCCTC GTGTGCCTGA AACCCAGTTC TCTCTATATA GCCATAGTAA
Sm13P6     CGAGAGTGAA GGGCTTCCTC GTTTGCCTGA AACCCAGTTC TCTCTATATA GCCATAGTAA     840

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     .......... .......... .......... .......... .......... ..........
hexA       TGAACAGAAT GAATCTGTTC TGACTGTTTT TAGTGCGATA GAGAATAAGA AAACCCCATA
Sm15P6     TGAACAGAAT GAATCTGTTC TGACTGTTTT NAGTGCGATA NAGAATAANA AANTNCCATA
Sm13P6     TGAACAGAAT GAATCTGTTC TGACTGTTTT TAGTGCGATA GAGAATAAGA AAACCCCATA     900

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     .......... .......... .......... .......... .......... ..........
hexA       CACTATATCG GGT.GTTTCT GAAGAACCGC TTTCTGATGA CGCACAAGTATT ATTGATGT
Sm15P6     CACGATATCG GGTTGTTTCT GNATANCCGC TTTCGGATGA CGCACATATT ATTNNTGAGT
Sm13P6     CACTATATCG GGT.GTTTCT GAAGAACCGC TTTCTGATGA CGCACATATT ATTGATGAGT     960

Sm13P7     .......... .......... .......... .......... .......... ..........
Sm15P7     .......... .......... .......... .......... .......... ..........
hexA       AA........ .......... .......... .......... .......... ..........
Sm15P6     AATATNGANG GGACTTACTT TTGCTAATNC CTCCATTATG GATGAAAGGA GGTANTAGCN
Sm13P6     AATATCTAAG GGACTTACTT TTGCTAATAC CTNCATTATG GATGAAAGGA GGTATTAGCT     1020
```

Alignment of sequences obtained from mutant strains with that of the *Photorhabdus temperate* K122 *hexA* gene. Sequences obtained using primers P6 or P7 as indicated. Consensus is highlighted. The site of transposon insertion for each mutant is also highlighted – Green for Sm13 and Blue for Sm15.

The *P. temperata* K122 genome has been sequenced (D. Clarke, personal communication) and the region of the *P. temperata* K122 genome containing *hexA* was identified and annotated (Fig 3.6). This was compared with the same region in the sequenced genomes of *P. luminescens* TTO1 and *P. asymbiotica* ATCC 43949. The *hexA* gene is present in other enteric bacteria (named *lrhA* in *E. coli*, *X. nematophila* and *Yersinia* spp.; *pecT* in *Erwinia (Pectobacterium)* spp.) and is always found next to the *nuo* operon (encoding NADH dehydrogenase I), indicating that the *hexA* gene is part of the enteric genetic backbone.

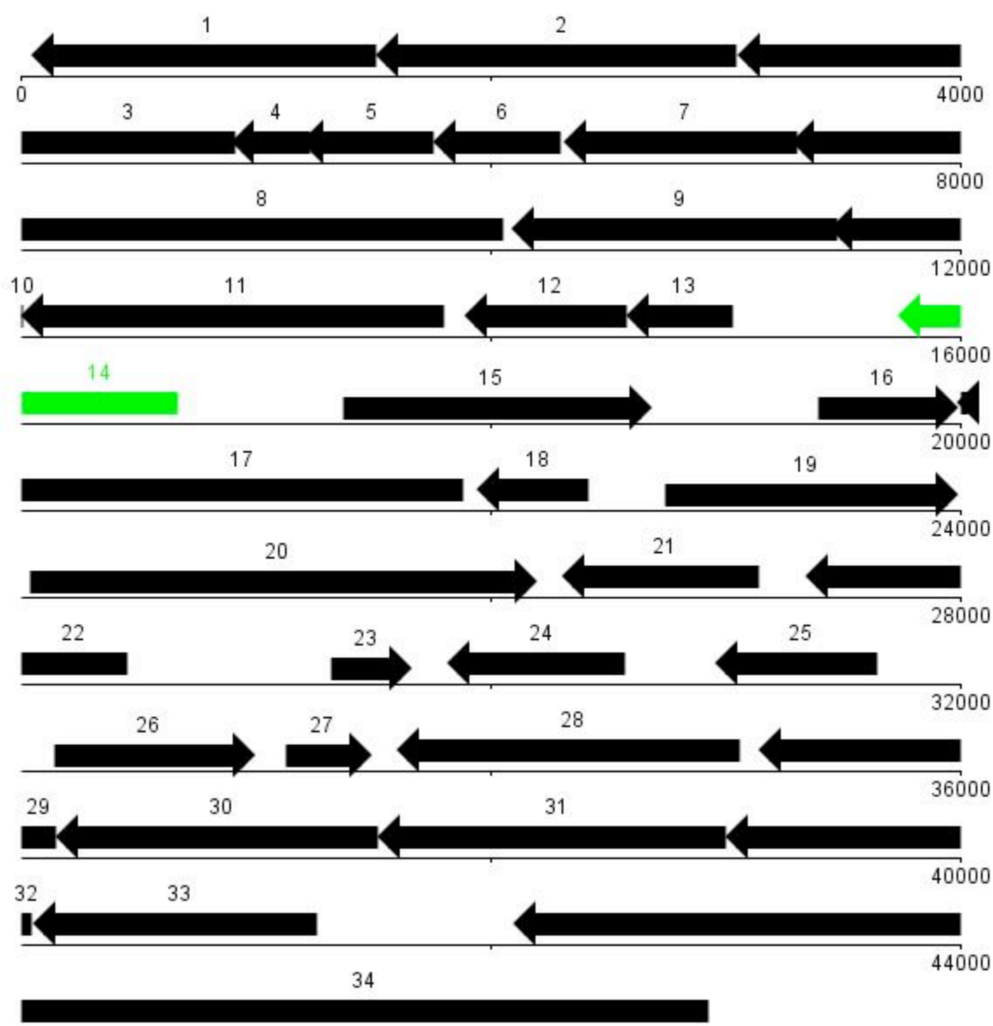


FIG. 3.6 Annotation of Genome Contig 17 of *P. temperata* K122, mutants Sm13 & 15 occur in gene 14 *hexA*, with mutant Pm55 occurring in gene 13, *nuoA*.

1) NADH dehydrogenase subunit N *nuoN*
2) NADH dehydrogenase subunit M *nuoM*
3) NADH dehydrogenase subunit L *nuoL*
4) NADH dehydrogenase subunit K *nuoK*
5) NADH dehydrogenase subunit J *nuoJ*
6) NADH dehydrogenase subunit I *nuoI*
7) NADH dehydrogenase subunit H *nuoH*
8) NADH dehydrogenase subunit G *nuoG*
9) NADH dehydrogenase subunit F *nuoF*
10) NADH dehydrogenase subunit E *nuoE*
11) NADH dehydrogenase subunit C *nuoC*
12) NADH dehydrogenase subunit B *nuoB*
13) NADH dehydrogenase subunit A *nuoA*
14) LysR Family transcriptional regulator *hexA*
15) Putative aminotransferase *alaT/yfbQ*
16) Hypothetical protein b2291/YfbR
17) Putative ion transport protein YfbS
18) Hypothetical protein b2295/YfbV
19) Acetate kinase *ackA*
20) Phosphate acetyltransferase *pta*
21) Put. inner membrane protein *yfcA*
22) Hypothetical protein
23) Plasmid stabilization protein RelE/ParE family
24) Hypothetical protein
25) Hypothetical protein
26) Hypothetical protein
27) Hypothetical protein
28) Serine/alanine/glycine abc transporter *cycA*
29) Succinyl glutamatedesuccinylase *astE*
30) Succinylarginine dihydrolase *astB*
31) Succinylglutamic semialdehyde dehydrogenase *astD*
32) Arginine n-succinyltransferase *astA*
33) Bifunctional succinylornithine transferase argM
34) Syringopeptin synthetase B *sypB*

P. temperata K122 Contig 17	*P. asymbiotica*	*P.luminescens* TTO1
1) NADH dehydrogenase subunit N *nuoN*	pau_01532	plu3077
2) NADH dehydrogenase subunit M *nuoM*	pau_01531	plu3078
3) NADH dehydrogenase subunit L *nuoL*	pau_01530	plu3079
4) NADH dehydrogenase subunit K *nuoK*	pau_01529	plu3080
5) NADH dehydrogenase subunit J *nuoJ*	pau_01528	plu3081
6) NADH dehydrogenase subunit I *nuoI*	pau_01527	plu3082
7) NADH dehydrogenase subunit H *nuoH*	pau_01526	plu3083
8) NADH dehydrogenase subunit G *nuoG*	pau_01525	plu3084
9) NADH dehydrogenase subunit F *nuoF*	pau_01524	plu3085
10) NADH dehydrogenase subunit E *nuoE*	pau_01523	plu3086
11) NADH dehydrogenase subunit C *nuoC*	pau_01522	plu3087
12) NADH dehydrogenase subunit B *nuoB*	pau_01521	plu3088
13) NADH dehydrogenase subunit A *nuoA*	pau_01520	plu3089
14) LysR Family transcriptional regulator *hexA*	pau_01518	plu3090
15) Putative aminotransferase *alaT/yfbQ*	pau_01517	plu3091
16) Hypothetical protein b2291/YfbR	pau_01516	plu3092
17) Putative ion transport protein YfbS	pau_01515	plu3093
18) Hypothetical protein YfbV	pau_01514	plu3094
19) Acetate kinase *ackA*	pau_01513	plu3095
20) Phosphate acetyltransferase *pta*	pau_01512	plu3096
21) Putative inner membrane protein YfcA	pau_01511	
22) Hypothetical protein	pau_01510	
23) Plasmid stability protein RelE/ParE family		
24) Hypothetical protein	pau_01505	plu3098
25) Hypothetical protein	pau_01503	plu3101
26) Hypothetical protein		
27) Hypothetical protein		
28) Serine/alanine/glycine abc transporter *cycA*	pau_01502	plu3105
29) Succinylglutamate desuccinylase *astE*	pau_01501	plu3106
30) Succinylarginine dihydrolase *astB*	pau_01500	plu3107
31) Succinylglutamic semialdehyde dehydrogenase	pau_01499	plu3108
32) Arginine n-sucinyltransferase *astA*	pau_01498	plu3109
33) Bifunctional succinylornithine transaminase *argM*	pau_01497	plu3110
34) Syringopeptin synthetase B *sypB*	pau_01491	plu3123

Table 3.2 List of genes on *P. temperata* K122 contig 17 with counterpart in *P. asymbiotica* & *P. luminescens* TTO1
With both the P. *asymbiotica* and P. *luminescens* TTO1 genomes fully, and sequentially annotated, gaps or non-sequential listings above indicated deletions/rearrangements that are present in the P. *temperata* K122 genome. The conservation of the *nuo* operon across species indicates its importance to the bacterial cell.

3.5 The HexA Lys-R Type Transcriptional Regulator

Whilst this work was being carried out, an independent study identified *hexA* as having a role in phenotypic variation in *P. temperata* K122. This paper confirmed the role of *hexA* in the maintenance of the secondary phenotype (Joyce & Clarke, 2003) and identified the *hexA* locus in *Photorhabdus temperata* K122 (Fig. 3.7) and through hybridisation studies, homologues were also found in the related strains *P. temperata* UK211, *P. luminescens* W14, *P. luminescens* TTO1 and the clinical strain *P. asymbiotica*. With the completion of the *P. luminescens* TTO1 genome sequencing project, a useful tool has been gained for genetic studies on the entire *Photorhabdus* species (Duchaud *et al.,* 2003). Analysis of this genome reveals *hexA* in a locus similar to that of *P. temperata* K122 (Fig. 3.8).

Given the differing methods/initial screens used to generate/isolate mutants compared to this study, it was decided to continue with the analysis of the Sm13 and Sm15 mutants to independently investigate the function of *hexA*. Differences/similarities between the sets of mutants could also help further the understanding of the actions/interactions of *hexA*.

With antibody to HexA available as a result of the work done by Joyce and Clarke (2003) Western blot analysis on late exponential/early stationary phase cultures of the Sm mutants was carried out to investigate the levels of HexA in the mutants (Fig. 3.9). As expected, the immunoblot did not detect the presence of the HexA protein in Sm13 and Sm15. The HexA protein was also not detected in *P. temperata* K122/1^0, suggesting that HexA is either not produced, or produced at a very low level in this variant. However, HexA is produced in the *P. temperata* K122/2^0 variant, adding support to the hypothesis that the *hexA* gene product has a role in repressing symbiosis factor expression and maintaining the secondary variant.

Interestingly, Sm11 gives a positive indication for HexA expression, suggesting that the secondary to primary variant switch in this strain must be occurring in a HexA-independent manner.

Figure 3.7 The *hexA* locus of *Photorhabdus temperate* K122. Adapted from Joyce & Clarke, 2003.

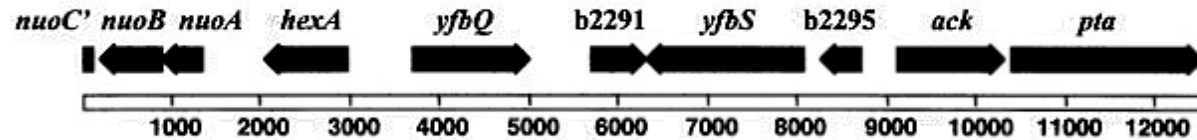
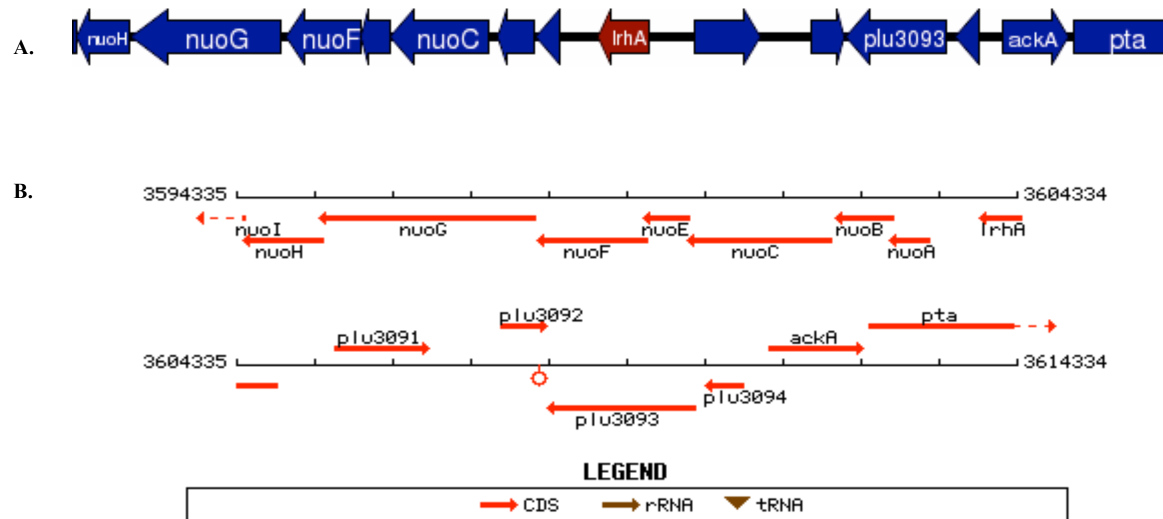


Figure 3.8. The *lrhA/hexA* locus of *Photorhabdus luminescens* TTO1.



A) Adapted from http://colibase.bham.ac.uk/.
B) Adapted from http://genolist.pasteur.fr/PhotoList/index.html. The basic gene arrangement of the locus is conserved between species.

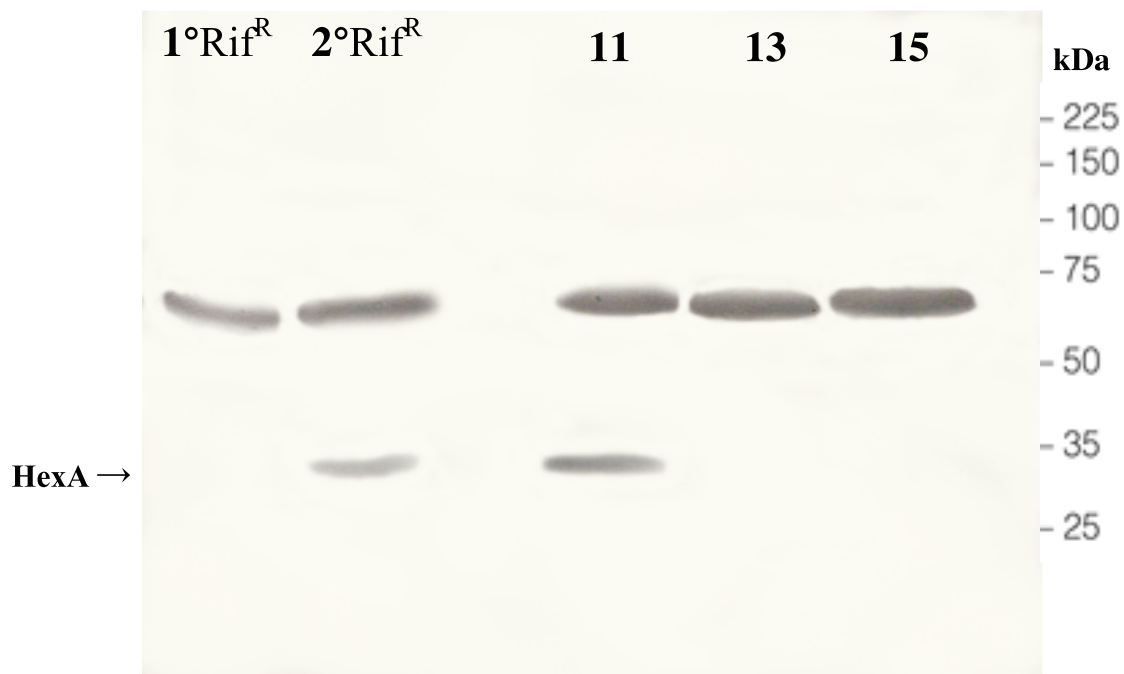


Figure 3.9 Immunoblot analysis of Sm mutants.

Cells were grown overnight in LB broth and whole cell protein extracts were separated by SDS-PAGE and proteins transferred to a nitrocellulose membrane. Membranes were incubated with a polyclonal anti-HexA antibody (a gift from S. Joyce). HexA is a protein of approximately 33kDa and is indicated by an arrow above. The second, higher molecular weight band is non-specific and can be used as a loading control.

3.6 Symbiosis and Pathogenicity

Given that it has been suggested that pathogenicity and symbiosis share common molecular mechanisms and that a bacterium-host interaction occurs as a result of reciprocal communication between the involved organisms (Hentschel *et* a*l*., 2000) it is therefore important that any genetic study of mutated *Photorhabdus* examines the effects on the pathogenic and symbiotic abilities of the bacterium. In *Photorhabdus*, the relationship between bacteria and nematode is of benefit to both organisms and as a result can be termed mutualism.

3.6.1 Mutualism

The ability to support a mutualistic symbiosis with nematodes of the *Heterorhabditis* genus is one of the major distinguishing characteristics between *Photorhabdus* phenotypic variants, with only the primary phenotypic variant capable of so doing. To assess the ability of the *Photorhabdus* Sm mutants isolated in this project to support nematode growth and development, a known number of surface sterilised nematodes was aliquoted onto lipid agar plates containing a lawn of the relevant bacterial strain. Development, growth and final yield of the nematode population was analysed to gain insight into whether or not the Sm mutants were restored to a fully functional symbiosis-supporting primary variant phenotype. From Table 3.3, it can be seen that the symbiotic phenotype has been restored in the Sm13 and Sm15 mutant strains, thereby further confirming a role for *hexA* in the regulation of the symbiosis factors produced by *Photorhabdus*. These data are also consistent with those obtained by Joyce and Clarke (2003). Mutualistic ability has also been restored in mutant Sm11, the *hexA* independent secondary to primary phenotypic variant, suggesting that *hexA* is not the only factor required for maintenance of a successful mutualistic relationship with *Heterorhabditis* nematodes.

RESULTS OF SYMBIOSIS ASSAY

Strain	IJ to Hermaphrodite	Hermaphrodite to ♀ & ♂	Fertilised Eggs	Juvenile Development	Viable IJ's returned
Primary Rif^R wt	Yes	Yes	Yes	Yes	Yes
Secondary Rif^R wt	No	No	No	No	No
Sm11	Yes	Yes	Yes	Yes	Yes
Sm13	Yes	Yes	Yes	Yes	Yes
Sm 15	Yes	Yes	Yes	Yes	Yes

Table 3.3, Analysis of nematode development in mutant strains in parallel with both Rif^R wild type variants of *Photorhabdus*

3.6.2 Pathogenicity

With both the primary and secondary variants of *Photorhabdus* being highly virulent towards a broad range of insect larvae (Dowds & Peters, 2002) it is to be expected that the Sm mutants generated in this study might also retain this insect-killing ability. To assess this, assays were performed whereby a known number (100) of each strain of bacterial cells (1^0, 2^0, Sm11, 13 and Sm15) were each injected into 10 *Galleria mellonella* larvae (Fig 3.10). From this assay it is clear that all three of the Sm mutant strains are unable to kill the insect host. This result is somewhat unusual given the retained virulence of the secondary form of *Photorhabdus*, however this data again corroborates that of Joyce and Clarke (2003) who reported that their *hexA* mutant strain was also attenuated for pathogenic ability, a phenomenon they attribute to the derepression of symbiosis factors caused by the *hexA* mutation. With *hexA*-independent mutant Sm11 also lacking pathogenic ability, this may indicate that reversion of the phenotypic switch has an affect on the bacterial strain's ability to act as an insect pathogen.

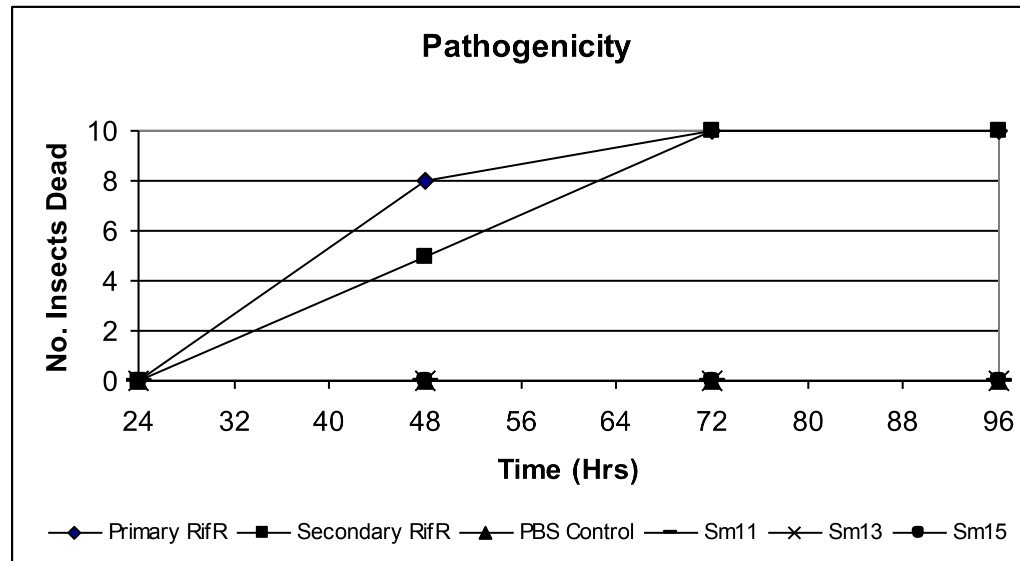


Figure 3.10. Comparison of pathogenicity of Sm mutants with *P. temperata* K122 primary and secondary Rif^R variants. 10µl of the relevant culture were injected directly into the haemocoel of each of 10 *Galleria mellonella* larvae, which were then incubated at 25^{0}C. control insects were injected with 10µl sterile PBS and the insects were monitored for insect death.

3.7 Complementation

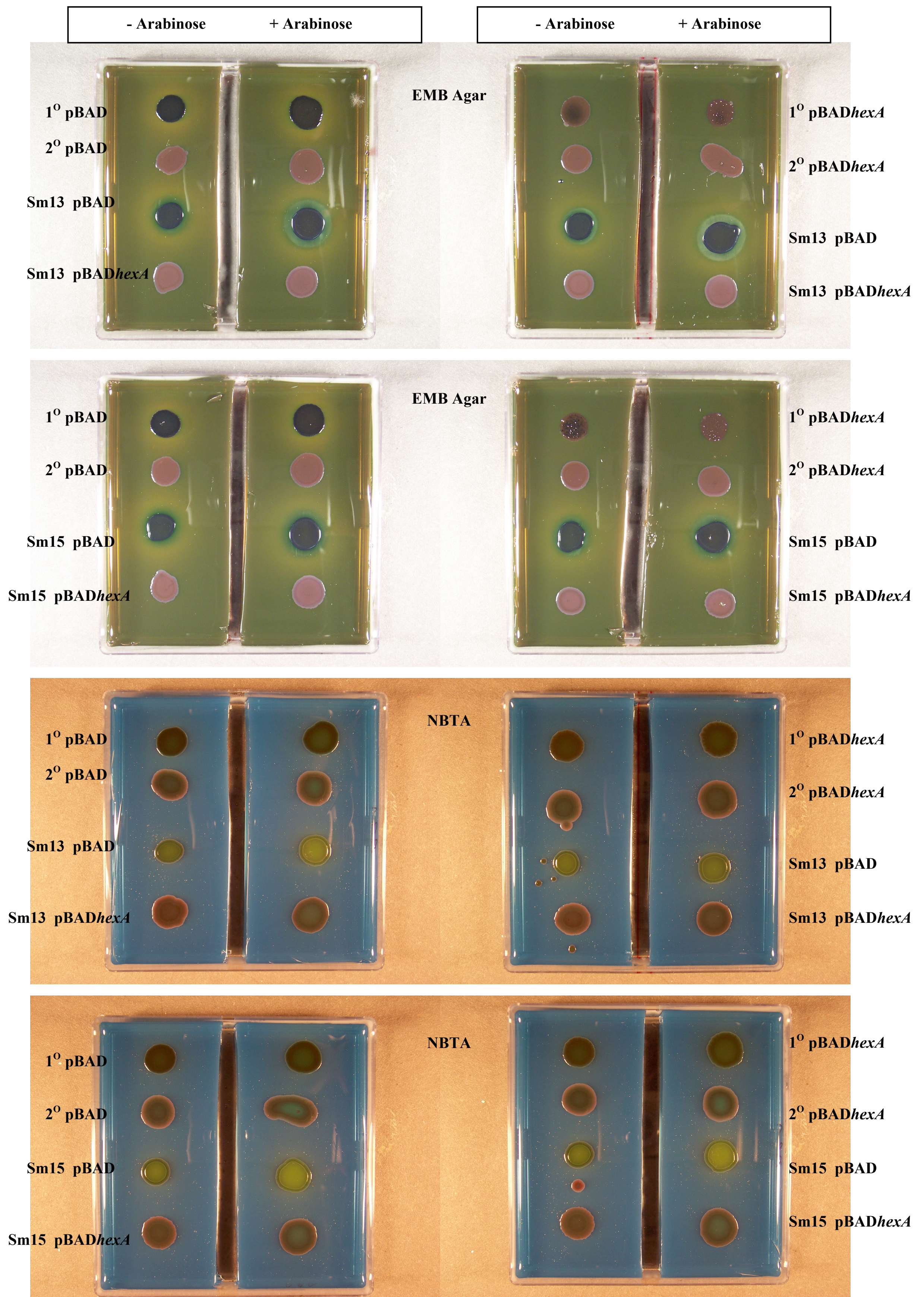
In order to confirm the role of the *hexA* in the observed phenotypic switching, complementation studies were carried out using a plasmid-borne copy of the *hexA* gene. The *hexA* gene from *P. temperata* K122 was cloned under the control of the pBAD promoter in the pBAD24 plasmid, resulting in pBAD24*hexA*, and this plasmid was transformed into the Sm mutants via electroporation. This plasmid was also transformed into the wild type primary and wild type secondary variants of *P. temperata* K122 cells to observe the effects of *hexA* overexpression. With the overproduction of HexA in the secondary being implicated in the maintenance of the secondary variant, the exogenous overproduction of HexA in the primary variant may result in the formation of a secondary variant-like colony.

Whilst the promoter of pBAD24*hexA* is arabinose inducible, it has been noted that 'leaky' expression from the promoter occurs and therefore for complementation experiments arabinose induction may not be necessary.

As a control, the plasmid pBAD24 (without *hexA*) was also electroporated into the strains. The resultant transformants were then phenotypically characterized on different agar based media in the presence and absence of arabinose (Fig 3.11, 3.12 & Table 3.4).

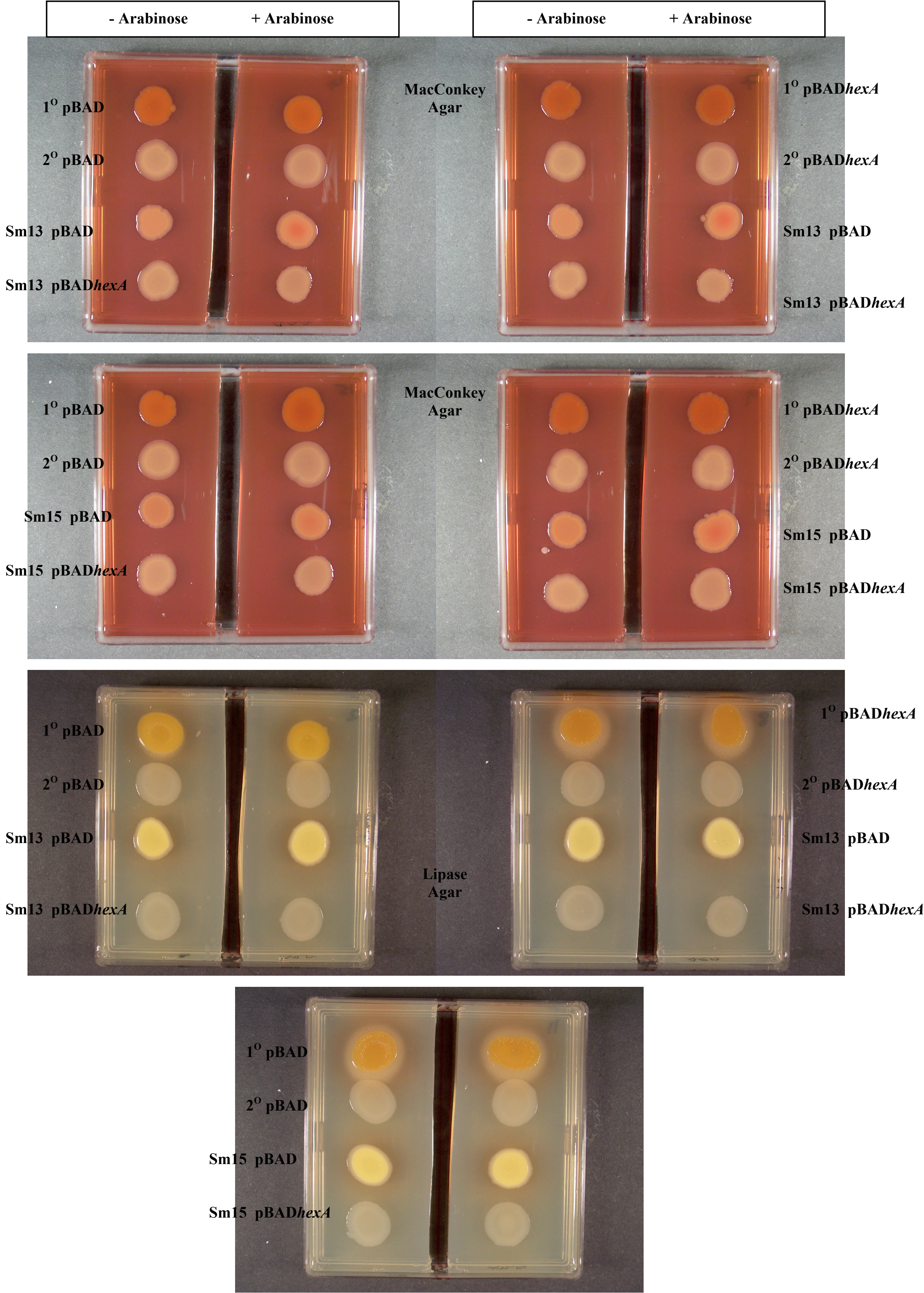
It is clear that the secondary-like phenotypes are restored in the Sm13 and Sm15 mutant strains carrying pBad24*hexA*. This is a similar situation to that of the BMM215 *hexA* mutant (Joyce & Clarke 2003), confirming the role of *hexA* expression in phase variation and symbiosis factor expression. Interestingly, for one of the phenotypes tested – dye adsorption on EMB media, the wild type primary strain expressing plasmid borne *hexA* appeared to have a slightly different adsorption profile to that of a normal wild type primary cell however exogenous expression of *hexA* in the primary variant does not result in a secondary variant-like cell. This may be as a result of insufficient levels of *hexA* expression occurring, even in the presence of arabinose-enriched media, as a result of the lack of arabinose uptake systems in *Photorhabdus*.

Figure 3.11. Examples of phenotypic testing carried out on complemented *hexA* mutants alongside Rif^R wt strains.



Arabinose activation not required for phenotypic reversion indicating residual expression from the 'leaky' promoter is sufficient to complement the gene knockout.

Figure 3.12. Examples of phenotypic testing carried out on complemented *hexA* mutants alongside Rif^R wt strains.



Arabinose activation not required for phenotypic reversion indicating residual expression from the 'leaky' promoter is sufficient to complement the gene knockout.

Strain	NBTA	LIA	MCA	EMB	Pigment	Biolum.	Antibiotic
K122 Prim Rif^R	Green/Red	++	Red	Green	Orange	+++	+++
Prim pBAD	Green	++	Red	Green	Orange	+++	+++
Prim *hexA*	Green/Red	-	Red	Dark Red	Orange	+++	+++
K122 Sec Rif^R	Red/Blue	-	White	Red	White	-	-
Sec pBAD	Red/Blue	-	White	Red	White	-	-
Sec *hexA*	Red/Blue	-	White	Red	White	-	-
Sm13	Green	++	Red	Green	Orange	+++	+++
Sm13pBAD	Green	++	Red	Green	Orange	+++	+++
Sm13hexA	Red/Blue	-	White	Red	White	-	-
Sm15	Green	++	Red	Green	Orange	+++	+++
Sm15pBAD	Green	++	Red	Green	Orange	+++	+++
Sm15hexA	Red/Blue	-	White	Red	White	-	-

Table 3.4 Results of phenotypic testing carried out on Rif^R wild type, mutant and *hexA* complemented strains of *Photorhabdus temperata* K122.

3.8 The role of *hexA* in the regulation of *csrB*

The *hexA* gene of the plant pathogenic bacterium *Erwinia carotovora* has been shown to have a role in the regulation of motility and in the expression of virulence factors (Harris *et al.,* 1998). The role of *hexA* in regulating these factors has been shown to involve the negative regulation of the alternate sigma factor (σ^S) produced by *rpoS* and via the negative regulation of the *rsmB* (*csrB*) small RNA (Fig 3.13) which itself has been show to be a global regulator of exoenzymes and secondary metabolites (Mukherjee *et al.,* 2000). Moreover, recent work has shown that *csrB* in *P. luminescens* appears to play a significant role in the regulation of many genes predicted to be involved in symbiosis, including bioluminescence, antibiotic production and pigment production (Krin *et al.,* 2008). To investigate whether HexA might also be involved in controlling the expression of *csrB*, a Northern blot was carried out on the Sm13 and Sm15 mutant strains. This blot was probed with the *csrB* gene of *E. coli*. It can be seen that there is a notable reduction in *csrB* expression in Sm13 and Sm15, which is partially restored by a plasmid borne copy of the *hexA* gene (see Fig. 3.14). Therefore, HexA appears to be a positive regulator of *csrB* expression, at least in the secondary variant. This is in sharp contrast to the situation in *Erwinia* where HexA negatively regulates the expression of the small RNA.

Both primary and secondary variants express *csrB* to the same levels suggesting that, despite its role in the regulation of putative symbiosis factors, this sRNA is not involved in controlling phenotypic variation (Fig. 3.14).

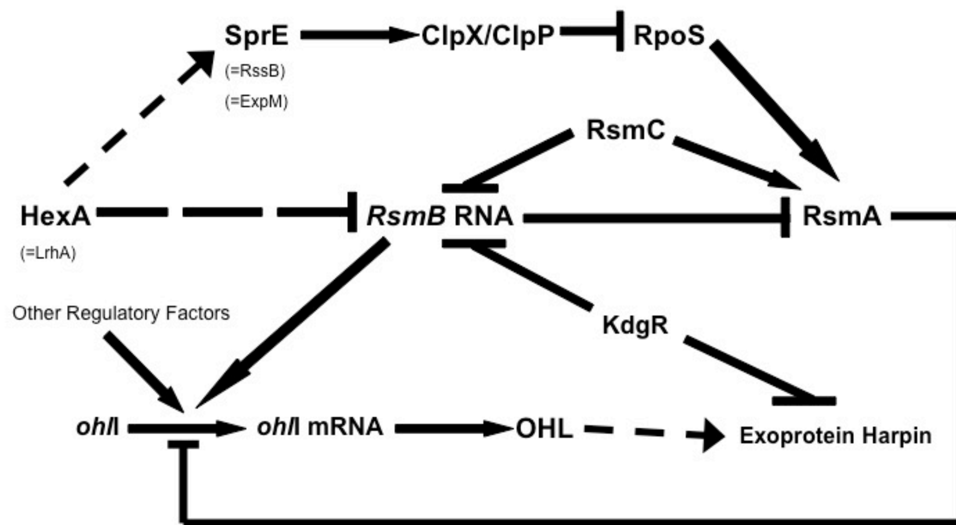
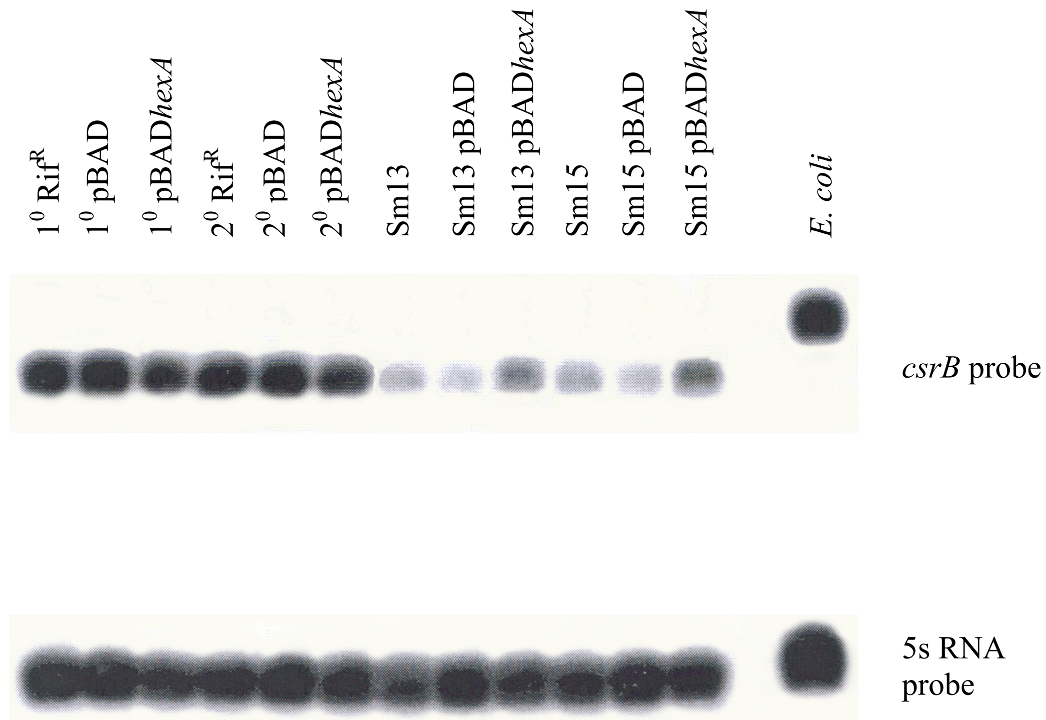


Fig. 3.13 Interactions of *hexA* in *Erwinia carotovora* (Mukherjee *et al*., 2000)
Note: RsmB/RsmC/RsmA= CsrB/RsmC/CsrA.

Fig. 3.14 Northern blot to investigate *csrB* expression in *P. temperata* K122 Rif^R wild type and mutant strains.



5s RNA including as loading control. Probe for *csrB* prepared by excision of *E. coli csrB* gene from plasmid pIM5. Expression of *csrB* is reduced in the *hexA* mutants, with a partial restoration of expression occurring in strains carrying a plasmid borne copy of the *hexA* gene - plasmid expression of *hexA* occurring at low levels without arabinose induction of the pBAD promoter

3.9 Identification of the role of Sm11 in *hexA* independent phenotypic variation

The Western analysis carried out on the mutant strains suggested that the phenotype of mutant Sm11 is independent of HexA (Fig. 3.7). To investigate this further, the Sm11 strain was assayed for its pathogenic and symbiotic abilities in comparison to both the wild type strains and the *hexA* mutants Sm13 & Sm15 (Fig. 3.2, 3.3 & 3.10; Table 3.6).

This data, in conjunction with previous phenotyping (Table 3.1) indicated Sm11 has a phenotype similar to that of the primary variant. It was therefore decided to attempt to identify the site of transposon insertion via a different method to that used previously, given that initial attempts were unsuccessful.

To this end, primers other than P6 and P7 were used to sequence the insert fragments. Primer pBR322F (5'- GTCATCCTCGGCACCGTCACCGTGG-3') was used to sequence forward from the plasmid into the cloned sequence and primer pBR322R (5'-CCAAAGCGGTCGGACAGTGCTCCGAG-3') to sequence in the reverse orientation from the plasmid backbone into the cloned sequence.

Analysis of the resultant sequences identified the region of insertion to be in a gene with homology to the *hipB* gene of *E. coli* (Fig 3.15 & 3.16). The *hipB* gene has been identified in other Gram negative organisms as well as in some Gram positive species including *S. aureus*, and is part of an operon including the *hipA* gene which has been shown to be directly required for development of a 'persistent' state (Correria *et al.,* 2006).

The high conservation of the operon indicates its central role in the development of persistence (Schumacher *et al.,* 2009). Its mode of action is thought to be similar to that of a Toxin-Antitoxin (TA) module. TA modules consist of a stable toxin (HipA) and an unstable antitoxin (HipB) and can act as regulators of macromolecular synthesis under conditions of nutritional stress (Korck & Hill, 2006). Expression of the toxin in excess of the antitoxin can sharply increase the frequency of persistence in *E. coli*. In the case of the *hipAB* operon, *hipA* expression in excess of *hipB* inhibits

protein, RNA and DNA synthesis, inducing a dormant state that lasts for more than 3 days in the vast majority of cells. HipB is also involved in autoregulation by acting as a transcriptional repressor of the *hipAB* operon (Black *et al.,* 1991; Black *et al.,* 1994). The phenomenon of persistence/tolerance is an epigenetic mechanism thought to be employed by many bacterial species to survive in the face of fluctuating environments (Balaban *et al.,* 2004). Indeed, such 'bet-hedging' behaviour is an ongoing process, with persister cells having been shown to pre-exist as non-growing cells in a population (Pedersen *et al.,* 2002). Persistent cells display a unique gene expression profile in comparison to both exponential and stationary phase cells and have therefore been described as a distinct, third physiological state of bacterial cells (Shah *et al.,* 2006). As previously mentioned, the occurrence of persisters can be seen as a form of 'bet-hedging' to maximize survival potential when environmental uncertainty exists (Cooper & Kaplan 1982; Seger & Brockmann, 1987; Donaldson-Mataschi *et al*., 2008).

Two types of bacterial persisters have been identified – Type I and Type II (Balaban *et al.,* 2004). Type I are a pre-existing population of non-growing cells generated during stationary phase. When inoculated into fresh medium from stationary phase, Type I persisters switch back to become growing cells after a characteristic time lag. Type II persisters are a subpopulation of slow growing cells that do not appear to originate from passage through stationary phase but are a normal component of the logarithmic growing phase cell population (Balaban *et al.,* 2004).

Overproduction of the HipA protein leads to drug tolerance in *E. coli* (Correia *et al.,* 2006). This may be similar to what occurs in the *P. temperata* K122 primary variant, with a lack of HipB causing an increased drug resistant state thereby allowing increased antibiotic production associated with the pathogenic phase of the nematode/bacterium life cycle. It is also possible that the HipA/HipB complex has a role in maintaining the secondary phenotype, with specific expression profiles and interactions of HipA and HipB necessary to maintain the secondary state.

The *hipB*-like gene sequenced from Sm11 shows close homology to the *hipB* gene of *P. luminescens* TTO1 and *P. asymbiotica*, however unlike these strains, in *P. temperata* K122 this *hipB*-like gene is not co-located with a partner *hipA* gene (Fig

3.15 & 3.16, Table 3.5). A more typical *hipAB* operon, with homology to *hipAB* from *Serratia* and *Xenorhabdus* spp., is found elsewhere on the *P. temperata* K122 genome (Fig. 3.17) but is absent from *P. luminescens* TTO1 and *P. asymbiotica*.

The expression of the *P. temperata* K122 orphan *hipB*-like gene, with no related *hipA* gene to regulate, may be specific to the secondary variant, with a role in the maintenance of the secondary phenotype by protein interaction or regulation of transcription. Such an interaction may occur with the other K122 *hipAB* operon, whereby the orphan *hipB* in conjunction with the second, *hipA* associated *hipB* regulates *hipA* expression, with the absence of this regulation resulting in the development of the primary phenotype.

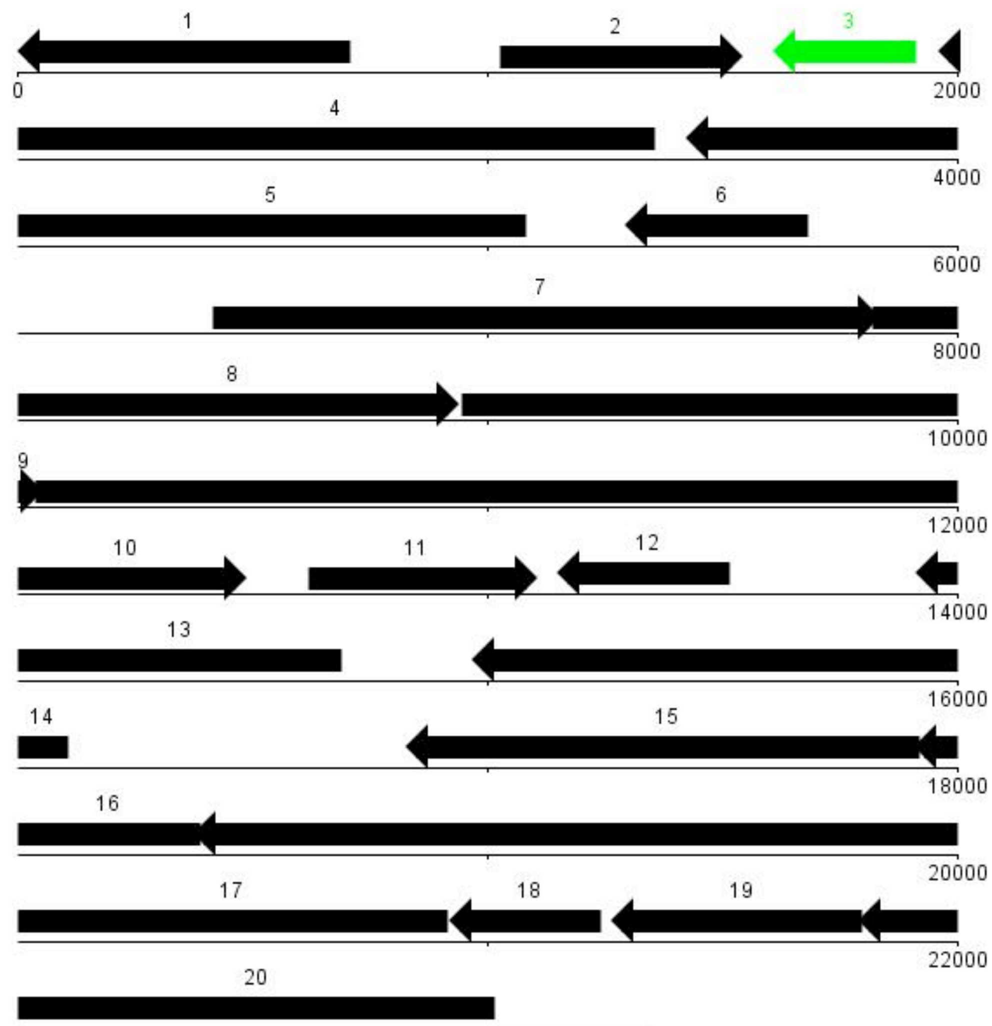


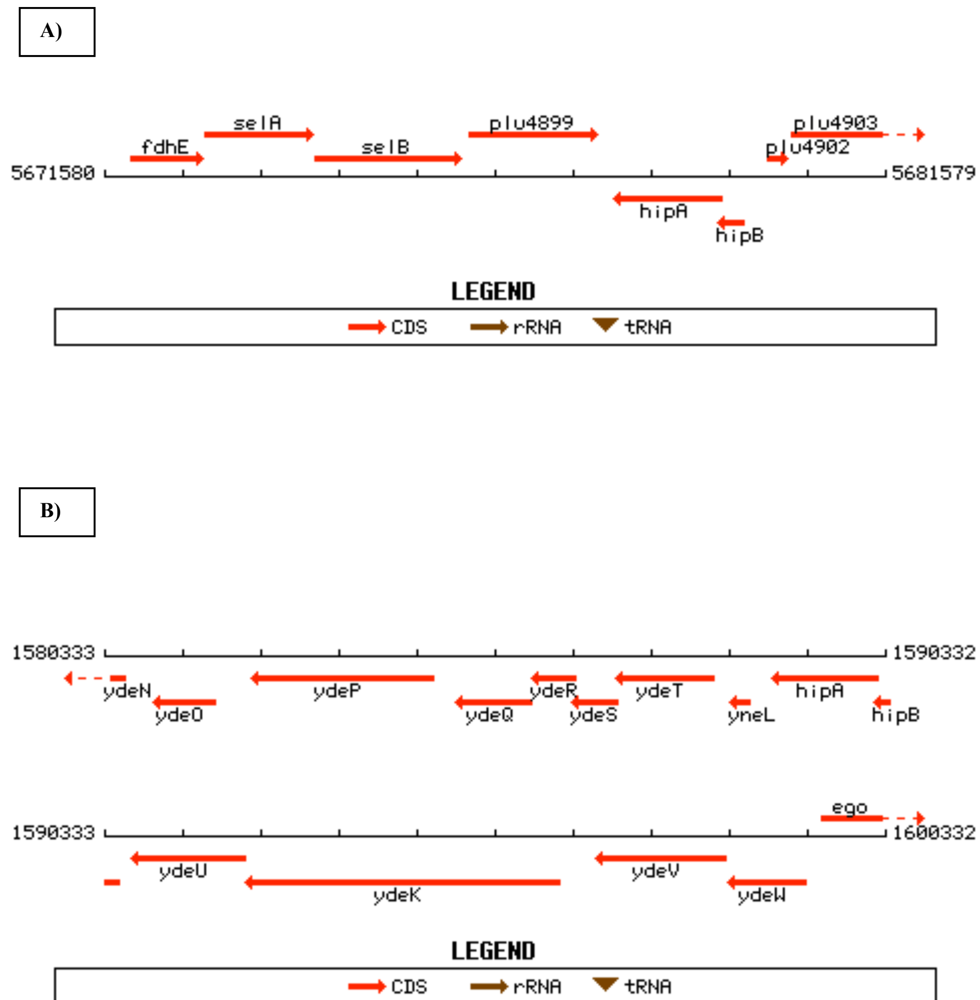
FIG. 3.15 Annotation of Genome Contig 507 of *P. temperata* K122, mutant Sm11 occurs in gene 3, encoding a HipB-like transcriptional regulator.

1) Hypothetical protein
2) Putative dienelactone hydrolase
3) HipB-like transcriptional regulator
4) tRNA modification GTPase TrmE
5) Putative inner membrane translocase YiC
6) *rnpA* Ribonuclease P
7) Chromosomal replication initiation protein DnaA
8) DNA polymerase III subunit beta DnaN
9) Recombination protein F RecF
10) DNA gyrase subunit B GyrB
11) Hypothetical protein
12) Hypothetical protein
13) Hypothetical protein
14) Aspartate-semialdehyde dehydrogenase Asd
15) Putative phage regulator
16) Tail fibre of prophage
17) Phage tail protein
18) Phage tail protein
19) Tail fibre component of prophage
20) Putative phage major tail sheath protein

Table 3.5 Genes on *P. temperata* K122 contig 507 with counterpart in *P. asymbiotica* & *P. luminescens* TTO1.
(Note: K122 has an orphan *hipB*, whilst the homologous counterpart in TTO1 and *asymbiotica* is paired with a counterpart *hipA* gene.)

	P. temperata **K122 Contig 507**	***P. asymbiotica***	***P.luminescens*** **TTO1**
1)	Hypothetical protein		plu4884
2)	Putative dienelactone hydrolase	pau_04394	
3)	HipB-like transcriptional regulator	pau_00435	plu4901
4)	tRNA modification GTPase TrmE	pau_04397	plu4905
5)	Putative inner membrane translocase YidC	pau_04398	plu4906
6)	RnpA Ribonuclease P	pau_04400	plu4908
7)	Chromosomal replication initiation protein DnaA	pau_00001	plu0001
8)	DNA Polymerase III subunit beta DnaN	pau_00002	plu0002
9)	Recombination protein F RecF	pau_00003	plu0003
10)	DNA gyrase subunit B GyrB	pau_00004	plu0004
11)	Hypothetical protein		plu0005
12)	Hypothetical protein		
13)	Hypothetical protein		plu0006
14)	Aspartate-semialdehyde dehydrogenase Asd	pau_00005	plu0007
15)	Putative phage regulator	pau_00007	plu0009
16)	Tail fibre of prophage	pau_00008	plu0010
17)	Phage tail protein	pau_00009	plu0011
18)	Phage tail protein	pau_00010	plu0013
19)	Tail fibre component of prophage	pau_00011	plu0014
20)	Putative phage major tail sheath protein	pau_00012	plu0015

Figure 3.16. Layout of the *hip* operon in *Photorhabdus luminescens* TTO1 and in *Escherichia coli*.



A) *Photorhabdus luminescens* TTO1 (from http://genolist.pasteur.fr/Photolist/) and **B)** *Escherichia coli* (from http://genolist.pasteur.fr/Colibri/). Surrounded by genes of undocumented function, HipB is involved in the autoregulation of the operon and acts as an antitoxin to HipA.

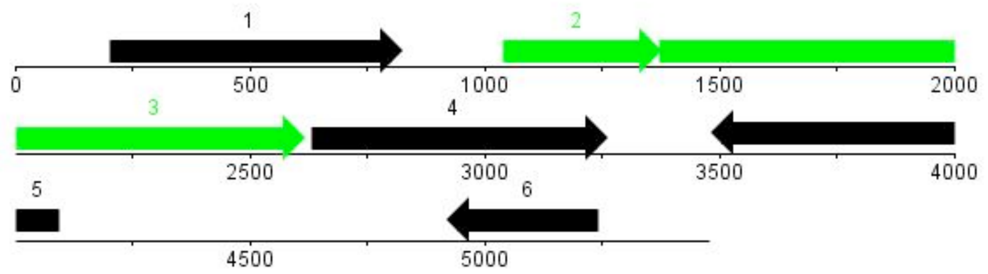


FIG. 3.17 Annotation of Genome Contig 217 of *P. temperata* K122.

1) MrfI recombinase, Type I fimbriae regulatory protein
2) Putative HipB like transcriptional regulator
3) HipA domain containing protein
4) Hypothetical protein
5) CDP-alcohol phosphatidyltransferase family YnbA
6) XRE family transcriptional regulator

Table 3.6 Genes on *P. temperata* K122 contig 217 with counterpart in *P. asymbiotica* & *P. luminescens* TTO1.

P. temperata K122 Contig 217		*P. asymbiotica*	*P.luminescens* TTO1
1)	MrfI recombinase, Type I fimbrial regulatory protein	pau_00724	plu4884
2)	Putative HipB-like transcriptional regulator		
3)	HipA domain-containing protein		
4)	Hypothetical protein	pau_00722	plu0767
5)	CDP-alcohol phosphatidyltransferasefamily YnbA		
6)	Putative XRE family transcriptional regulator		plu0808

3.10 Summary

Ths study confirmed a role for the LysR type transcriptional regulator HexA in the regulation of mutualism, pathogenicity and phenotyic variation in *P. temperata* K122, and has identified a role for HexA in the regulation of the small global RNA regulator *csrB*, with the reduction in *csrB* expression observed in the *hexA* Sm mutants Sm13 & Sm 15 indicative of a role for HexA, in the secondary variant, in the positive regulation of *csrB* expression. *csrB* has, in *P. luminescens* TT01, been linked with the regulation of many genes potentially involved in symbiosis, including bioluminescence, antibiotic production and pigment production (Krin *et al*., 2008). *csrB* has also been shown to have a role as a global regulator of exoenzymes and of secondary metabolites (Mukherjee *et al*., 2000). However, with both primary and secondary variants expressing *csrB* to similar levels, there does not appear to be a role for the sRNA in the control of phenotypic variation.

This study has also identified a phenotypic mutant whose phenotypic reversion occurs in an *hexA* independent manner. This mutant (Sm11) is disrupted in a gene that is a *hipB* homologue, a gene that has been shown to be part of a two gene operon that is directly required for the development of a 'persistent' state (Correria *et al*., 2006). With phenotypic variation in *Photorhabdus* having been observed after prolonged growth in stationary phase and/or under osmotically stressful conditions (Akhurst 1980; Boemare and Akhurst 1988; Krasomil-Osterfeld 1995), it has been suggested that the phenotypic switch occurs as an extreme reaction to ensure survival in the face of harsh environmental conditions (Boemare *et al*., 1997; ffrench-Constant *et al*., 2003). The identification of this *hipB* gene's apparent role in phenotypic variation is consistent with the idea that the switch occurs as a stress response to ensure survival and suggests that the *Photorhabdus* secondary variant may be a form of Type I persister cell (Balaban *et al*., 2004) generated during stationary phase.

Chapter 4

4. Primary to secondary phenotypic variation

4.1 Introduction

The primary to secondary phenotypic shift that occurs in *Photorhabdus* after prolonged stationary phase culturing has been shown to affect the bacterium's ability to produce factors required for mutualism with the nematode (Ehlers *et al.,* 1990; Forst *et al.,* 1997). These factors include antimicrobial agents, lipases, phospholipases and proteases. Other primary specific factors affected by phenotypic variation include pigment and bioluminescence production, motility, colony morphology and size, dye adsorption patterns and development of intracellular crystal protein inclusions (Forst *et al.,* 1997). Proteomic studies have identified 32 membrane proteins and 54 cytoplasmic proteins as having altered levels of synthesis in the secondary variant, with proteins affected including those involved in adaptation to stress, molecular chaperones, transport and binding of nutrients, secondary metabolites, cell envelope-related proteins, energy metabolism, translation and proteins of unknown function (Turlin *et al.,* 2006). The global regulator H-NS was also observed to be present at higher levels in the secondary variant cells (Turlin *et al.,* 2006). These factors are known to individually affect mutualism indicating that mutualism may be an evolved, complex process when compared to pathogenicity. Indeed, pathogenicity may be conferred on nonpathogenic *E. coli* by means of the introduction of a single, cloned gene from *Photorhabdus* – one such gene is *mcf* (makes caterpillars floppy) that, when exogenously expressed, is sufficient to enable previously non-pathogenic *E. coli* to persist within and kill an insect host (Daborn *et al.,* 2002). Other, similarly potent virulence factors have been identified by Rapid Virulence Annotation (RVA) using gain of toxicity assays (Waterfield *et al.,* 2008). Mutualism has not been conferred in this manner and with so many factors having influence on the *Photorhabdus* bacterium's ability to partake in the mutualistic complex with the nematode, it would suggest the presence of a complex, multi-gene regulatory pathway overseeing the expression and regulation of mutualism.

The primary to secondary phenotypic switch has been observed following prolonged growth (Akhurst 1980; Boemare and Akhurst 1988) with secondary variant cells recovering more quickly from starvation than their primary counterparts (Bleakley & Nealson 1988; Boemare *et al.,* 1997). This agrees with the observation that there are altered levels of synthesis of proteins involved in energy production, transport and biosynthesis in the different variants (Turlin *et al.,* 2006). As a result of these changes, it has been hypothesized that the phenotypic switch occurs as a response to unfavourable environmental conditions (ffrench-Constant *et al.,* 2003). Therefore, the phenotypic switch may be a survival strategy whereby a shut down of all systems not required for bacterial survival occurs – with phenotypic variation occurring as a 'bet-hedging' strategy to prepare for life without the nematode.

In this chapter, genetic analyses were undertaken to further characterize the molecular mechanisms involved in controlling the phenotypic switch from primary to secondary variant that occurs in *Photorhabdus*. In the previous chapter, we identified *hexA* as a gene required for the maintenance of the secondary variant. Additionally, a two component signal-transduction system AstR-AstS has also been shown to have a role in the transition to the secondary variant phenotype in *P. luminescens* (Derzelle *et al.,* 2004a). Therefore, further investigation of the primary to secondary phenotypic switch may help to elucidate the factors involved in the phenotypic switching process.

4.2 Identification of candidate genes with roles in primary to secondary phenotypic switching

In order to identify the genetic loci that determine primary to secondary phenotypic variation, a similar method to that used for secondary to primary screening was employed. Briefly, random transposon mutagenesis was applied to investigate the genetics involved in the primary to secondary type phenotypic switch of *Photorhabdus*, with Tn*5* mutagenesis being carried out on a primary starter culture. As described previously, rifampicin resistant *P. temperata* K122 were conjugated with *E. coli* S-17λ*pir* carrying the pUTkm2 suicide plasmid containing a transposable (mini-Tn*5*) kanamycin resistance cassette

(section 2.2.15, Materials and Methods). In this case, the starter cultures were primary phenotypic variants. Exconjugants were initially screened for loss of pigment and 127 candidate phenotypically switched colonies were isolated from a screen of 13,000 mutants. These mutants were termed 'Pm' mutants (Primary mutated).

A control conjugation (section 2.5.15) was performed, to eliminate the possibility that any observed phenotypic switch occurred as a result of environmental stress factors/growth conditions encountered during the mutagenesis procedure i.e. to determine the background rate of primary to secondary switching. Plasmids pHMW011 and pHMW014 were used to enable recreation of the experimental conditions in the absence of a transposon insertion event. In total, 20,000 exconjugant colonies were screened for loss of pigmentation (i.e. primary to secondary variant switch). No phenotypically switched or secondary like colonies were isolated from this procedure, suggesting a very low background level of phenotypic variation and indicating that any phenotypic switch observed after mini-Tn*5* mutagenesis was not a result of experimental conditions but as a result of the transposon insertion event.

4.3 Phenotypic characterisation of mutant strains

The 127 mutants were then subjected to further in-depth phenotypic analysis to confirm that a primary to secondary switch had taken place. This analysis identified 9 transposon mutants that were strongly secondary-like in all the phenotypic tests performed (Table 4.1). These mutants (Pm9, 20, 53, 55, 58, 76, 85, 92 and 100) were confirmed to have loss of pigmentation relative to the parent wild-type primary variant. All candidate colonies appeared blue on NBT agar, white on MacConkey agar and white in the presence of Congo Red, all of which indicated cell surface changes when compared to the wild type parent primary phenotype which displayed green, red and red dye adsorption patterns respectively. Furthermore, these Pm mutants also had a non-mucoid colony morphology and were lacking in both antibiotic production and lipase activity.

These 9 mutants were subjected to further analysis, whilst the remaining 118 Pm mutants, which exhibited intermediate phenotypes, will be discussed further in Chapter 5.

4.3.1 Growth and Bioluminescence

Bioluminescence normally occurs only in the primary variant, upon entry into stationary phase. The 9 Pm strains were cultured in parallel with the primary and secondary variants and measurements of growth (Fig. 4.1) and bioluminescence (Fig. 4.2) were taken as described in Materials and Methods. It can be seen from these data that the Pm strains have lost their ability to produce light when compared to the wild type primary variant parent strain and also have growth profiles that are similar to that of the secondary variant, with the exception of Pm58 which displays a retarded growth rate in comparison to the wild-type strains.

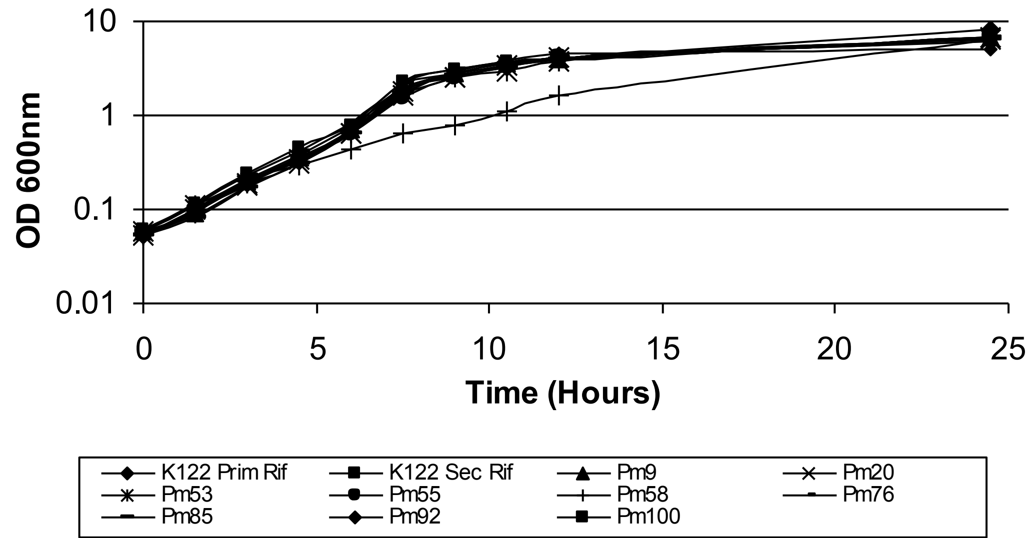


Fig. 4.1, Growth curve of Pm mutants compared to *P. temperata* K122 Rif^R primary and secondary variants. Cultures were grown overnight and inoculated (1:100) into fresh LB broth. Growth of the bacteria at 28^0C was measured by observing the OD_{600nm} of the culture over time.

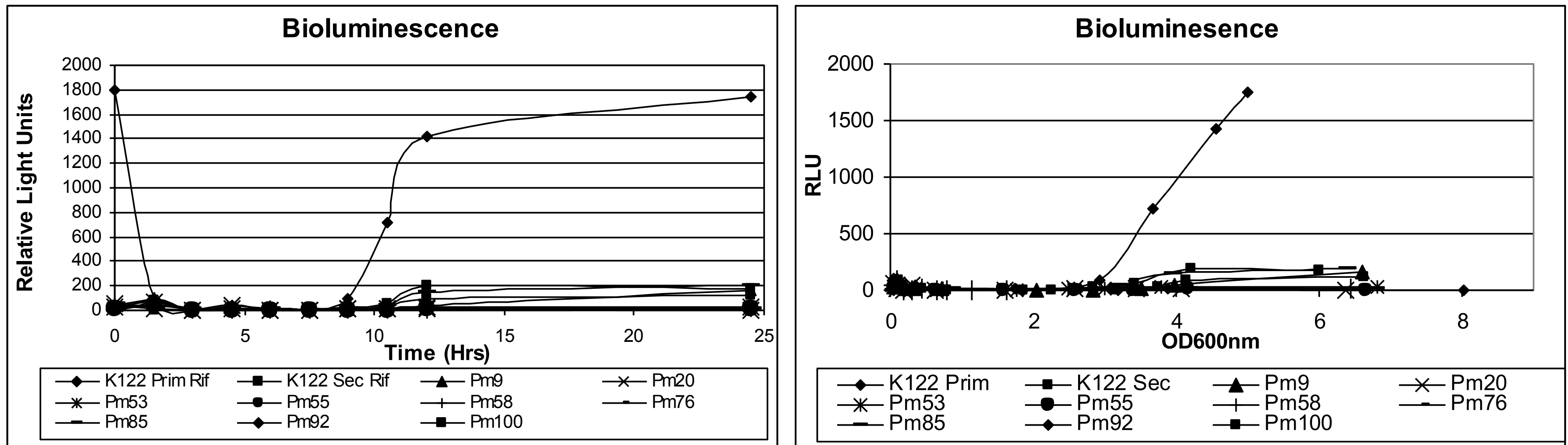


Fig. 4.2, Bioluminescence of Pm mutants compared to *P. temperata* K122 primary and secondary variants, shown as Relative light Units vs. Time (Left) and Relative light units vs. OD600nm (Right)

Bioluminescence data comparing primary like mutants to wild type strains. Relative Light Units (RLU) are a function of bioluminescence units per OD_{600nm} of cells, where an $OD_{600nm} = 1$, corresponding to approximately $2x10^8$ cells/ml (Clarke, 1993). The initial luminescence seen is as a result of inoculating test cultures from stationary phase overnights. Levels of luminescence of test cultures are expected to be higher overnight (12-20hrs – readings not taken) returning to lower levels when final reading is taken the following day.

Table 4.1 Initial phenotypic characterization of selected Primary Mutants.

	Colour on LB[a]	Bioluminescence[b]	Colour on NBTA[c]	Colour on Congo Red[d]	Colour on MCA[e]	Antibiosis[f]	Lipase Activity[g]	Colony Morphology	Stability[h]
K122 1° Rif^{R}	Orange	Yes	Green	Red/Orange	Red	++	++	Mucoidy	Yes
K122 2° Rif^{R}	White	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm9	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm20	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm53	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm55	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm58	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm76	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm85	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm92	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes
Pm100	White/Cream	No	Blue	White	White	-	-	Non-mucoidy	Yes

(a) Pigment observed after 48 hours growth on LB agar. **(b)** Bioluminescence observed on a Fujifilm Intelligent Dark Box II **(c)** Colour on NBTA- Nutrient agar supplemented with bromothymol blue (BTB) and triphenyltetrazolium chloride (TTC). Phase I colonies adsorb BTB, obscuring the red colour produced by the reduction of TTC to formazan. Phase II colonies do not. **(d)** Congo Red Agar- LB agar with 0.01% Congo Red, a planar hydrophobic compound that binds lipids and lipoproteins. Differences in Congo Red staining between strains has been interpreted to indicate differences in cell wall components, which in turn may lead to changes in virulence and drug susceptibility **(e)** Colour on MacConkey Agar (MCA) – Phase I colonies are red due to adsorption of the neutral red dye in the medium. Phase II colonies remain off-white **(f)** Antibiosis – 2 day old *Photorhabdus* cultures that have been spot inoculated onto LB agar plates are overlaid with a soft agar containing an inoculum of 24hr old *Micrococcus luteus*. After incubation a clear zone of inhibition around the *Photorhabdus* colony indicates antibiotic production. Phase I colonies produce much higher quantities of agar diffusible antibiotics than Phase II **(g)** Lipase activity – Cultures are grown on a calcium agar incorporating Tweens (fatty acids combined with polyoxyethylenesorbitan). The fatty acids form a white precipitate indicative of lipolysis around colonies producing lipases. **(h)** Stability – refers to the reculturability of the strain without the occurrence of variations in phenotype

4.3.2 Mutualism and Pathogenicity

Mutualism

Given that one of the major differences between the wild-type primary and secondary variants is the unique ability of the former to support mutualism/symbiosis with nematodes of the *Heterorhabditis* genus, and with the loss of many of the primary characteristics that was observed in the selected Pm mutants, symbiosis assays were performed to identify any effect the insertional mutagenesis may have had on mutualistic ability. The results of these assays are summarised in Table 4.2. Three of the strains tested, Pm53, 85 and 100 retained their mutualistic ability, resulting in the production of living IJs at the end of the assay. In all of the other strains however, nematode development did not progress past the F_o hermaphrodite stage, indicating a loss of the ability of strains Pm9, 20, 55, 58, 76 and 92 to support nematode growth and development. Of note is that whilst the secondary variant does not support IJ recovery to the hermaphrodite stage, the Pm mutants do. With the antibiotic 3-5-dihydroxy-4-isopropylstilbene (ST) having been show to be a necessary requirement for IJ to hermaphrodite recovery (Joyce *et al.,* 2008; Lango & Clarke 2010) it would be expected that the non-halo forming/non-antibiotic producing Pm mutants (Table 4.1) should be lacking in the ability to initiate IJ recovery. However, given that ST alone in the absence of bacteria is insufficient for recovery (Joyce *et al.,* 2008) other required factors may still be present in the Pm mutants which enable hermaphrodite development. Also, given that the assay used is not overly sensitive, ST may still be produced by the mutant strains but not at a sufficient level to kill the *M. luteus* reporter strain.

Table 4.2 Analysis of nematode development in *P. temperata* K122 Pm mutant strains.

Strain	IJ to Hermaphrodite	Hermaphrodite to ♀ & ♂	Fertilised Eggs	Juvenile Development	Viable IJ's returned
Primary Rif^R wt	Yes	Yes	Yes	Yes	Yes
Secondary Rif^R wt	No	No	No	No	No
Pm9	Yes	No	No	No	No
Pm20	Yes	No	No	No	No
Pm53	Yes	Yes	Yes	Yes	Yes
Pm55	Yes	No	No	No	No
Pm58	Yes	No	No	No	No
Pm76	Yes	No	No	No	No
Pm85	Yes	Yes	Yes	Yes	Yes
Pm92	Yes	No	No	No	No
Pm100	Yes	Yes	Yes	Yes	Yes

Pathogenicity

Although the primary and secondary variants are both highly virulent towards a broad range of insect larvae, the *hexA* mutants isolated previously in this study were shown to be significantly attenuated in virulence. To investigate whether any of the 9 selected Pm mutants had undergone changes to their pathogenic ability, pathogenicity assays were carried out (Section 2.4.9 Materials and Methods). From the results obtained (Fig 4.3) all insects were dead by 72 hours, regardless of inoculated strain. Despite there being inherent variability in rates of insect killing in assays of this type, there was some notable variance in the rate of killing by some strains, with both Pm9 & 100 displaying a reduced rate of killing compared to the wild type Rif^R parent strains. Interestingly, Pm58, the strain displaying a reduced rate of growth (Fig. 4.1) does not appear to be affected in pathogenic ability as a result of the retarded growth displayed by this strain.

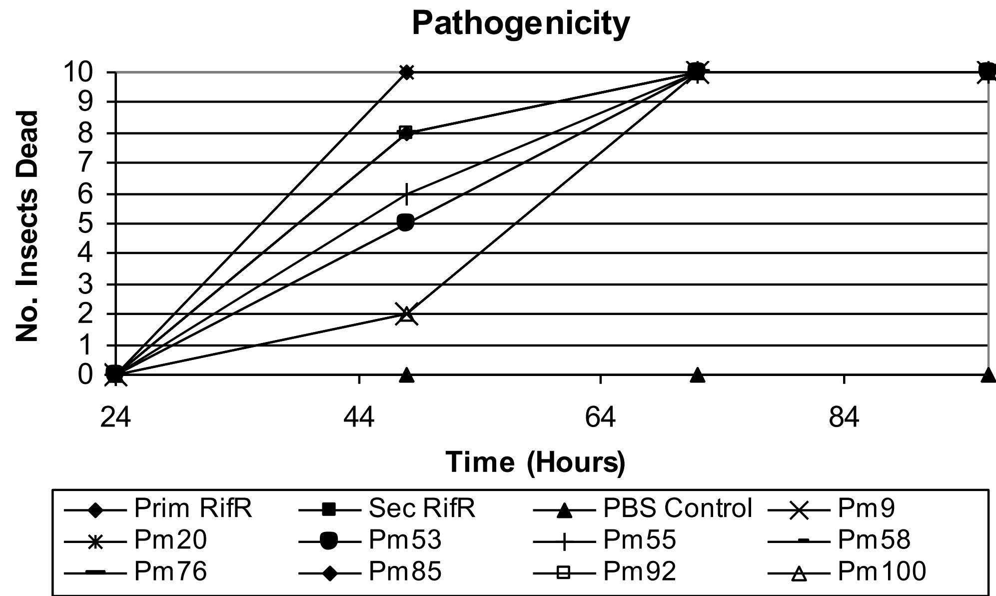


Fig. 4.3, Pathogenicity of Pm mutant strains compared to *P. temperata* K122 primary and secondary Rif^R variants 10μl of the relevant culture were injected directly into the haemocoel of each of 10 *Galleria mellonella* larvae, which were then incubated at 25^0C. control insects were injected with 10μl sterile PBS and the insects were monitored for insect death.

4.4 Effect of mutagenesis effected phenotypic switch on *hexA* expression

HexA has been characterised as having a role in the maintenance of the secondary variant in *Photorhabdus*. Therefore it was formally possible that the secondary variant phenotype observed in the Pm mutants could be attributed to an increase in the production of HexA in these mutants. To investigate, Western Blot analysis was carried out upon the Pm mutants in conjunction with the wild-type primary variant parent strain and the secondary phenotypic variant included as a positive control (Fig. 4.4). Only the wild-type secondary variant strain expressed HexA to a high level. The levels of HexA production in the Pm strains did not match those of the wild-type secondary cells, confirming that the phenotypic switch observed in these strains is via a HexA independent mechanism.

4.5 Regulation of the primary to secondary phenotypic switch

With the wide range in types of gene identified by this study as having a role in the primary to secondary phenotypic switch undergone by *P. temperata* K122, the process is likely to be complex and multifactorial, potentially under the control of a master regulator. One potential candidate for the regulation of so many factors is the previously mentioned *csrA/csrB* system. To identify any effects of the identified mutations on the *csrB* regulatory process, Northern blot analysis was carried out on mutants Pm 9, 53, 58 and 76 to examine *csrB* expression in these mutants as compared to the wild-type strains (Fig. 4.5). The results are indicative of no role for *csrB* in these mutant strains, with expression levels similar to that of the wild-type parent strain. This is consistent with the HexA independent nature of the Pm mutants.

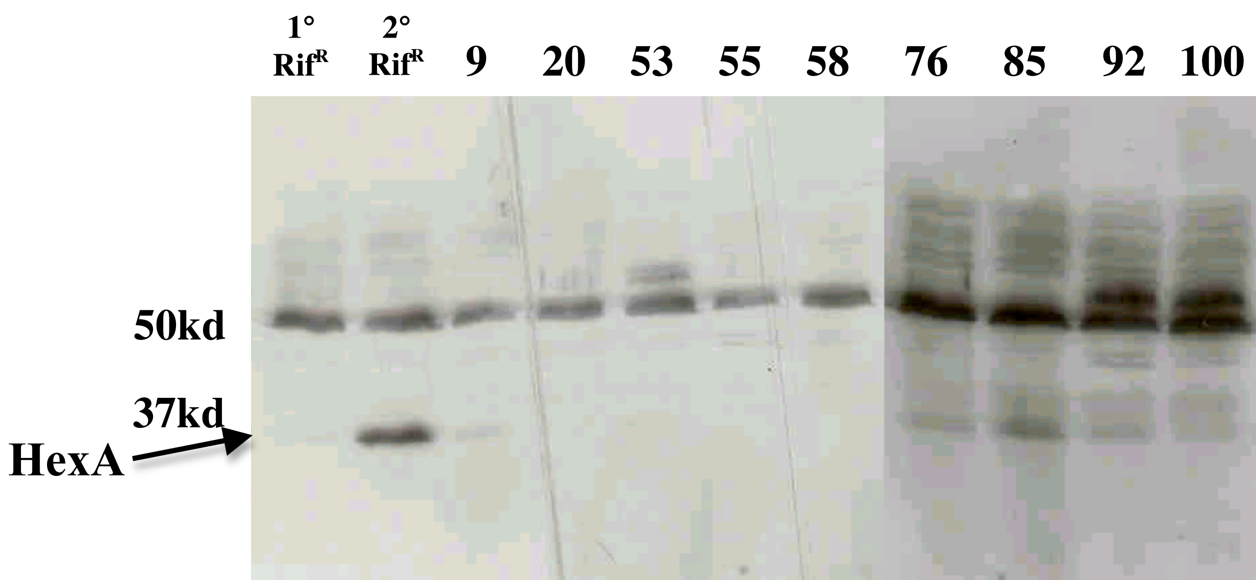
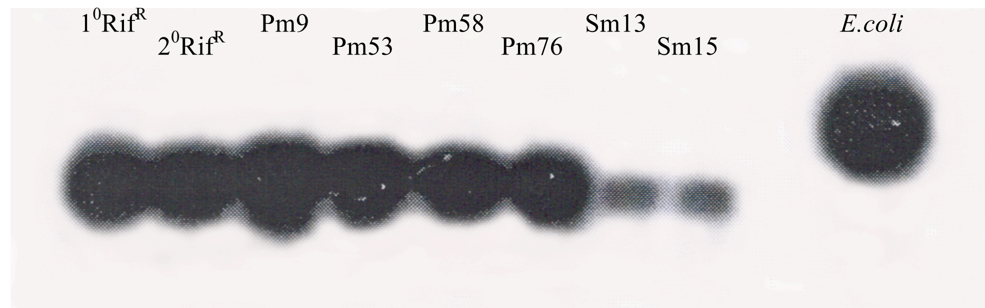


Fig. 4.4, Western analysis of Primary mutants probed with HexA. The HexA band is indicated by arrow. The 50kd band is a non-specific band that can function as a loading control.

A)



B)

Fig. 4.5, Northern Blot analysis of selected Primary mutants.
A) Blot probed with *E. coli csrB* excised from plasmid pIM5. Secondary to Primary mutants Sm13 & 15 included to illustrate difference in expression profiles of *csrB* in *hexA* mutant. Primary mutants 9, 53, 58 & 76 appear unaffected for *csrB* expression.
B) Same blot using 5S RNA probe to provide a loading control.

4.6 Confirmation of transposon insertion and sequencing of Pm mutants

To ensure the integrity of the transposon insertion event, Southern Blot analysis was performed on *Eco*RV restricted genomic DNA from these mutants. This analysis is undertaken to confirm that the mutant strain carries only one, intact, copy of the mini-Tn*5* cassette, thereby enabling the site/gene of insertion to be identified. The kanamycin cassette was used as probe to confirm the presence of a single transposon insertion in each of the mutants. From this analysis, single copy insertions were confirmed for each of the mutants (Fig. 4.6), thereby enabling the site/gene of insertion to be identified by PCR and sequencing.

The Southern Blot analysis also led to the identification of 6 putative classes of phenotypic mutant based on band size detected after *Eco*RV restriction:

- Class I – Primary mutants Pm9 & Pm 20
- Class II – Pm53
- Class III – Pm55
- Class IV – Pm58
- Class V – Pm76
- Class VI – Pm85, Pm 92 & Pm100

Each of the mutants was then sequenced as described in Materials and Methods. The sequence results obtained confirmed the initial classification from the Southern analysis data (Table 4.3). The genes into which the insertions had occurred were identified by BLASTN and BLASTX searching against the genome sequence of *P. luminescens* TTO1 and the general NCBI sequence database. As before, these genes were also identified on the relevant *P. temperata* K122 genome contigs and the contigs were annotated. (Figs. 4.8, 3.6, 4.9, 4.12 & 4.13). Comparisons of gene organisation were also made between *P. temperata* K122, *P. luminescens* TTO1 and *P. asymbiotica* (Tables 4.4, 3.2, 4.5, 4.6 & 4.7).

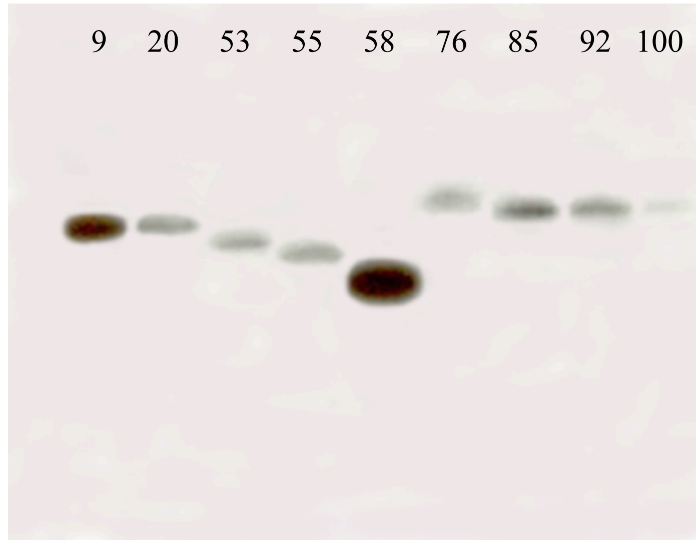


Fig. 4.6, Southern analysis of Primary mutants probed with km cassette.

Table 4.3 Sequencing of Primary→Secondary mutants:

	Genes	Role of putative protein	Nearest known homologue
Class I	*sciC / impG*	Putative cytoplasmic protein operon involved in interactions with eukaryotic cells	*Salmonella enterica, Rhizobium leguminosarum*
Class II	*sciB / impH*	Putative temperature dependent secretion protein	*Salmonella enterica, Rhizobium leguminosarum*
Class III	*nuoA*	NADH dehydrogenase (metabolism)	*Photorhabdus temperata*
Class IV	*serB*	Serine & enterobactin siderophore biosynthesis	*Yersinia pestis*
Class V	*lysR*	LysR type transcriptional regulator	*Pseudomonas aeruginosa*
Class VI	*flhB*	Flagellar biosynthesis/Type III secretion	*Yersinia pestis*

4.6.1 Class I & II Primary mutants

Sequencing of the Class I mutants - Pm9 and Pm20, revealed the site of transposon insertion in a gene encoding for a homologue of a putative cytoplasmic protein - SciC/ImpG. Previously isolated in *Salmonella enterica* and *Rhizobium leguminosarum*, the SciC/ImpG protein has been implicated in interactions with eukaryotic cells through their involvement in the recently described type VI secretion system (T6SS). Interestingly, Pm9 displays reduced a reduced rate of killing when assayed for pathogenicity (Fig. 4.3) which may result from a loss of SciC function and reduced ability to interact with the insect. T6SSs are found throughout the proteobacteria (Fig. 4.7) and have an important role in the virulence of many human and fish pathogens (Zheung & Leung 2007; Bingle *et al.,* 2008), facilitating bacterial survival and propagation in the harsh environment of the eukaryotic host, which is rendered more habitable by the exported proteins (Cascales, 2008).

Sequencing of the single Class II mutant, Pm53, also indicated involvement with a T6SS, with the site of transposon insertion occurring in a gene encoding for a homologue of the SciB/ImpH protein which, as before, has also been isolated in *Salmonella enterica* and *Rhizobium leguminosarum*, where its role has been suggested to be that of a temperature dependent secretion protein.

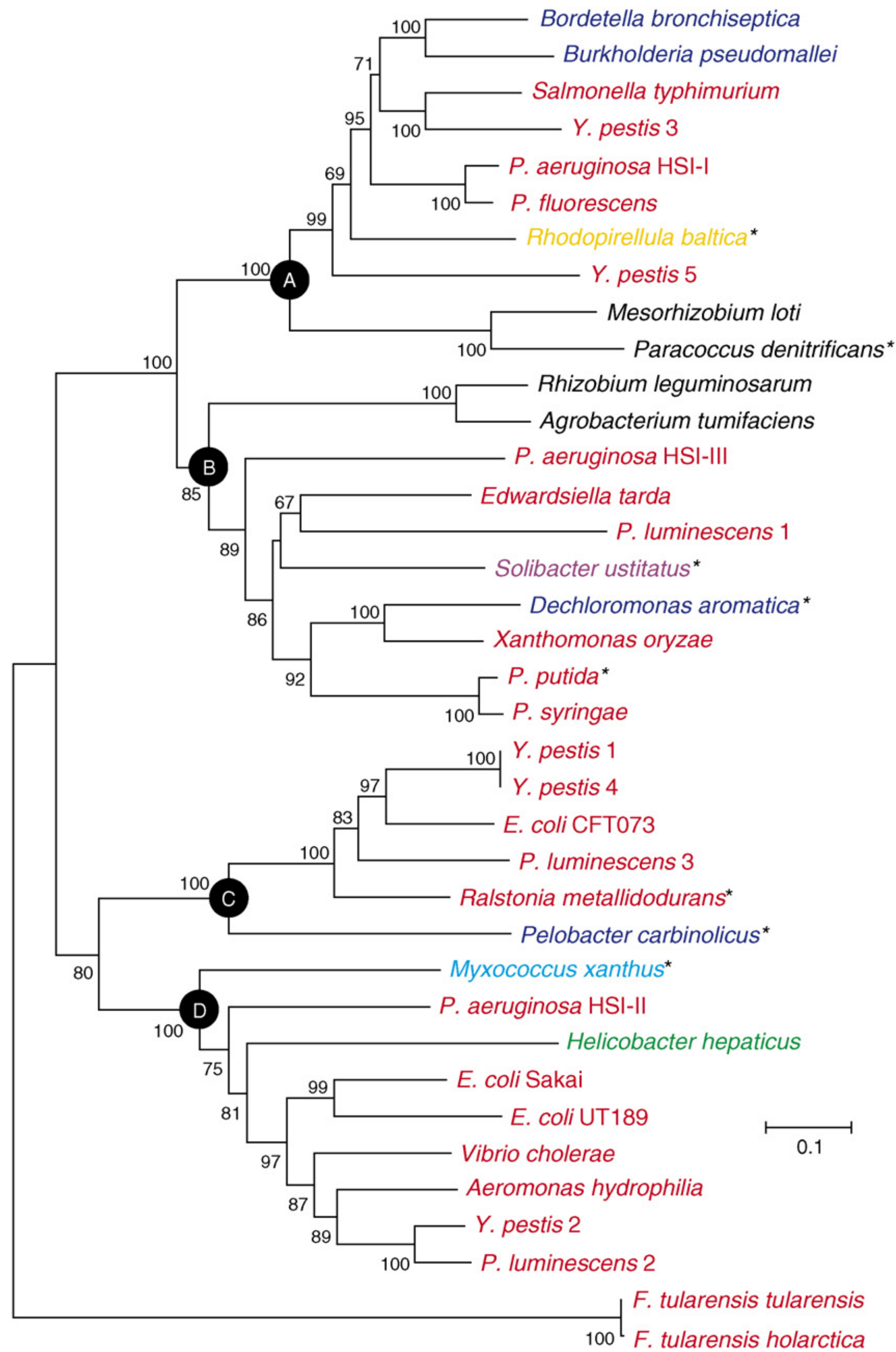
Both Class I and Class II mutants mapped to contig no. 176 of the *Photorhabdus temperata* K122 genome (Fig. 4.8) which illustrates the T6SS gene arrangement is similar to that seen in other bacterial species. Table 4.4 compares the gene organisation of K122 contig 176 to the corresponding genes on the genomes of *P. asymbiotica* and *P. luminescens* TTO1, and whilst there are several differences between the strains, the genes comprising the T6SS apparatus are conserved. With 4 Type VI secretion systems identified in *P. luminescens* TTO1 (Rodou *et al.*, 2010), BLAST analysis would suggest a similar number present in *P. temperata* K122, with homology to known T6SS proteins occurring on not only contig 176 but also on 3 further contigs (nos. 8, 78 & 491).

Like other secretion systems, the T6SS are defined by a set of original components. Originally named IAHP (IcmF-associated homologous proteins) because of the presence of a gene encoding an IcmF-like protein (Das & Chaudhuri 2003), T6SS components are encoded within gene clusters that vary in organization, usually with between 12 and 25 proteins produced, 2 of which are also found in type 4 secretion systems (T4SS) – DotU and the aforementioned IcmF-like protein. The DotU protein has been shown to be involved, in conjunction with IcmF, a cytoplasmic membrane protein, in conferring stability on the *Legionella* type IV secretion system and protecting the secretion apparatus from degradation and thereby playing a role in the intracellular replication of *L. pneumophila* (Sexton *et al.,* 2004).

The majority of the proteins associated with the T6SS locus are of unknown function, however, some do have known characteristics. To provide energy to the transport process, secretion systems frequently employ ATPases. T6SSs are no exception, with a gene coding for a member of the ClpV protein family most often found. ClpV proteins are homologues of the ClpB AAA+ family of ATPases (Neuwald *et al.,* 1999). The ClpV ATPases locate at specific sites in the cell envelope when the T6SS machinery is active with a hypothesized role as the driving force for direct translocation through the cell envelope (Economou *et al.,* 2006). Another gene commonly found in the T6SS cluster encodes for a putative outer membrane lipoprotein, the ultimate function of which remains to be elucidated.

Two other proteins commonly involved and most likely to be secreted by the T6SSs – Hcp and VgrG are not necessarily contained within the T6SS locus (Pukatzki *et al.,* 2006; Filloux *et al.,* 2008). The function of Hcp remains to be elucidated, although, along with its T6SS secretion, it may also be involved in the assembly of a conduit at the bacterial cell surface, through which other effector molecules may be transported to the host cell (Filloux *et al.,* 2008). Pukatzki *et al.,* (2006) have demonstrated the T6SS-dependent secretion of VgrG protein in *V. cholerae*. Despite being secreted substrates however, mutations in the genes *vgrG1* and *vgrG2* result in a lack of Hcp secretion, suggesting an additional role for the protein in the functioning of the T6SS (Pukatzki *et al.,* 2007). Filloux *et al* (2008) have proposed not only a role for Hcp and Vgr in T6SS

Fig. 4.7 Evolutionary relationships of type VI secretion systems (from Bingle *et al.,* 2008).



Evolutionary relationships of type VI secretion systems. The distance tree (neighbour-joining) shown here was calculated from an alignment of concatenated DUF770 and DUF877 protein sequences from all classes of bacteria possessing T6SS. Bootstrap values (% from 1000 replicates) over 50% are indicated at the nodes. The same four major groups (labelled A–D at their basal nodes) were obtained in a maximum parsimony analysis of the same data: bootstrap support values A, 99%; B, 94%; C, 98%; D, 100%. Bacterial taxons are indicated by font colour: Alphaproteobacteria, black; Betaproteobacteria, blue; Gammaproteobacteria, red; Deltaproteobacteria, turquoise; Epsilonproteobacteria, light green; Acidobacteria, purple; Planctomycetales, yellow. Asterisk indicates species that are not considered to be pathogens or symbionts. The scale bar indicates 0.1 substitutions per site.
The relationships of 3 out of 4 T6SS identified in *Photorhabdus luminescens* are illustrated.

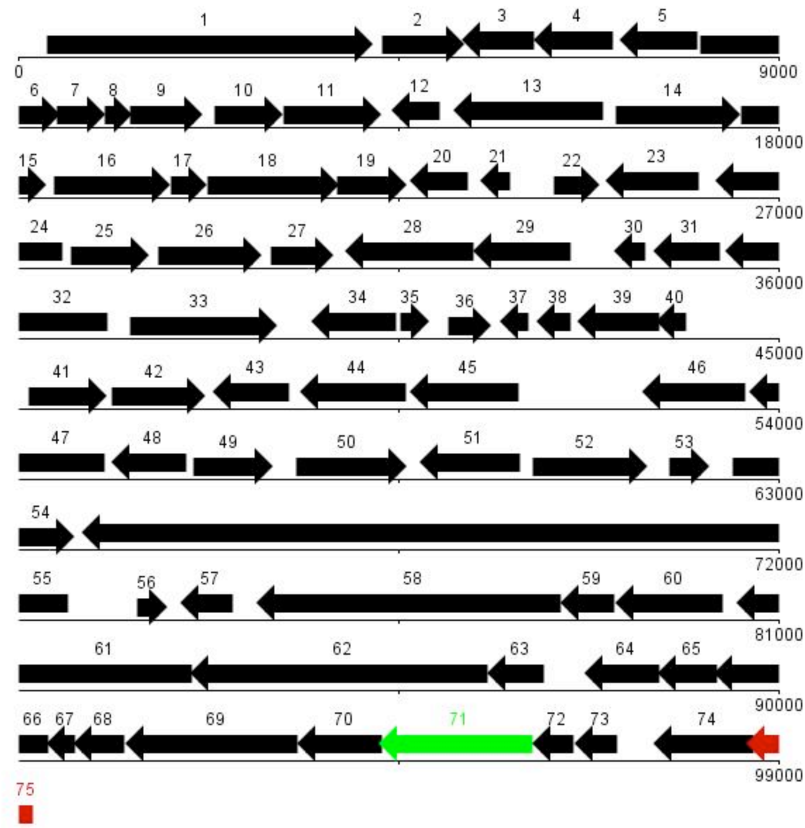


FIG. 4.8 Annotation of Genome Contig 176 of *P. temperata* K122, mutants Pm 9&20 occur in gene 71 SciC/ImG, mutant Pm53 in gene 75 SciB/ImpH.

1) Putative indigoidine synthase protein *indC*
2) Putative indigoidine synthase protein *indA*
3) 2-keto-4-pentanoate hydratase *mhpD*
4) Putative alpha/beta hydolase protein *ohpC*
5) DNA-binding transcriptional regulator *hcaR*
6) 3-phenylpropionate dioxygenase alpha subunit *hcaE*
7) 3-phenylpropionate dioxygnease beta subunit *hcaF*
8) 3-phenylpropionate dioxygenase ferredoxin reductase subunit *hcaC*
9) 2,3-duhydroxy-2,3-dihydrophenylpropionate dehydrogenase *hcaB*
10) 3-(2,3-dihydroxyphenyl) propionate dioxygenase *mhpB*
11) Phenylpropionate dioxygenase ferredoxin reductase subunit *hcaD*
12) Hypothetical protein
13) Periplasmic oligopeptide binding protein *oppA*
14) Putative betaine aldehyde dehydrogenase *ydcW*
15) Hypothetical protein
16) 4-aminobutyrate aminotransferase *goaG*
17) Putative inner membrane protein *yhaH*
18) FAD dependent oxidoreductase
19 & 20) Hypothetical proteins
21) Putative mazF-like growth inhibitor/transcriptional regulator
22 & 23) Hypothetical proteins
24) Putative transport protein *ynfM*
25) Putative LysR-type transcriptional regulator *ynfL/alsR*
26) Making large colonies protein *mlc*
27) Putative dethiobiotin synthase *ynfK*
28) Xylulose kinase *xylB*
29) Putative D-xylulose reductase *ydjJ*
30) Hypothetical protein
31) 3-hydroxy acid dehydrogenase *ydfG*
32) Putative histidine ammonia lyase
33) Exochitinase
34) Peptidase S58 *dmpA*
35 & 36) Hypothetical proteins
37) Putative transposase
38) Transposase Tn5045
39 & 40) Hypothetical proteins pSB102_p69 & 68
41) 2,5-diketo-D-gluconate reductase B *dkgB*
42) Putative NADH oxidase
43) LysR-type transcriptional regulator *yafC*
44) Membrane secretion protein *hlyD*
45) Macrolide export ATP-binding permease
46) d-Aminopeptidase d*mpA*
47) Beta-alanine pyruvate transaminase
48) PzF family phenazine biosynthesis protein *pab*
49) AraC-family transcriptional regulator
50) O-glycosyl hydrolase family protein
51) Xylose operon regulatory protein *xylR*
52) Xylose isomerase *xylA*
53) Hypothetical protein
54) Putative oxalate decarboxylase *oxdD*
55) Makes caterpillars floppy *mcf*
56) Plasmid stabilization protein *parE*
57) Putative CMP/dCMP deaminase
58) Virulence sensor protein *bvgS/pvpS*
59) Two-component response regulator *bvgR/pvpR*
60) Hypothetical protein
61) ClpA/B TypeVI secretion chaperone ATPase
62) T6SS icmF-related protein *evpO/sciS/impL*
63) T6SS transmembrane protein *evpN/dotU/impK*
64) T6SS protein *evpM/impJ/sciO/vasE*
65) T6SS lipoprotein *evpL/vasD*
66) T6SS secretion associated protein *evpK/impA*
67) T6SS protein *evpJ*
68) Hypothetical protein
69) T6SS protein *evpI* (Vgr family)
70) T6SS protein *evpG/impH/vasB/sciB*
71) T6SS protein *evpF/impG/sciC*
72) T6SS protein *evpE/tssE/impF*
73) T6SS effector protein *evpC*
74) T6SS protein *evpB/impC/sciI*
75) T6SS protein *evpA/impB/sciH*

P. temperata K122 Contig 176	*P. asymbiotica*	*P. luminescens* TTO1
1) Putative indigoidine synthase protein *indC*	pau_02362	plu2186
2) Putative indigoidine synthase protein *indA*	pau_02361	plu2187
3) 2-keto-4-pentanoate hydratase *mhpD*	pau_02360	plu2201
4) Putative alpha/beta hydolase protein *ohpC*	pau_02359	plu2202
5) DNA-binding transcriptional regulator *hcaR*	pau_02358	plu2203
6) 3-phenylpropionate dioxygenase alpha subunit *hcaE*	pau_02357	plu2204
7) 3-phenylpropionate dioxygnease beta subunit *hcaF*	pau_02356	plu2205
8) 3-phenylpropionate dioxygenase ferredoxin reductase subunit *hcaC*	pau_02355	plu2206
9) 2,3-duhydroxy-2,3-dihydrophenylpropionate dehydrogenase *hcaB*	pau_02354	plu2207
10) 3-(2,3-dihydroxyphenyl) propionate dioxygenase *mhpB*	pau_02353	plu2208
11) Phenylpropionate dioxygenase ferredoxin reductase subunit *hcaD*	pau_02352	plu2209
12) Hypothetical protein		
13) Periplasmic oligopeptide binding protein *oppA*	pau_02342	plu2350
14) Putative betaine aldehyde dehydrogenase *ydcW*	pau_01173	plu2349
15) Hypothetical protein	pau_01174	plu2348
16) 4-aminobutyrate aminotransferase *goaG*	pau_01177	plu2347
17) Putative inner membrane protein *yhaH*	pau_01178	plu2346
18) FAD dependent oxidoreductase	pau_01179	plu2345
19) Hypothetical protein	pau_02339	plu2343
20) Hypothetical protein	pau_02338	
21) Putative mazF-like growth inhibitor/transcriptional regulator		
22) Hypothetical protein		
23) Hypothetical protein		plu2223
24) Putative transport protein *ynfM*	pau_02180	plu2224
25) Putative LysR-type transcriptional regulator *ynfL/alsR*	pau_02181	plu2225
26) Making large colonies protein *mlc*	pau_02182	plu2226
27) Putative dethiobiotin synthase *ynfK*	pau_02183	plu2227
28) Xylulose kinase *xylB*		plu1959
29) Putative D-xylulose reductase *ydjJ*	pau_02204	plu1960
30) Hypothetical protein	pau_02185	plu2232
31) 3-hydroxy acid dehydrogenase *ydfG*	pau_02186	plu2233
32) Putative histidine ammonia lyase	pau_02187	plu2234
33) Exochitinase	pau_02188	plu2235
34) Peptidase S58 *dmpA*	pau_02144	plu2239
35) Hypothetical protein		
36) Hypothetical protein	pau_00623	
37) Putative transposase		
38) Transposase Tn5045		
39) Hypothetical protein pSB102_p69		
40) Hypothetical protein pSB102_p68		

Table 4.4, List of genes on *P. temperata* K122 contig 176 and identified homologues in *P. asymbiotica* and *P. luminescens* TTO1 (continued below).

***P. temperata* K122 Contig 176**	***P. asymbiotica***	***P.luminescens* TTO1**
41) 2,5-diketo-D-gluconate reductase B *dkgB*		plu1527
42) Putative NADH oxidase		plu1526
43) LysR-type transcriptional regulator *yafC*		plu1525
44) Membrane secretion protein *hlyD*	pau_02197	plu2645
45) Macrolide export ATP-binding permease	pau_02199	plu2647
46) d-Aminopeptidase d*mpA*	pau_02203	plu2258
47) Beta-alanine pyruvate transaminase	pau_02208	plu2260
48) PzF family phenazine biosynthesis protein *pab*	pau_02222	plu2271
49) AraC-family transcriptional regulator		
50) O-glycosyl hydrolase family protein		plu2272
51) Xylose operon regulatory protein *xylR*		plu2274
52) Xylose isomerase *xylA*		plu2275
53) Hypothetical protein		
54) Putative oxalate decarboxylase *oxdD*	pau_02307	plu2325
55) Makes caterpillars floppy *mcf*	pau_03369	plu4142
56) Plasmid stabilization protein *pare*		plu0251
57) Putative CMP/dCMP deaminase	pau_02223	
58) Virulence sensor protein *bvgS/pvpS*	pau_02265	plu2284
59) Two-component response regulator *bvgR/pvpR*	pau_02266	plu2285
60) Hypothetical protein	pau_02267	plu2286
61) ClpA/B TypeVI secretion chaperone ATPase	pau_02268	plu2287
62) T6SS icmF-related protein *evpO/sciS/impL*	pau_02269	plu2288
63) T6SS transmembrane protein *evpN/dotU/impK*	pau_02270	plu2289
64) T6SS protein *evpM/impJ/sciO/vasE*	pau_02271	plu2290
65) T6SS lipoprotein *evpL/vasD*	pau_02272	plu2291
66) T6SS secretion associated protein *evpK/impA*	pau_02273	plu2292
67) T6SS protein *evpJ*	pau_02274	plu2293
68) Hypothetical protein	pau_02275	plu2294
69) T6SS protein *evpI* (Vgr family)	pau_02276	plu2295
70) T6SS protein *evpG/impH/vasB/sciB*	pau_02277	plu2296
71) T6SS protein *evpF/impG/sciC*	pau_02278	plu2297
72) T6SS protein *evpE/tssE/impF*	pau_02279	plu2298
73) T6SS effector protein *evpC*	pau_02280	plu2299
74) T6SS protein *evpB/impC/sciI*	pau_02281	plu2300
75) T6SS protein *evpA/impB/sciH*	pau_02282	plu2301

Table 4.4 (contd.), Continuation of listing of genes on *P. temperata* K122 contig 176 and identified homologues in *P. asymbiotica* and *P. luminescens* TTO1.

equipment assembly, but also a role for both proteins in the manufacture of a conduit to enable injection of further proteins into host cells. Other proteins have also been proposed to be secreted in a T6SS-dependent manner but identification of their subsequent function requires additional investigation (Filloux *et al.,* 2008).

4.6.2 Class III Primary mutants

The identification of the gene *nuoA* (Pm55) as the cause of the Class III mutant secondary phenotype indicates that the function of the respiratory chain has a role to play in phenotypic variation. The *nuo* operon, mapping to *P. temperata* K122 contig no. 17 (Fig. 3.6 & Table 3.2) is a conserved locus across the enterobacteria and has been shown to code for the energy-conserving NADH dehydrogenase Complex I (NDH-1) of both *Escherichia coli* and *Salmonella typhimurium* (Falk-Krezesinski and Wolfe 1998).

Located in the cytoplasmic membrane, NADH-dehydrogenases, in particular NDH-1, are part of the aerobic respiratory chain, the function of which is to generate a protein gradient (proton motive force – PMF) across the cytoplasmic membrane (Anraku and Gennis 1987; Calhoun and Gennis, 1993).

NADH dehydrogenase II (NDH-2) is encoded by the *ndh* gene and expressed at enhanced levels during exponential phase growth (Jackson *et al.,* 2004). NDH-2 does not play a role in the generation of PMF but oxidises NADH and feeds electrons into the respiratory chain (Matsushita *et al.,* 1987).

The PMF drives part of the cellular processes that require energy (e.g. ATP synthesis, active transport and motility) (Larsen *et al.,* 1974) and plays a central role in sensory transduction in response to oxygen, light and other effectors (Taylor 1983). PMF may also be required for insertion of proteins into membranes and the translocation of proteins across membranes (Date *et al.,* 1980; Gasser *et al.,* 1982). *E. coli* mutants lacking NDH-1 have been shown to have a competitive disadvantage in stationary phase (Zambrano & Kolter 1993) and are affected in growth on mixed amino acids as a

result of the inhibition of TCA enzymes that occurs in the absence of NDH-1(Prüß *et al.,* 1994). These defects of NDH-I mutants can be suppressed by serine (Dhamdhere & Zgurskaya 2010).

NDH-1 in the plant root colonizing bacterium *Pseudomonas fluorescens* has been shown to have a role in the root-tip colonization process. Mutants lacking NDH-1 functionality have a competitive disadvantage in root-tip colonization (Camacho-Carvajal *et al.,* 2002). A parallel to this can be seen in *Photorhabdus*, with mutualistic ability impaired in the *nuoA* mutant strain identified in this study. Lango and Clarke (2010) isolated a mutant in malate dehydrogenase (*mdh*) which identified the TCA cycle as having a role in the change of *Photorhabdus* from pathogen to mutualist. One of the functions of the TCA cycle is to provide intermediate compounds (including NADH) which donate protons and electrons to the electron transport chain. With differences in PMF and respiratory enzyme activity previously having been shown to occur between the primary and secondary variant phenotypes (Smigielski *et al*., 1994) it is conceivable that a mutation in genes affecting the provision of energy by the electron transport chain may also affect phenotypic variation. More recently, a *P. luminescens nuo* mutant has been identified that is affected in both motility and biofilm formation, with both reduced below wildtype levels (Amos *et al.,* 2011).

4.6.3 Class IV Primary mutants

The *serB* gene (Contig 22, Fig 4.9) encodes 3-phosphoserine phosphatase, an enzyme required for the biosynthesis of serine (Fig. 4.10). *Photorhabdus* species maintain the *serB* gene organisation seen in other enterobacteria, where *serB* is colocated with a *radA* gene encoding a DNA repair protein (Table 4.5). In *Escherichia coli*, serine and glycine availability are essential for the metabolism of several diverse classes of cellular components (Fig. 4.11) (Ravnikar & Somerville 1987). It has been established that, in *E. coli*, glucose is a precursor of serine (Roberts *et al.,* 1955) and that during growth on glucose, as much as 15% of the assimilated carbon in *E. coli* has been estimated to involve serine or its metabolites. (Pizer & Potochny 1964).

In *P. temperata*, reduced serine bioavailability may have far reaching effects on symbiosis and pathogenicity, both directly and indirectly, as a result of the diverse classes of cellular components dependent on serine biosynthesis in the Enterobacteriaceae. Lango and Clarke (2010) have shown that during growth on LB media, *P. luminescens* rapidly assimilates the available serine confirming the important role this amino acid plays in *Photorhabdus* growth. The disruption of *serB* in Pm58 may be the cause of the observed growth retardation phenotype observed in this strain (Fig. 4.1).

The exhaustion of available serine also indicates a need for the bacterial cells to manufacture serine to meet further demand. An example of this may occur during enterobactin siderophore biosynthesis, which requires the enzymatic activation of serine by EntF in enterobacteria (Reichart *et al.,* 1992). An EntF homologue, PhbG has been identified in *Photorhabdus* (Ciche *et al*., 2003) presumably with a similar role in the biosynthesis of the photobactin siderophore of *Photorhabdus*. In *P. temperata*, iron scavenging/acquisition has been shown to have a key role in the bacteria-nematode-insect interaction, with strains that are deficient in iron-scavenging unable to support nematode growth and development (Watson *et al.,* 2005), however, Photobactin itself is not required for a successful mutualism between the bacterium and the nematode (Ciche *et al.,* 2003; Watson *et al.,* 2005).

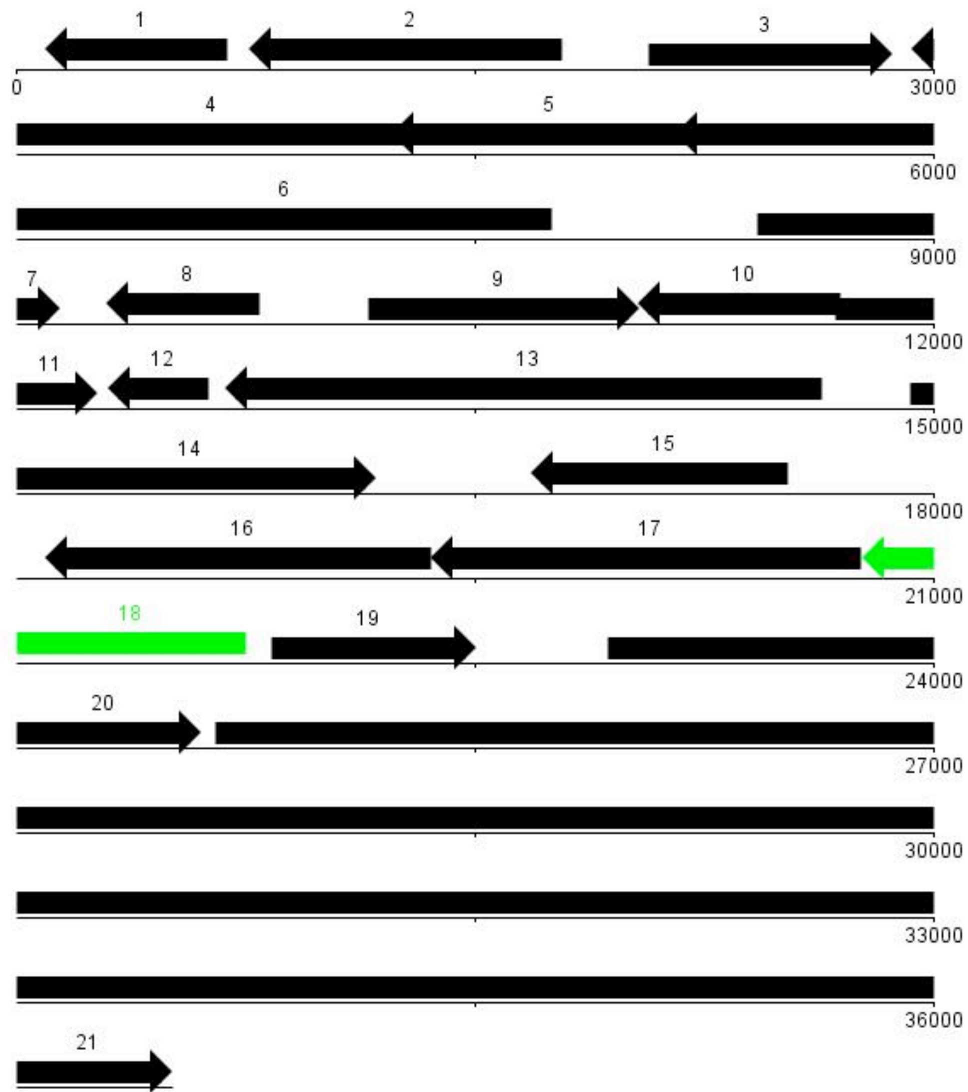


FIG. 4.10 Annotation of Genome Contig 22 of *P. temperata* K122, mutant Pm58 occurs in gene 18, *serB* phosphoserine phosphatase.

1) Molybdenum cofactor biosynthesis protein *mogA*
2) Transaldolase B *talB*
3) YaaA-like hypothetical protein
4) Threonine synthase *thrC*
5) Homoserine kinase *thrB*
6) Bifunctional aspartokinase I/homoserine dehydrogenase I *thrA*
7) Two compononent response regulator *arcA*
8) Conserved hypothetical protein *creA*
9) Right origin binding protein *rob*
10) Phosphoglycerate mutase *gpmB*
11) NTPase *yjjX*
12) Trp operon repressor *trpR*
13) Lytic murein transglycosylase *slt*
14) ABC-transporter ATP binding protein *yjjK*
15) Putative ABC transporter protein
16) Nicotinamide-nucleotide adenylyl transferase transcriptional regulator *nadR*
17) DNA repair protein *radA*
18) Phosphoserine phosphatase *serB*
19) Hypothetical protein *smp*
20) Putative haemolysin activator *hlyB*
21) Hypothetical protein

***P. temperata* K122 Contig 22**	***P. asymbiotica***	***P.luminescens* TTO1**
1) Molybdenum cofactor biosynthesis protein *mogA*	pau_00539	plu0569
2) Transaldolase B *talB*	pau_00538	plu0568
3) YaaA-like hypothetical protein	pau_00536	plu0566
4) Threonine synthase *thrC*	pau_00535	plu0565
5) Homoserine kinase *thrB*	pau_00534	plu0564
6) Bifunctional aspartokinase I/homoserine dehydrogenase I *thrA*	pau_00533	plu0563
7) Two compononent response regulator *arcA*	pau_00532	plu0562
8) Conserved hypothetical protein *creA*	pau_00531	plu0561
9) Right origin binding protein *rob*	pau_00530	plu0560
10) Phosphoglycerate mutase *gpmB*	pau_00529	plu0559
11) NTPase *yjjX*	pau_00528	plu0558
12) Trp operon repressor *trpR*	pau_00527	plu0557
13) Lytic murein transglycosylase *slt*	pau_00526	plu0556
14) ABC-transporter ATP binding protein *yjjK*	pau_00524	plu0555
15) Putative ABC transporter protein	pau_00523	plu0554
16) Nicotinamide-nucleotide adenylyl transferase *nadR*	pau_00522	plu0553
17) DNA repair protein *radA*	pau_00521	plu0552
18) Phosphoserine phosphatase *serB*	pau_00520	plu0551
19) Hypothetical protein *smp*	pau_00519	plu0550
20) Putative haemolysin activator *hlyB*	pau_01486	plu0549
21) Hypothetical protein	pau_01485	plu0548

Table 4.5, Listing of genes on *P. temperata* K122 contig 22 and identified homologues in *P. asymbiotica* and *P. luminescens* TTO1.

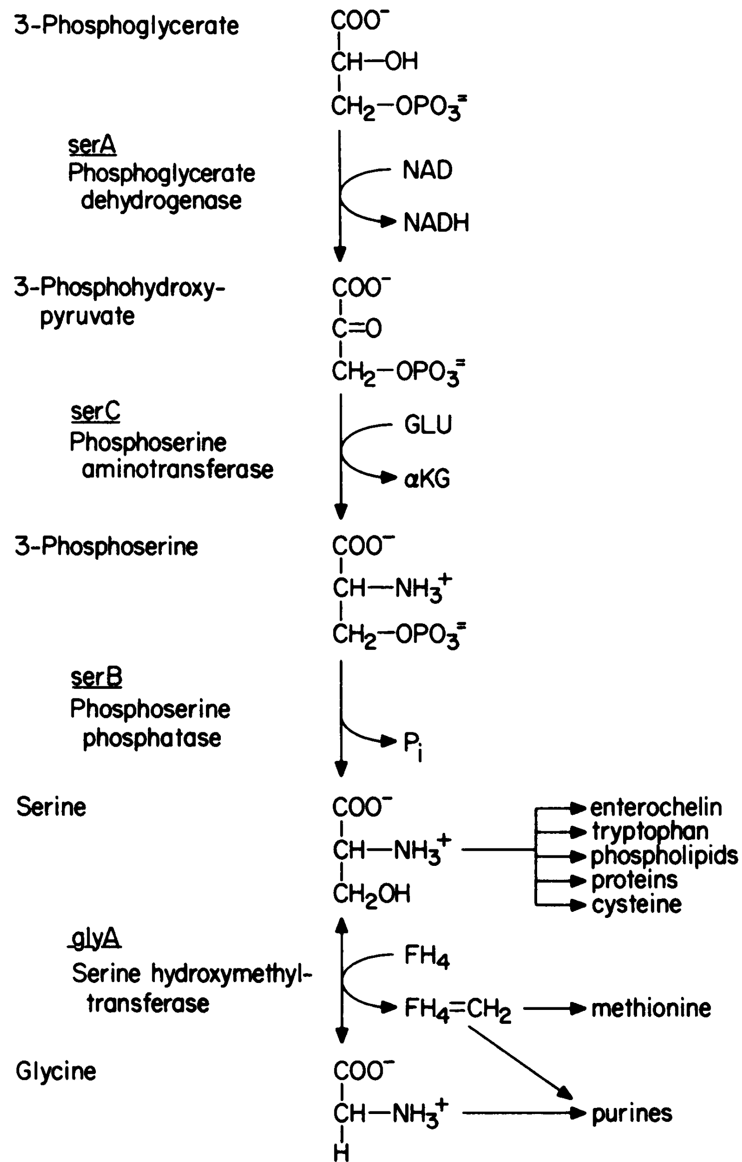


Fig. 4.10, Conventional pathway of serine and glycine biosynthesis (Ravnikar & Somerville 1987).

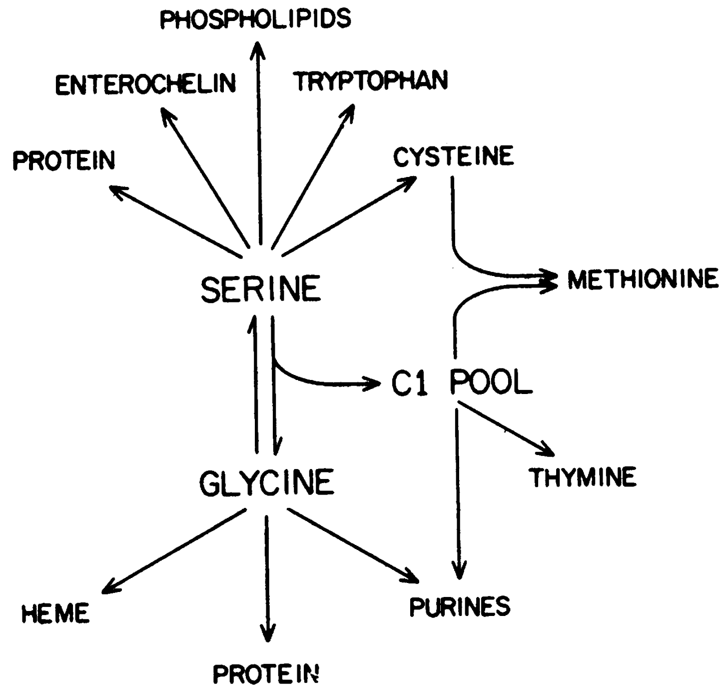


Fig. 4.11, Metabolites derived from serine and glycine (Ravnikar & Somerville 1987)

4.6.4 Class V Primary mutants

The LysR family of genes is the largest known and consists of a wide range of autoregulatory transcriptional regulators (Schell 1993). With the LysR type transcriptional regulator (LTTR) HexA already identified as having a role in the secondary to primary phenotypic switch, it is interesting to note a potentially similar LTTR - that identified in Pm76 (Fig. 4.12, contig 177) – may be involved in the primary variant to secondary variant phenotypic switch. Also of note is that whilst this LTTR is a common feature of K122 and TTO1, it is absent in *P. asymbiotica* (Table 4.6). With *P. asymbiotica* having some secondary-like characteristics (e.g. lack of pigment production) and a knockout in K122 resulting in a secondary-like mutant, the absence of this LTTR may be one of the reasons for the observed differences between *P. asymbiotica* and other *Photorhabdus* strains.

4.6.5 Class VI Primary mutants

Located on *P. temperata* K122 contig no. 238 (Fig.4.13), the *flhB* gene encodes a protein that acts as the export apparatus component of flagellar assembly.
Best studied in the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium, flagellar motility has an important role to play in both the pathogenic and symbiotic ability of many strains of bacteria (Graf *et al*., 1994; Feldman *et al*., 1998; Millikan & Rubi 2002; Ferris & Minamino, 2006; Gauger *et al*., 2007; Shimoyama *et al*., 2009). The bacterial flagellum is a complex self-assembling nanomachine that contains its own secretion/protein export apparatus. Upon completion of early flagellar structure, this apparatus switches substrate specificity to export late structural subunits thereby coupling sequential flagellar gene expression with flagellar assembly. The switch is achieved by a conformational change of the export apparatus component FlhB (Williams *et al*., 1996; Minamino & Macnab 2000), driven by the flagellar hook-length control protein FliK (Patterson-Delfield *et al*., 1973; Hirano *et al*., 1994), therefore any disruption of the *flhB* gene could potentially affect flagellar formation and/or component secretion, thereby affecting motility, with subsequent affects on pathogenicity and symbiosis.

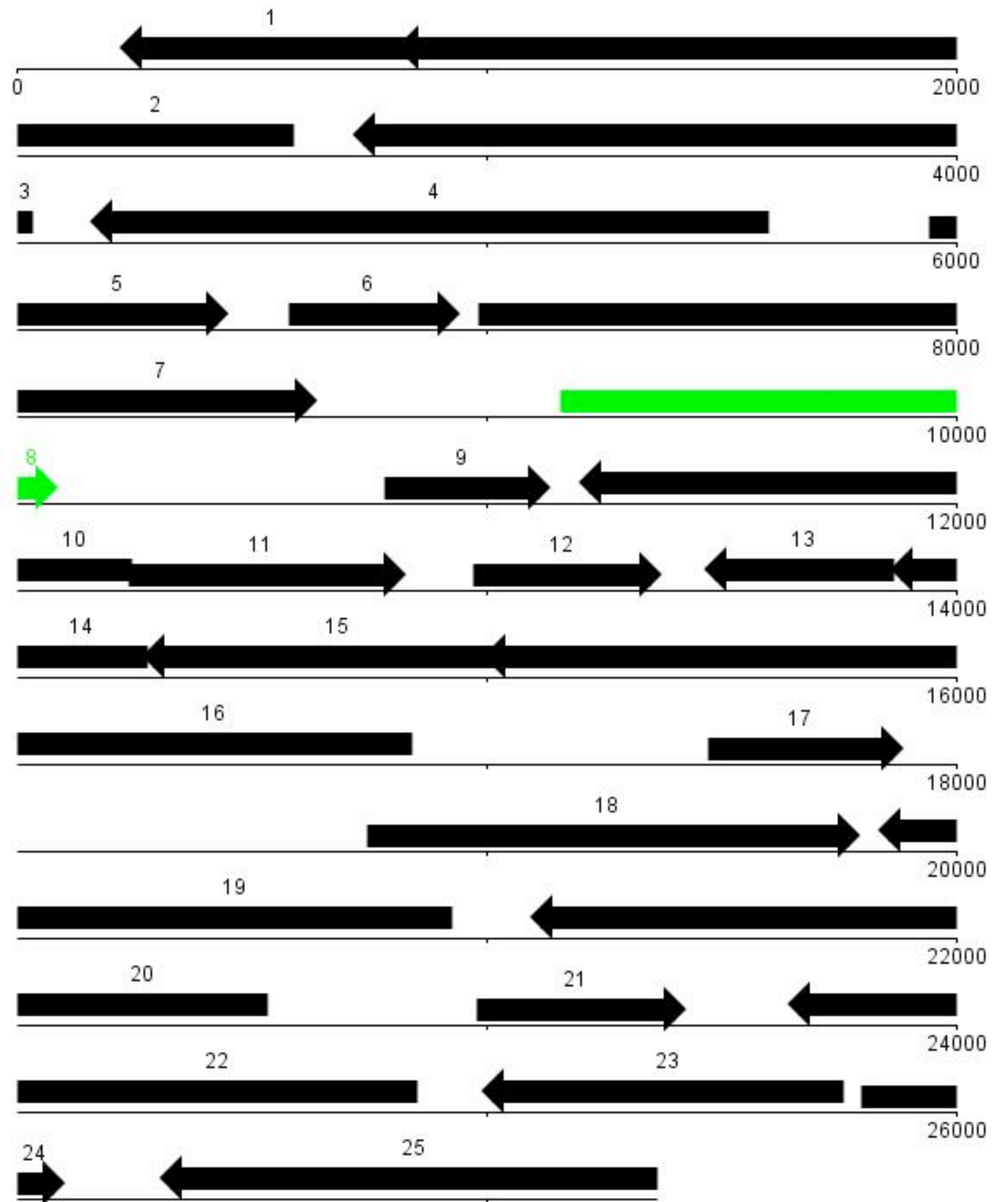


FIG. 4.12 Annotation of Genome Contig 177 of *P. temperata* K122, mutant Pm76 occurs in gene 8, a LysR type transcriptional regulator.

1) TetR family HTH transcriptional regulator *yjdC*
2) Thiol-disulfide interchange protein precursor *dipZ*
3) Anaerobic C4-dicarboxylase transporter *dcuA*
4) Aspartate ammonia-lyase *aspA*
5) Inner membrane protein *fxsA*
6) GroES co-chaperonin
7) GroEL chaperonin
8) LysR family transcriptional regulator
9) Putative lipoprotein
10) Putative KamA family iron-sulfur protein *yjeK*
11) Elongation factor P *efp*
12) Quaternary ammonium compound resistance *sugE*
13) Fumarate reductase subunit D *frdD*
14) Fumarate reductase subunit C *frdC*
15) Fumarate reductase iron-sulfur subunit B *frdB*
16) Fumarate reductase flavoprotein subunit *frdA*
17) Hypothetical protein
18) Lysyl-tRNA synthetase protein *poxA/genX*
19) Glycerophosphodiester phosphodiesterase *glpQ*
20) sn-Glycerol-3-phosphate transporter *glpT*
21) Hypothetical protein
22) Glycerol dehydrogenase *gldA*
23) Hypothetical protein
24) Sulfur transport protein *sirA/tusA*
25) Zinc/cadmium/mercury/lead-transporting ATPase *zntA*

P. temperata K122 Contig 177	*P. asymbiotica*	*P.luminescens* TTO1
1) TetR family HTH transcriptional regulator *yjdC*	pau_03764	plu4140
2) Thiol-disulfide interchange protein precursor *dipZ*	pau_03763	plu4139
3) Anaerobic C4-dicarboxylase transporter *dcuA*	pau_03764	plu4138
4) Aspartate ammonia-lyase *aspA*	pau_03760	plu4137
5) Inner membrane protein *fxsA*	pau_03758	plu4136
6) GroES co-chaperonin	pau_03757	plu4135
7) GroEL chaperonin	pau_03756	plu4134
8) LysR family transcriptional regulator		plu4133
9) Putative lipoprotein	pau_03755	plu4132
10) Putative KamA family iron-sulfur protein *yjeK*	pau_03754	plu4132
11) Elongation factor P *efp*	pau_03753	plu4130
12) Quaternary ammonium compound resistance *sugE*	pau_03752	plu4129
13) Fumarate reductase subunit D *frdD*	pau_03751	plu4127
14) Fumarate reductase subunit C *frdC*	pau_03750	plu4126
15) Fumarate reductase iron-sulfur subunit B *frdB*	pau_03749	plu4125
16) Fumarate reductase flavoprotein subunit *frdA*	pau_03748	plu4124
17) Hypothetical protein	pau_03746	plu4122
18) Lysyl-tRNA synthetase protein *poxA/genX*	pau_03743	plu4121
19) Glycerophosphodiester phosphodiesterase *glpQ*	pau_03742	plu4120
20) sn-Glycerol-3-phosphate transporter *glpT*	pau_03741	plu4119
21) Hypothetical protein	pau_03740	plu4118
22) Glycerol dehydrogenase *gldA*	pau_03738	plu4115
23) Hypothetical protein	pau_03737	plu4110
24) Sulfur transport protein *sirA/tusA*	pau_03736	plu4109
25) Zinc/cadmium/mercury/lead-transporting ATPase *zntA*	pau_03733	plu4108

Table 4.6, Detail of genes on *P. temperata* K122 contig 17 and identified homologues in *P. asymbiotica* and *P. luminescens* TTO1. The LysR type transcriptional regulator mutated in Pm76 is absent in the *P. asymbiotica* strain.

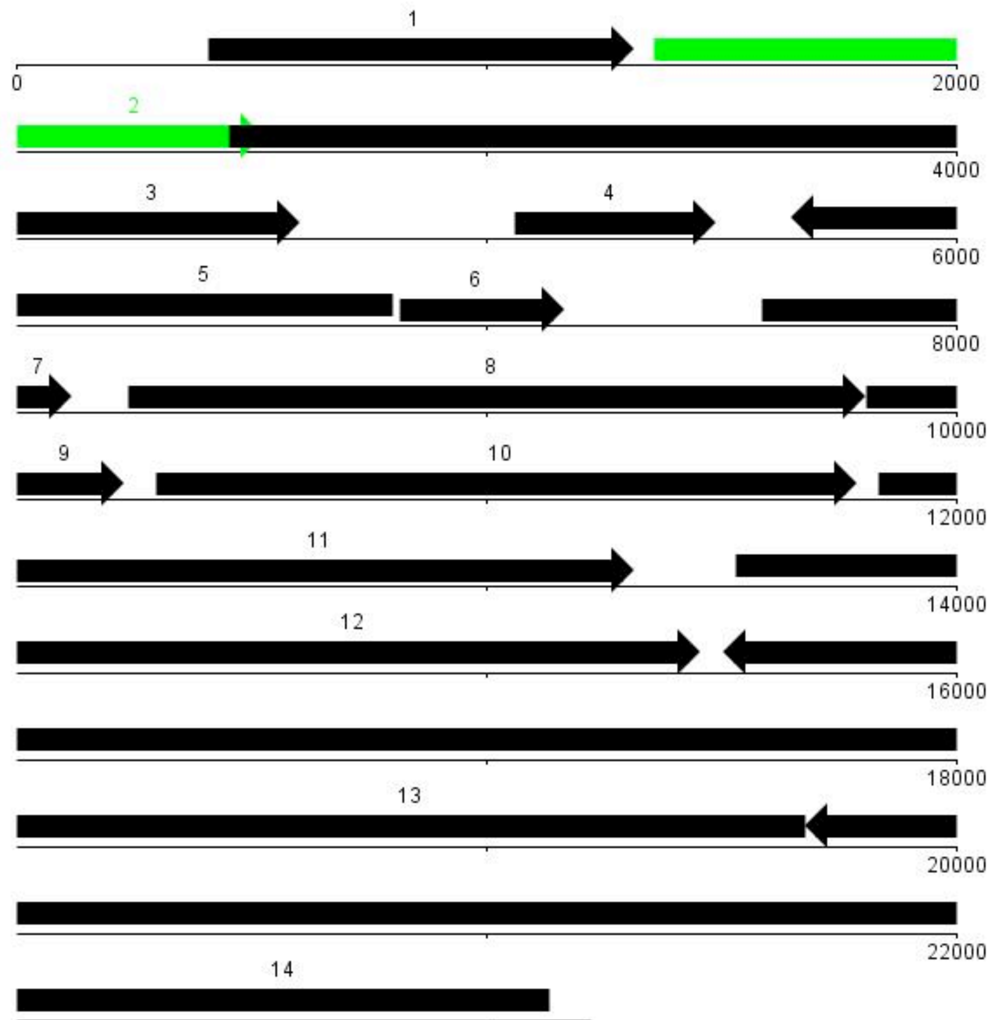


FIG. 4.13 Annotation of Genome Contig 238 of *P. temperata* K122, mutants Pm85, 92 & 100 occur in gene 2, *flhB*.

1) Putative ABC transporter ATPase
2) Flagellar biosynthesis protein *flhB*
3) Flagellar biosynthesis protein *flhA*
4) Hypothetical protein
5) Putative Fic (Filamentation induce by cAMP) family protein
6) Hypothetical protein
7) Hypothetical protein
8) Hypothetical protein
9) Hypothetical protein
10) Hypothetical protein
11) Hypothetical protein
12) Exochitinase-like protein *chiB2*
13) Insecticidal toxin complex protein *tccB2*
14) Insecticidal toxin complex protein *tccA2*

P. temperata K122 Contig 238	*P. asymbiotica*	*P.luminescens* TTO1
1) Putative ABC transporter ATPase		
2) Flagellar biosynthesis protein *flhB*	pau_02666	plu1895
3) Flagellar biosynthesis protein *flhA*	pau_02665	plu1896
4) Hypothetical protein	pau_02664	plu1897
5) Putative Fic (Filamentation induce by cAMP) family protein		plu4284
6) Hypothetical protein		
7) Hypothetical protein	pau_02663	plu1899
8) Hypothetical protein	pau_02662	plu1900
9) Hypothetical protein	pau_02661	plu1901
10) Hypothetical protein	pau_02660	plu1902
11) Hypothetical protein	pau_02659	plu1903
12) Exochitinase-like protein *chiB2*	pau_02059	plu2458
13) Insecticidal toxin complex protein *tccB2*	pau_02058	plu2459
14) Insecticidal toxin complex protein *tccA2*	pau_02557	plu2460

Table 4.7, Continuation of listing of genes on *P. temperata* K122 contig 238 and identified homologues in *P. asymbiotica* and *P. luminescens* TTO1.

flhB has also been shown to have a role in the regulation of the alternate sigma factor FliA (σ^{28}) with an *flhB* mutant of the plant pathogenic bacterium *Xanthomonas campestris* producing much decreased levels of FliA protein (Yang *et al*., 2009). FliA is involved in the regulation of TTSS and associated virulence genes in a wide range of bacteria, including *Yersinia*, *Erwinia* and *Salmonella* spp. (Jahn *et al.,* 2008). In *Xenorhabdus nematophila*, FliA, whilst not essential for maintenance of virulence, has been shown to have a role not only in flagella synthesis, but also in exoenzyme and antibiotic production also (Park and Forst 2006). *flhB* also is homologous with *yscU*, the last gene of the *virB* locus found in pathogenic *Yersinia* species. YscU is a membrane protein involved in the secretion of the antihost Yop proteins of such pathogenic *Yersinia* (Allaoui *et al.,* 1994).

The pathways involving *flhB* and *yscU* are both examples of Type III secretion systems (TTSS) (Fig. 4.14). In general, Type Three Secretion (T3S) is characterised by (i) host-contact mediated TTSS induction, (ii) energy requirement for protein secretion and translocation into host cells, (iii) secretion-regulated expression of genes encoding proteins secreted downstream in the pathway and (iv) dedicated cytoplasmic chaperones for some secreted proteins, although the flagellar secretions system does not share the first characteristic nor the ability to translocate proteins into eukaryotic cells, with other TTSS. (Blocker *et al.,* 2003) TTSS enable bacteria to inject bacterial proteins (effectors) into eukaryotic host cells across both the bacterial and host cell membranes, from where they can manipulate/direct host cell function. However, with the *P. temperata* K122 TTSS identified on a separate contig (David Clarke, personal communication & Fig. 5.11) it is probable that the *flhB* identified here is involved in flagellar formation and motility. Easom and Clarke (2008) identified a competitive advantage for *Photorhabdus* with motile ability during insect infection, which is somewhat consistent with the finding that mutant Pm100 displays reduced rates of insect killing when compared to the Rif^{R} wild type parent strains (Fig 4.3).

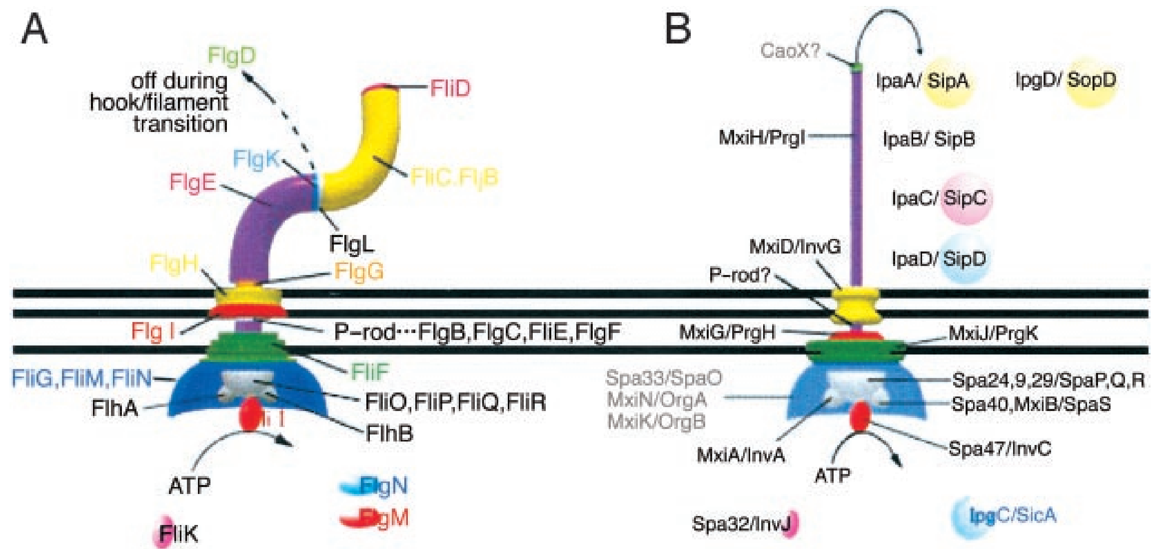


Fig. 4.14, From Blocker *et al.,* 2003. Diagrams of known positions of major flagellar components (A) and established and hypothetical TTSS functional homologs (B). Functions of proteins conserved in both systems are marked by similar position, shading, and coloring whether they share sequence homologies or not. Those TTSS components for which no sequence homology or experimental evidence exists to establish their relation to the similarly positioned and colored flagellar components in A are shown transparently in B.

4.7 Summary

The work in this chapter identified 9 mutants that were switched phenotypically from primary to secondary-like. A range of genes, involved in Type VI secretion, metabolism, biosynthesis, motility and transcriptional regulation were implicated in effecting this phenotypic transition, indicative of a complex range of interactions leading to the occurrence of a phenotypic switch in *Photorhabdus.*

Whilst the effect of the mutations introduced to these strains may be acting either directly on gene function, or indirectly via changes in the expression of genes downstream of the site of transposon insertion, work by Gaudrialt *et al* (2008) in *P. luminescens* TTO1 suggests that whilst genomic rearrangements can affect individual phenotypes, the coordinated phenotypic switch observed during *Photorhabdus* primary to secondary variation can occur independently of global genomic architecture, with deletions, additions/duplications and rearrangements within the genome all occurring without the large scale switching of phenotypes that is observed during the transition between the primary and secondary variants.

It is however, also possible that the phenotypic switches observed in the 9 mutants identified in this chapter may still be occurring indirectly as a result of the gene knockouts, with the loss of gene function affecting cell fitness and resulting in a stressed bacterial cell, with subsequent activation of the phenotypic switch to ensure survival. Irrespective of the mode of activation of the switch, the range of factors which influence the occurrence of the switch seem to indicate that the switch is an important mechanism in reacting to the fitness of the bacterial cell and adapting the cell for survival under stressful conditions.

Chapter 5

5. Characterisation of primary mutants with intermediate phenotypes

5.1 Introduction

With several genes having heretofore been identified as having an involvement in the phenotypic switching of *P. temperata* K122, it was decided to examine the remainder of the previously generated Pm mutants that had been characterized as partially switched. The results of the phenotypic tests carried out on these mutants are listed in Table 5.1. From these data, it appears that whilst all of the mutants are similar to the secondary variant in that they lack pigment production, there are no set links between the different phenotypic characters tested, with variable phenotypes occurring for each mutant, giving a further indication of the genetic complexity involved in the phenotypic switch. These data would also hint at the hierarchical nature of the regulatory network that controls phenotypic variation and at the existence of phenotype specific regulatory circuits (Table 5.2). From these data, it can be seen that the mutants fall into 30 different phenotypic groupings, with a bias toward the occurrence of pigment mutants as a result of the selection method used. A trend can be seen in the rate of commonality of occurrence of each mutant phenotype with for example, the Pigment-Lipase phenotype most prevalent out of the groupings identified. However, with the exception of catalase, which occurs exclusively in the presence of the lipase phenotype, there does not appear to be any overarching linkage between the phenotypes tested, and no step-wise progression of the occurrence of the phenotypic switch is apparent.

Table 5.1 Phenotypic characterization of the primary to secondary Pm mutant strains.

	Colour on LB	Antibiosis	Bioluminescence	Colour on NBTA	Colour on EMB	Lipase Activity	Catalase Activity	Motility
K122 1°	Orange	+++	Yes	Green	Black	+++	+++	Yes
K122 2°	White	-	No	Blue	Red	-	-	No
Pm1	White	+++	Yes	Green	Black	-	+++	Yes
Pm2	White	+++	Yes	Green	Black	-	+++	Yes
Pm3	White	+++	Yes	Green	Black	+++	+++	Yes
Pm4	White	+++	Yes	Green	Black	-	+++	Yes
Pm5	White	+++	Yes	Green	Black	-	+++	Yes
Pm6	White	-	Yes	Green	Black	+++	+++	Yes
Pm7	White	++	Yes	Green	Black	+++	+++	Yes
Pm8	White	+++	Yes	Green	Black	-	+++	Yes
Pm10	White	-	Yes	Green	Black	-	+	Yes
Pm11	White	-	Yes	Green	Black	+++	+++	Yes
Pm12	White	-	Yes	Green	Black	+++	+++	Yes
Pm13	White	+++	Yes	Green	Black	-	+++	Yes
Pm14	White	-	No	Green	Black	+++	+++	Yes
Pm15	White	++	Yes	Green	Black	+++	+++	Yes
Pm16	White	+++	Yes	Green	Black	-	+++	Yes
Pm17	White	+++	Yes	Green	Black	+++	+++	Yes
Pm18	White	-	No	Blue	Red	-	++	No
Pm19	White	+++	Yes	Green	Black	+++	+++	Yes
Pm21	White	+++	Yes	Green	Black	-	+++	Yes
Pm22	White	-	Yes	Green	Black	+++	+++	Yes
Pm23	White	+++	Yes	Green	Black	-	+++	Yes
Pm24	White	++	Yes	Green	Black	+++	+++	Yes
Pm25	White	+++	Yes	Green	Black	+++	+++	Yes
Pm26	White	+++	Yes	Green	Black	-	+++	Yes
Pm27	White	++	Reduced	Green	Red	-	+++	Yes
Pm28	White	+++	Yes	Green	Black	-	+++	Yes
Pm29	White	+++	Yes	Green	Black	+++	+++	Yes
Pm30	White	-	Yes	Green	Black	+++	+++	Yes
Pm31	White	+++	Yes	Green	Black	+	+++	Yes
Pm32	White	+++	Yes	Green	Black	+++	+++	Yes
Pm33	White	+++	Yes	Green	Black	-	+++	Yes
Pm34	White	-	Yes	Green	Black	+++	+++	Reduced
Pm35	White	-	Yes	Green	Black	+++	+++	Yes
Pm36	White	+++	Yes	Green	Black	-	+++	Yes
Pm37	White	+++	Yes	Green	Black	-	+++	Yes
Pm38	White	+++	Yes	Green	Black	-	+++	Yes
Pm39	White	++	Yes	Green	Black	+++	+++	Yes
Pm40	White	+++	Yes	Green	Black	-	+++	Yes
Pm41	White	-	Yes	Green	Black	+++	+++	Yes
Pm42	White	+++	Yes	Green	Black	-	+++	Yes

Table 5.1 (contd.) Phenotypic characterization of the primary to secondary Pm mutant strains.

	Colour on LB	Antibiosis	Bioluminescence	Colour on NBTA	Colour on EMB	Lipase Activity	Catalase Activity	Motility
K122 1°	Orange	+++	Yes	Green	Black	++	+++	Yes
K122 2°	White	-	No	Blue	Red	-	-	No
Pm43	White	+++	Yes	Green	Black	+++	+++	No
Pm44	White	-	Yes	Green	Black	+++	+++	Yes
Pm45	White	-	No	Green	Black	+++	+++	No
Pm46	White	+++	Yes	Green	Black	-	+++	Yes
Pm47	White	+++	Reduced	Green	Black	-	++	Yes
Pm48	White	+++	Yes	Green	Black	-	+++	Yes
Pm49	White	+++	Yes	Green	Black	-	+++	No
Pm50	White	-	Reduced	Green	Black	+	+++	Yes
Pm51	White	-	Yes	Green	Black	-	+++	Yes
Pm52	White	-	Yes	Green	Black	+++	+++	Yes
Pm54	White	-	Yes	Green	Black	+++	+++	Yes
Pm56	White	+++	Yes	Green	Black	-	+++	Yes
Pm57	White	+++	Reduced	Green	Black	-	+++	Yes
Pm59	White	+++	Yes	Green	Black	+++	+++	Yes
Pm60	White	-	No	Blue	Red	-	+++	Yes
Pm61	White	+++	Yes	Green	Black	-	+++	Yes
Pm62	White	-	Reduced	Green	Black	+++	+++	Yes
Pm63	White	+++	Yes	Green	Black	-	+++	Yes
Pm64	White	-	Reduced	Green	Black	+++	+++	Yes
Pm65	White	+++	Yes	Green	Black	-	+++	Yes
Pm66	White	-	Yes	Blue	Black	-	+++	Yes
Pm67	White	++	Yes	Green	Black	-	+++	Yes
Pm68	White	++	Yes	Green	Black	-	+++	Yes
Pm69	White	+++	Yes	Green	Black	-	+++	Yes
Pm70	White	+++	Yes	Green	Red	+++	+++	Yes
Pm71	White	-	Reduced	Green	Black	+++	+++	Yes
Pm72	White	-	Reduced	Blue	Red	+++	+++	Yes
Pm73	White	+++	Yes	Green	Black	-	+++	Yes
Pm74	White	-	Reduced	Green	Black	+++	+++	Yes
Pm75	White	++	Yes	Green	Black	-	+++	Yes
Pm77	White	-	Yes	Green	Black	+++	+++	Yes
Pm78	White	-	Yes	Green	Red	-	+++	Yes
Pm79	White	+++	Yes	Green	Black	-	+++	Yes
Pm80	White	+++	Yes	Green	Black	-	+++	Yes
Pm81	White	-	Yes	Green	Black	+	+++	Yes
Pm82	White	-	Reduced	Green	Black	+	+++	Yes
Pm83	White	+++	Yes	Green	Black	-	+++	Yes
Pm84	White	++	Reduced	Blue	Red	-	+++	Yes
Pm86	White	++	Yes	Blue	Red	-	+++	Yes
Pm87	White	-	Yes	Green	Black	-	+++	Yes

Table 5.1 (contd.) Phenotypic characterization of the primary to secondary Pm mutant strains.

	Colour on LB	Antibiosis	Bioluminescence	Colour on NBTA	Colour on EMB	Lipase Activity	Catalase Activity	Motility
K122 1°	Orange	+++	Yes	Green	Black	++	+++	Yes
K122 2°	White	-	No	Blue	Red	-	-	No
Pm88	White	+++	Reduced	Green	Black	+++	+++	Yes
Pm89	White	+++	Yes	Green	Black	-	+++	Yes
Pm90	White	++	Yes	Green	Black	-	+++	Yes
Pm91	White	-	Reduced	Green	Black	+++	+++	Yes
Pm93	White	-	Yes	Blue	Red	+++	+++	Yes
Pm94	White	-	Yes	Green	Red	+++	+++	Yes
Pm95	White	+++	Yes	Green	Red	-	+++	Yes
Pm96	White	+++	Yes	Green	Black	-	+++	Yes
Pm97	White	+++	Yes	Green	Black	-	+++	Yes
Pm98	White	-	Reduced	Green	Black	-	+++	Yes
Pm99	White	-	Yes	Green	Black	+++	+++	Yes
Pm101	White	+++	Yes	Blue	Red	-	+++	Yes
Pm102	White	+++	Yes	Green	Black	-	+++	Yes
Pm103	White	-	Yes	Green	Black	-	+++	Yes
Pm104	White	-	Reduced	Blue	Black	-	+++	No
Pm105	White	+++	Yes	Green	Black	-	+++	Yes
Pm106	White	+++	Yes	Green	Black	-	+++	Yes
Pm107	White	-	No	Green	Red	-	+++	Yes
Pm108	White	-	Yes	Green	Red	-	+++	Yes
Pm109	White	+++	Yes	Green	Black	-	+++	Yes
Pm110	White	+++	Yes	Green	Black	-	+++	Yes
Pm111	White	++	No	Green	Red	-	-	Yes
Pm112	White	+++	Yes	Green	Black	-	+++	Yes
Pm113	White	+++	Yes	Green	Black	-	+++	Yes
Pm114	White	+++	Yes	Green	Black	-	+++	Yes
Pm115	White	+++	Yes	Green	Black	-	+++	Yes
Pm116	White	++	Yes	Green	Red	-	-	Yes
Pm117	White	+++	Yes	Green	Black	-	+++	Yes
Pm118	White	+++	Yes	Green	Black	-	+++	Yes
Pm119	White	+++	Yes	Green	Black	-	+++	Yes
Pm120	White	+++	Yes	Green	Black	-	+++	Yes
Pm121	White	+++	Yes	Green	Black	-	+++	Yes
Pm122	White	-	Reduced	Green	Red	-	-	No
Pm123	White	++	Yes	Green	Black	-	+++	Yes
Pm124	White	+++	Yes	Green	Black	-	+++	Yes
Pm125	White	-	Reduced	Green	Black	-	+++	Yes
Pm126	White	-	Yes	Blue	Red	-	-	Yes
Pm127	White	-	Yes	Blue	Red	-	-	No

Table 5.2 Distribution of secondary-like phenotypes amongst the Pm mutants.

A)

No. Secondary-like phenotypes	Secondary-like phenotypes	No. out of 118 Mutants
1	Pigment	10
2	Pigment, Lipase	52
2	Pigment, Antibiotic	12
2	Pigment, Motility	1
2	Pigment, Biolum.	2
2	Pigment, EMB	1
3	Pigment, Antibiotic, Biolum.	6
3	Pigment, Antibiotic, Motility	2
3	Pigment, Biolum, Lipase	2
3	Pigment, Lipase, Motility	1
3	Pigment, Antibiotic, Lipase	4
3	Pigment, Antibiotic, EMB	1
3	Pigment, EMB, Lipase	1
4	Pigment, Antibiotic, Lipase, Catalase	1
4	Pigment, Biolum, EMB, Lipase	1
4	Pigment, Antibiotic, Biolum, Lipase	4
4	Pigment, Antibiotic, NBTA, Lipase	1
4	Pigment, Antibiotic, EMB, Lipase	2
4	Pigment, NBTA, EMB, Lipase	2
4	Pigment, Antibiotic, NBTA, EMB	1
4	Pigment, EMB, Lipase, Catalase	1
5	Pigment, Antibiotic, Biolum, NBTA, EMB	1
5	Pigment, Biolum, NBTA, EMB, Lipase	1
5	Pigment, Biolum, EMB, Lipase, Catalase	1
6	Pigment, Antibiotic, Biolum, NBTA, EMB, Lipase	2
6	Pigment, Antibiotic, Biolum, NBTA, Lipase, Motility	1
6	Pigment, Antibiotic, NBTA, EMB, Lipase, Catalase	1
7	Pigment, Antibiotic, Biolum, NBTA, EMB, Lipase, Motility	1
7	Pigment, Antibiotic, Biolum, EMB, Lipase, Catalase, Motility	1
7	Pigment, Antibiotics, NBTA, EMB, Lipase, Catalase, Motility	1

B)

Pigment	30/30
Lipase	20/30
Antibiotic	17/30
EMB	15/30
Bioluminescence	12/30
NBTA	10/30
Motility	7/30
Catalase	6/30

A) Distribution of Secondary like phenotypes of the 118 Pm strains

B) Frequency of occurrence of each phenotype within the 30 groupings identified

Of these 118 primary to secondary variant phenotypic mutants, 12 were selected (Pm3, 6, 7, 24, 25, 35, 39, 59, 77, 81, 102 & 112) for sequencing in order to investigate whether the intermediate phenotypic variants could provide evidence for a general sequence of changes leading to the development of the full secondary phenotype. The resultant sequences were subjected to BLAST analysis using the BLASTN and BLASTX database queries. The sequences were also compared to the *P. temperata* K122 contig database to gain further insight into the genomic arrangement surrounding each transposon insertion event. The corresponding regions of the *P. luminescens* TTO1 and *P. asymbiotica* genomes were also examined for conservation of gene arrangement and similarity of function (summarised in Table 5.3).

5.2 Mutant Pm3

Deficient in pigment production but primary variant-like for all other phenotypes tested, sequencing of mutant Pm3 revealed the transposon insertion to be in a DNA-N6-adenine methyl transferase, found on K122 contig 58 (Fig. 5.1). Lacking a counterpart in *P. asymbiotica*, a homologous gene (*plu3449*) was found on the *P. luminescens* TTO1 genome (Table 5.4) where it constitutes part of a 47.5kb phage locus running from *plu3423-plu3489* (Wilkinson *et al*., 2009). Bacteriophage play important roles in both bacterial phenotypic variation and virulence. For example, in *Pseudomonas aeruginosa*, the activity of phage Pf4 has been shown to be involved in the development of small colony phenotypic variant cells (SVCs) exhibiting increased adhesion properties, indicative of a role for such phenotypic variants in biofilm development (Webb *et al*., 2004). Such SCVs are often more resistant to antibiotics than wild type cells (Mooij *et al*., 2007). The virulence factors of many pathogenic bacteria have also been observed to be located on prophage loci, suggesting a role for prophages in conferring pathogenicity on the host bacterium (Waldor, 1998; Davis & Waldor 2000; Boyd & Brüssow 2002).

Mutant	Gene/Protein Identified	K122 Contig	*asymbiotica*	TTO1
Pm3	DNA N-6-adenine methyl transferase (DNA Methylase)	58		*plu3449*
Pm6	*galK* (galactokinase)	57	*pau_00469*	*plu0576*
Pm7	RtxA toxin	194	*pau_02098*	*plu2400*
Pm24	*trkA* (Potassium uptake)	384	*pau_04151*	*plu4671*
Pm25	Hypothetical protein	168	*pau_00387*	*plu0480*
Pm35	*rarD*	384	*pau_04113*	*plu4632*
Pm39	Hypothetical protein	776	*pau_03019*	*plu2670*
Pm59	DNA Polymerase II *dinA/polB*	145	*pau_00754*	*plu0616*
Pm77	Transcriptional regulator *sdiA*	487	*pau_04062*	*plu4562*
Pm81	*aroQ* – 3 dehydroquinate dehydratase	134	*pau_03705*	*plu4073*
Pm102	Putative lipoprotein LscW;HscY;ExsC	162	*pau_01067*	*plu3751*
Pm112	Similar to protein gp29 of Enterobacteria phage Mu	58		*plu3441*

Table 5.3 Summary of sequenced Pm mutants, with the identified genes, K122 contig location and appropriate homologues in *P. asymbiotica* and *P. luminescens* TTO1.

Indeed, many of the regions of the *Photorhabdus* genome identified as containing virulence cassettes with insecticidal activity are phage-related loci (Waterfield *et al*., 2004; Yang *et al*., 2006). Such phage-conferred factors include, but are not limited to extracellular toxins, proteins altering antigenicity, invasion effector proteins, enzymes and adhesion proteins (Brüssow *et al*., 2004). It has previously been suggested that the phage elements in the *Photorhabdus* genome may participate in the release of molecules involved in pathogenesis via partial lysis of the bacteria (Duchaud *et al*., 2003). A similar situation to this has been observed during biofilm formation in *Steptococcus pneumoniae* whereby an initial lysis of cells occurs, facilitating the release of extracellular DNA, important for structural stability of the biofilm (Whitchurch *et al*., 2002; Carrolo *et al*., 2010).

Phage have been implicated in the adaptation of pathogens to new hosts and according to Brüssow *et al* (2004) may affect the fitness of a bacterium in any one of at least five different ways including:

- Acting as anchor points for genome rearrangement
- Causing gene disruption
- Providing protection against lytic infection
- Lysis of competing strains by prophage infection
- The introduction of new genes conferring new fitness factors

Disruption of prophage may result in the loss of any adaptation conferred by that phage and thereby result in an inability to exploit a specific environment.

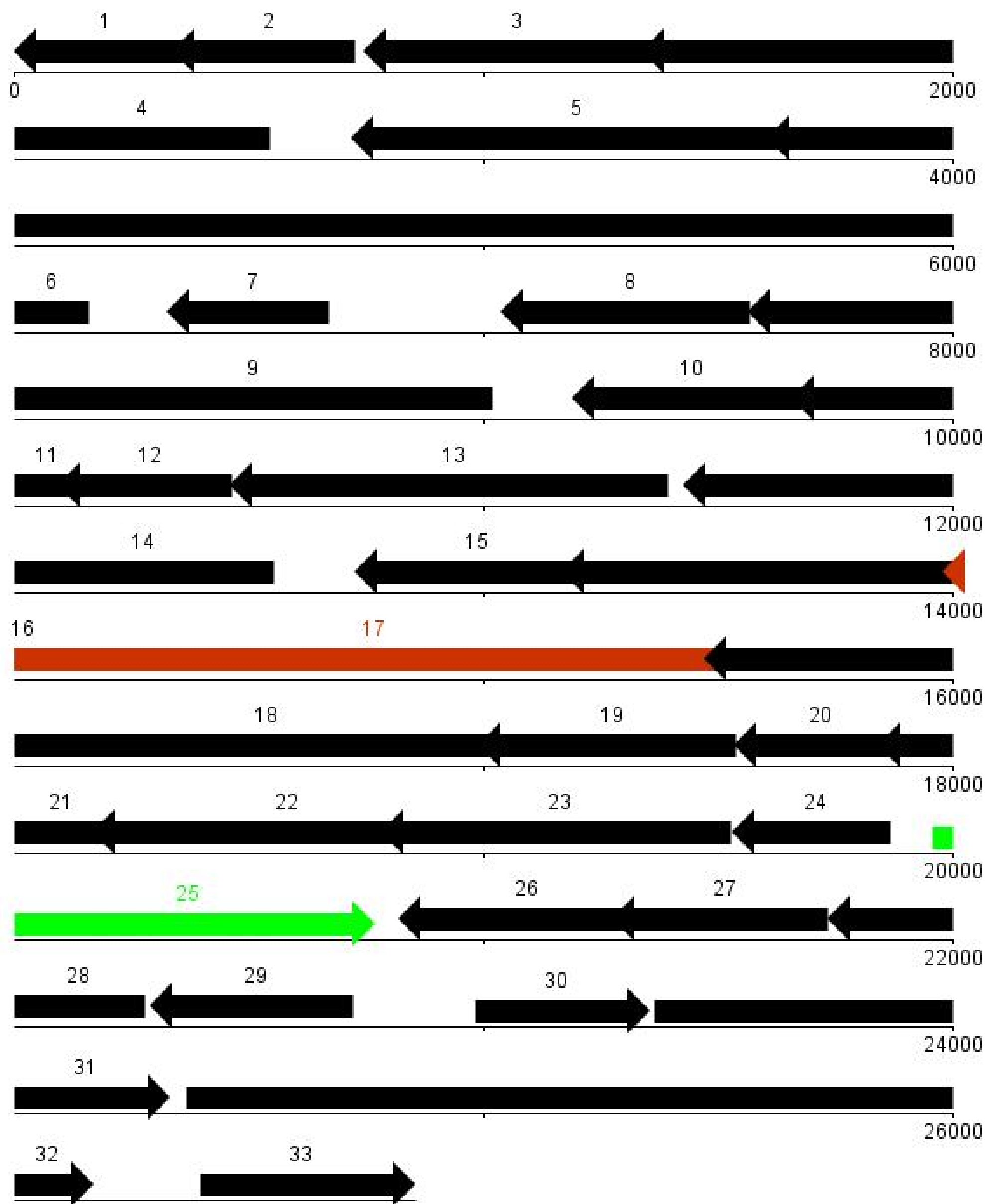


FIG. 5.1 Annotation of Genome Contig 58 of *P. temperata* K122, mutant Pm3 occurs in gene 25, with mutant Pm112 in gene 17.

1) Phage baseplate assembly protein
2) Phage baseplate protein
3) Phage baseplate assembly protein V
4) Putative Phage regulatory protein
5) Put. Methyl-accepting phage chemotaxis protein
6) Putative bacteriophage tail protein
7) Putative phage related protein
8) Phage tail core protein
9) Phage tail sheath protein
10) Putative phage related protein
11) Putative phage related protein
12) Putative phage related export protein
13) Putative phage related protein
14) Hypothetical bacteriophage protein
15) Putative phage related protein
16) Phage head assembly protein
17) Phage portal protein
18) Phage terminase large subunit
19) Phage terminase small subunit
20) Hypothetical phage protein
21) Putative phage related export protein
22) Putative phage lipoprotein
23) Putative phage related murein transglycosylase
24) Put. phage related membrane protein
25) DNA-N6-adenine methyl transferase
26) Hypothetical protein
27) Putative phage related lipoprotein
28) Put. phage related membrane protein
29) Put. phage related DNA binding protein
30) IclR type HTH protein
31) Hypothetical phage related protein
32) Putative phage transposase
33) Put. phage DNA binding protein ExeA

***P. temperata* K122 Contig 58**	***P. asymbiotica***	***P.luminescens* TTO1**
1) Phage baseplate assembly protein		plu3424
2) Phage baseplate protein		plu3425
3) Phage baseplate assembly protein V		plu3426
4) Putative Phage regulatory protein		plu3427
5) Put. Methyl-accepting phage chemotaxis protein		plu3428
6) Putative bacteriophage tail protein		plu3429
7) Putative phage related protein		plu3430
8) Phage tail core protein		plu3431
9) Phage tail sheath protein		plu3432
10) Putative phage related protein		plu3434
11) Putative phage related protein		plu3435
12) Putative phage related export protein		plu3436
13) Putative phage related protein		plu3437
14) Hypothetical bacteriophage protein		plu3438
15) Putative phage related protein		plu3439
16) Phage head assembly protein		plu3440
17) Phage portal protein		plu3441
18) Phage terminase large subunit		plu3442
19) Phage terminase small subunit		plu3443
20) Hypothetical phage protein		plu3444
21) Putative phage related export protein		plu3445
22) Putative phage lipoprotein		plu3446
23) Putative phage related murein transglycosylase		plu3447
24) Put. phage related membrane protein		plu3448
25) DNA-N6-adenine methyl transferase		plu3449
26) Hypothetical protein		plu3450
27) Putative phage related lipoprotein		plu3451
28) Put. phage related membrane protein		
29) Put. phage related DNA binding protein		
30) IclR type HTH protein		plu3455
31) Hypothetical phage related protein		plu3456
32) Putative phage transposase		plu3457
33) Put. phage DNA binding protein ExeA		plu3458

Table 5.4 List of identified genes on *P. temperata* K122 contig 58 and counterparts in *P. asymbiotica* and *P. luminescens* TTO1.

5.3 Mutant Pm6

Mutant Pm6 displays a phenotype that is negative for pigment and antibiotic production, but primary variant-like for all other phenotypes tested. Identified as an insertion in *galK,* encoding galactokinase, and located on K122 contig 57 (Fig. 5.2), homologues are found in *P. asymbiotica* (*PAU_00469*) and *P. luminescens* TTO1 (*plu0576*) (Table 5.5). The TTO1 region surrounding this gene (*plu0567-plu0577*) has been identified as having sugar transport, metabolism and amino acid synthesis activities (Gaudriault *et al*., 2006). Galactokinase is involved in the galactose metabolic pathway (Leloir pathway) whereby galactose is converted to glucose. This pathway utilizes 3 gene products - those of *galT* (galactose-1-phosphate uridylyltransferase), *galK* (galactokinase) and *galE* (UDP-glucose-4-epimerase) to metabolise galactose. In *E. coli*, whilst *galT* and *galK* are colocalised and translationally coupled (Schumperli *et al*., 1982), the *galE* gene is not. This is also the case in *Photorhabdus*, with *galT* and *galK* together on an operon with *galE* elsewhere in the genome (plu4831 in TTO1, coding sequence K122_4979 in K122 and PAU_04343 in *P. asymbiotica*). In *Salmonella, galE* mutants manufacture incomplete (i.e. rough) LPS and are subsequently less virulent, due to a lack of the UDP-glucose intermediate normally produced by GalE activity, which interconverts UDP glucose and UDP galactose. This mutation is complemented in the presence of exogenous galactose as a result of the activities of GalK and GalT which can synthesize UDP-galactose from galactose via galactose-1-phosphate (Nnalue & Stocker, 1986). *galE* and *galU* mutants of *Photorhabdus* have been isolated which are affected in transmission to the infective juvenile (Easom *et al*., 2010). These mutants are also avirulent to insects and sensitive to insect produced antimicrobial peptides (Easom *et al*., 2010), suggesting that LPS is involved in both symbiosis and pathogenicity.

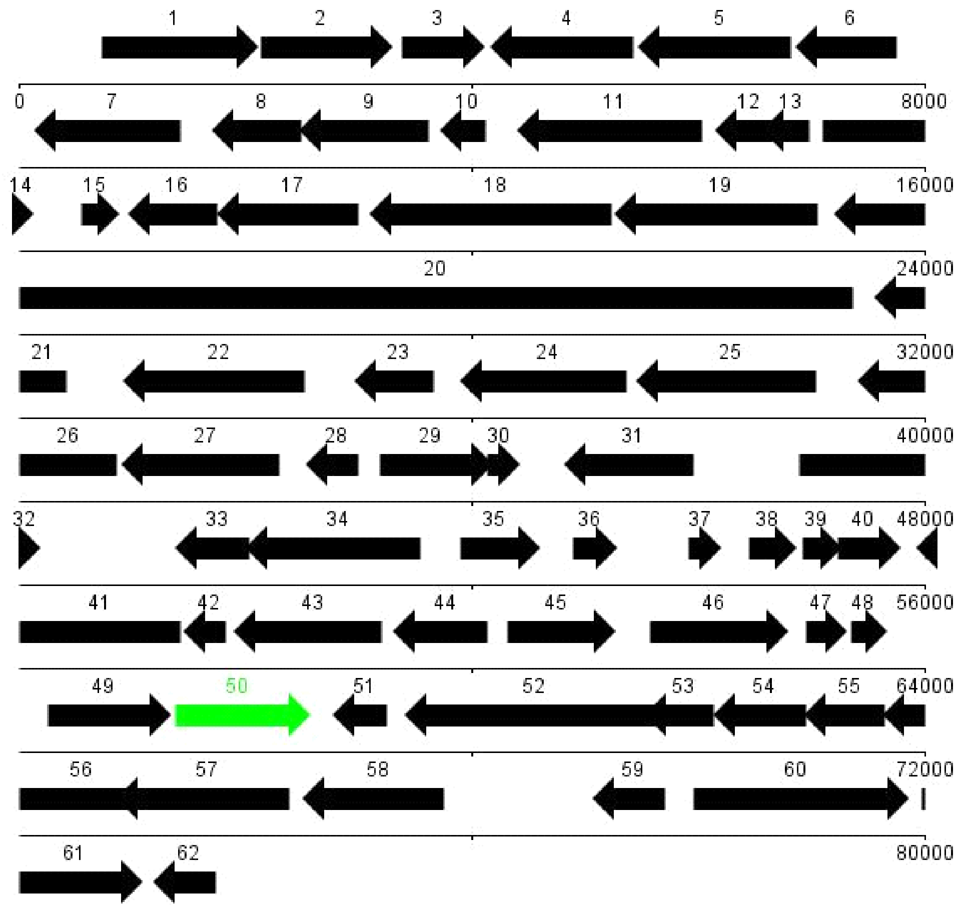


FIG. 5.2 Annotation of Genome Contig 57 of *P. temperata* K122, mutant Pm6 occurs in gene 50, *galK* galactokinase.

1) Cystathionine beta synthase
2) Cystathinone gamma lyase
3) *deoD* Purine nucleoside phosphorylase
4) *deoB* Phosphopentomutase
5) *deoA* Thymidine phosphorylase
6) deoC Deoxyribose phosphate aldolase
7) Putative NupC family transport protein
8) *yjjV* Putative hydrolase
9) *yjjU* Transcriptional regulator
10) *osmY* Osmotically inducible protein
11) *prfC* Peptide chain release factor 3
12) *rimI* Ribosomal-protein-alanine N-acetyltransferase
13) *holD* DNA polymerase III subunit psi
14) *rsmC* 16S ribosomal RNA m2G1207 methyl transferase
15) putative membrane protein
16) Hydantoin racemase
17) Put. major facilitator superfamily drug resistance transporter
18) Hypothetical protein
19) Putative carbamoyl transferase
20) Putative ribosomal peptide synthase
21) Hypothetical protein
22) Putative tryptophan halogenase
23) *attM/aiiB* Zn-dependent hydrolase-like protein
24) *phoA* Alkaline phosphatase
25) *fes* Enterochelin esterase-like protein
26) 4-hydroxyphenylacetate 3-monooxygenase
27) *tnaA* tryptophan deaminase
28) Putative glutathione-S transferase
29) Putative 2,4-diaminobutyrate-4-transferase
30) *orfA* IS200 transposase
31) LacI-family transcriptional regulator
32) Put. major facilitator superfamily (mfs) protein
33) Hypothetical protein
34) Put. amino acid transporter membrane protein
35) *maoR* Putative LuxR family transcriptional regulator
36) Hypothetical protein
37) Hypothetical protein
38) Hypothetical protein
39) Hypothetical protein
40) Putative GnaT family acety transferase protein
41) *feaB* phenylacetaldehyde dehydrogenase
42) Hypothetical protein
43) Putative oxidoreductase *ordL*
44) LuxR-family transcriptional regulator
45) *iunH* inosine-uridine preferring nucleoside hydrolase
46) Hypothetical protein
47) XRE-Family transcriptional regulator
48) Putative DNA-binding protein
49) *galT* Galactose-phosphate uridylyl transferase
50) *galK* Galactokinase
51) Tn*3*-family transposase
52) Hypothetical protein
53) Puromycin N-acetyltransferase
54) Monophosphatase
55) N-methyl-transferase
56) AtaP4 protein
57) Hypothetical protein
58) Put. N-acetyl puromycin N-acetylkinase precursor
59) Putative *yeaH*-like inner membrane protein
60) *dnaK* Molecular chaperone
61) *dnaJ* chaperone protein
62) Hypothetical protein

P. temperata K122 Contig 57	*P. asymbiotica*	*P.luminescens* TTO1
1) Cystathionine beta synthase	pau_00518	plu0524
2) Cystathinone gamma lyase	pau_00517	plu0523
3) *deoD* Purine nucleoside phosphorylase	pau_00516	plu0522
4) *deoB* Phosphopentomutase	pau_00515	plu0521
5) *deoA* Thymidine phosphorylase	pau_00514	
6) deoC Deoxyribose phosphate aldolase	pau_00513	plu0520
7) Putative NupC family transport protein	pau_00512	plu0519
8) *yjjV* Putative hydrolase	pau_00493	plu0517
9) *yjjU* Transcriptional regulator	pau_00492	plu4247
10) *osmY* Osmotically inducible protein	pau_00491	plu4248
11) *prfC* Peptide chain release factor 3	pau_00490	plu4249
12) *rimI* Ribosomal-protein-alanine N-acetyltransferase	pau_00489	plu4250
13) *holD* DNA polymerase III subunit psi	pau_00488	plu4251
14) *rsmC* 16S ribosomal RNA m2G1207 methyl transferase	pau_00487	plu4252
15) putative membrane protein	pau_00485	plu4253
16) Hydantoin racemase		plu4254
17) Put. major facilitator superfamily drug resistance transporter		plu4255
18) Hypothetical protein	pau_00483	
19) Putative carbamoyl transferase	pau_00482	
20) Putative ribosomal peptide synthase		
21) Hypothetical protein		
22) Putative tryptophan halogenase		
23) *attm/aiiB* Zn-dependent hydrolase-like protein	pau_00481	plu2238
24) *phoA* Alkaline phosphatase	pau_00480	plu4256
25) *fes* Enterochelin esterase-like protein	pau_00479	plu4257
26) 4-hydroxyphenylacetate 3-monooxygenase	pau_00478	plu4258
27) *tnaA* tryptophan deaminase		plu0799
28) Putative glutathione-S transferase		plu4259
29) Putative 2,4-diaminobutyrate-4-transferase		plu4263
30) *orfA* IS200 transposase		
31) LacI-family transcriptional regulator		
32) Put. major facilitator superfamily (mfs) protein		plu0728
33) Hypothetical protein	pau_00477	plu4272
34) Putative amino acid transporter membrane protein	pau_00476	plu4273
35) *maoR* Putative LuxR family transcriptional regulator	pau_00475	plu4274
36) Hypothetical protein		
37) Hypothetical protein	pau_00944	plu0350
38) Hypothetical protein	pau_00256	plu1929
39) Hypothetical protein	pau_02177	plu3889
40) Putative GnaT family acety transferase protein	pau_02178	plu3890
41) *feaB* phenylacetaldehyde dehydrogenase	pau_00470	plu4285
42) Hypothetical protein	pau_00469	plu4286
43) Putative oxidoreductase *ordL*	pau_00468	plu4287
44) LuxR-family transcriptional regulator	pau_00467	plu4288
45) *iunH* inosine-uridine preferring nucleoside hydrolase	pau_00466	plu4287
46) Hypothetical protein	pau_00464	plu4291
47) XRE-Family transcriptional regulator	pau_00460	plu4293
48) Putative DNA-binding protein	pau_00459	plu4294
49) *galT* Galactose-phosphate uridylyl transferase	pau_00540	plu0575
50) *galK* Galactokinase	pau_00469	plu0576
51) Tn*3*-family transposase		
52) Hypothetical protein		
53) Puromycin N-acetyltransferase		
54) Monophosphatase		
55) N-methyl-transferase		
56) AtaP4 protein		
57) Hypothetical protein		
58) Put. N-acetyl puromycin N-acetylkinase precursor		
59) Putative *yeaH*-like inner membrane protein	pau_00542	plu0578
60) *dnaK* Molecular chaperone	pau_00543	plu0579
61) *dnaJ* chaperone protein	pau_00545	plu0580
62) Hypothetical protein	pau_00546	plu0581

Table 5.5 List of identified genes on *P. temperata* K122 contig 57 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

5.4 Mutant Pm7

With slightly reduced antibiotic production and lacking in pigment, the otherwise primary-like (Table 5.1) mutant Pm7 displays a mutation in an RtxA toxin-like gene found on K122 contig 194 (Fig. 5.3). A homologue is found in *P. luminescens* TTO1 (*plu2400*, Table 5.6) that is located on the *Photorhabdus* virulence cassette PVCttO1-lopT described by Waterfield *et al* (2004). The *plu2400* gene has been shown to be induced upon insect infection and has putatively been assigned dermonecrotic activity (Münch *et al*., 2008). A further homologue is also found on the *P. asymbiotica* genome (*PAU_02098*, Table 5.5), which again has been described on a virulence cassette – PVCpa43949-lopT. The gene arrangement of K122 at this site differs somewhat to *P. luminescens* TTO1 and *P. asymbiotica* indicating some rearrangements have taken place, and as a result the gene order of the PVC is not conserved. This region of the K122 genome has been described as a possible horizontal acquisition 'hotspot' (Waterfield *et al*., 2004).

5.5 Mutant Pm24

Mutated in a potassium (K^+) uptake gene, *trkA*, unpigmented Pm24 displays reduced antibiotic production but is otherwise phenotypically primary-like (Table 5.1). The *trkA* gene is found on K122 contig 384 (Fig 5.4), and homologues to the *trkA* gene are found in both *P. asymbiotica* and *P. luminescens* TTO1 (*PAU_04151* and *plu4671* respectively, Table 5.7). The Trk K^+ constitutive transport system of *E. coli* is well characterized, and requires high proton motive force and intracellular ATP levels (Rhoads & Epstein 1977). It has also been shown to be regulated by cell turgor pressure (Rhoads & Epstein 1978). Genes involved in Trk activity have been found dispersed through out the *E. coli* chromosome with 5 loci identified (*trkA, D, E, G & H*), encoding 3 separate systems termed TrkD, TrkG and TrkH. (Epstein & Kim, 1971; Dosch *et al*., 1991). In bacteria, K^+ is necessary for the activation of enzymes, cell turgor pressure homeostasis and for salt tolerance (Csonka & Epstein, 1996; Stumpe *et al.,*1996). K^+ is an accumulated element and as such, uptake systems are a

necessity (Nakamura *et al*., 1998). TrkA is a membrane bound protein and has been shown to bind NAD^+ (Bossemeyer *et al.,* 1989; Schlosser *et al.,*1993). The function of two of the Trk uptake systems, TrkG and TrkH require the *trkA* gene product (Dosch *et al*., 1991).

Mutations in *E. coli trkA* abolish Trk activity leading to reduced K^+ uptake (Bossemeyer *et al.,* 1989; Dosch *et al*., 1991; Walderhaug *et al*., 1987). If a similar situation occurs in *Photorhabdus*, the lack of pigment seen in Pm24 may be as a result of a metabolic deficiency caused by limited K^+ reserves. Osmolarity induced stress caused by a decreased cellular K^+ levels may also be a factor, which would be consistent with the observation that osmotically stressful conditions can result in phenotypic variation (Krasomil-Osterfeld 1995).

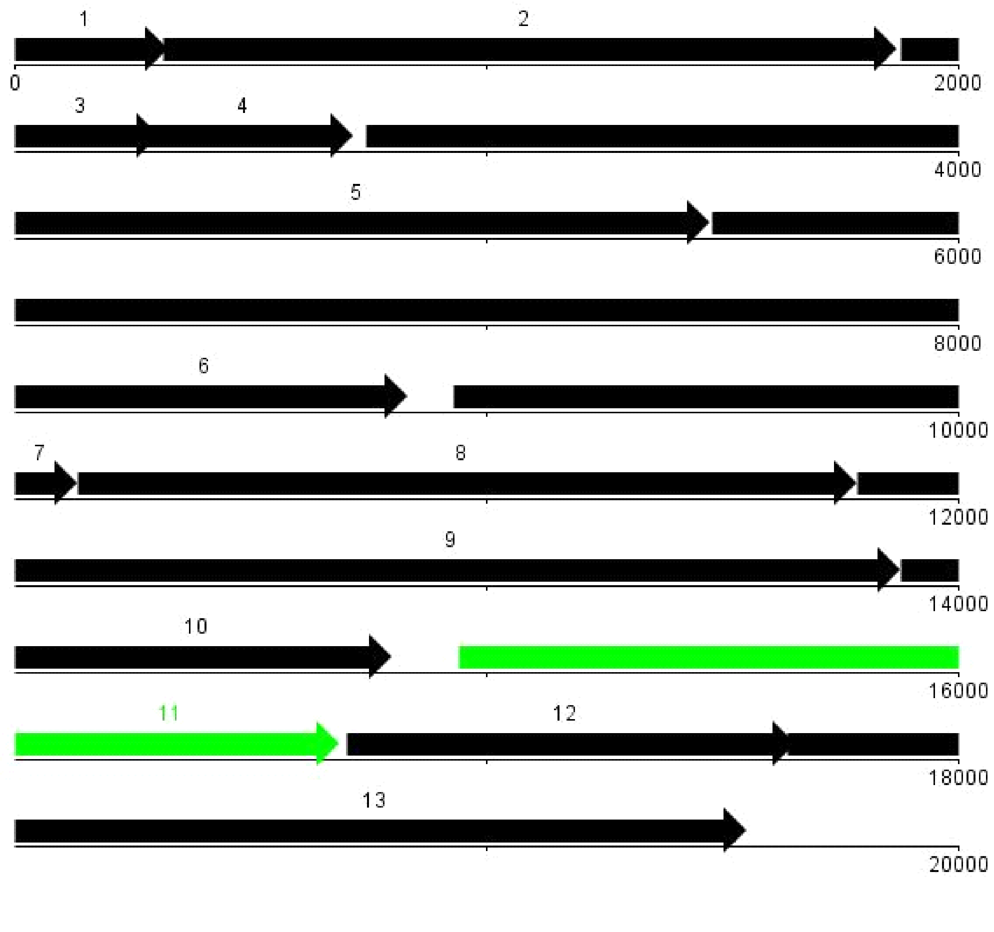


FIG. 5.3 Annotation of Genome Contig 194 of *P. temperata* K122, mutant Pm7 occurs in gene 11, *rtxA* - RtxA toxin.

1) Hypothetical protein
2) VgrG-like hypothetical protein
3) Phage baseplate assembly protein
4) Hypothetical protein
5) Hypothetical protein
6) Hypothetical protein
7) Hypothetical protein
8) Hypothetical protein
9) Hypothetical protein
10) Hypothetical protein
11) *rtxA* RtxA toxin
12) Hypothetical protein
13) RtxA-like Rtx toxin

P. temperata K122 Contig 194	*P. asymbiotica*	*P.luminescens* TTO1
1) Hypothetical protein	pau_02795	plu1661
2) VgrG-like hypothetical protein	pau_02796	plu1660
3) Phage baseplate assembly protein	pau_02797	plu1659
4) Hypothetical protein	pau_02798	plu1658
5) Hypothetical protein	pau_02799	plu1657
6) Hypothetical protein	pau_02800	plu1656
7) Hypothetical protein	pau_02801	plu1655
8) Hypothetical protein	pau_02802	plu1654
9) Hypothetical protein	pau_02803	plu1653
10) Hypothetical protein	pau_02804	plu1652
11) *rtxA* RtxA toxin	pau_02098	plu2400
12) Hypothetical protein	pau_02097	plu1656
13) RtxA-like Rtx toxin	pau_02800	plu3324

Table 5.6 List of identified genes on *P. temperata* K122 contig 194 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

5.6 Mutant Pm25

Another mutant secondary-like in pigment only, the mutation in Pm25 occurs in a hypothetical protein found on K122 contig 168 (Fig 5.5). Corresponding homologues are found on the *P. asymbiotica* (PAU_00387) and *P. luminescens* TTO1 genomes (*plu0480*) (Table 5.8). Interestingly, the TTO1 homolog has been identified previously as a stationary phase extracellular protein with a carbohydrate-binding domain (Turlin *et al*., 2006). This gene has also been identified as being differentially transcribed during exponential phase growth between a variant TTO1 primary phenotype cell (TTO1α) and a delayed pathogenicity TTO1 variant (VAR*). The difference in transcription was not maintained once cells entered stationary phase (Lanois *et al*., 2011).

5.7 Mutant Pm35

Deficient in both pigment and antibiotic production, whilst remaining primary-like for other tested phenotypes, mutant Pm35 occurs in the *rarD* gene. Found on K122 contig 384 (Fig 5.4), homologues occur in both *P. asymbiotica* (PAU_04113) and *P. luminescens* TTO1 (*plu4632*) (Table 5.7). An adjacent region in TTO1 (*plu4621-4630*) has been identified as being a siderophore biosynthesis operon (Gaudriault *et al*., 2006; Wilkinson *et al.,* 2009). Involved in chloramphenicol resistance, *rarD* (*r*ecombination *a*nd *r*epair) in *E.coli* is transcriptionally regulated by the activity of the ferric nitrate reductase global regulator protein (FNR) in response to anaerobiosis (Salmon *et al*., 2003). A role in *rarD* regulation has also been putatively assigned to ArcA (Salmon *et al*., 2005), part of the ArcAB two-component regulatory system that has been characterized as a second global regulator of anaerobic gene response (Gunsalus & Park, 1994; Lynch & Lin 1996; Bauer *et al*., 1999). During anaerobic growth, *rarD* expression levels are increased, with a decrease occurring in an *arcA* deficient strain (Salmon *et al*., 2005). ArcA regulation has been shown to be an important regulator of the modulation of starvation induced gene expression, with a requirement for ArcA to enable reduced respiratory activity in glucose

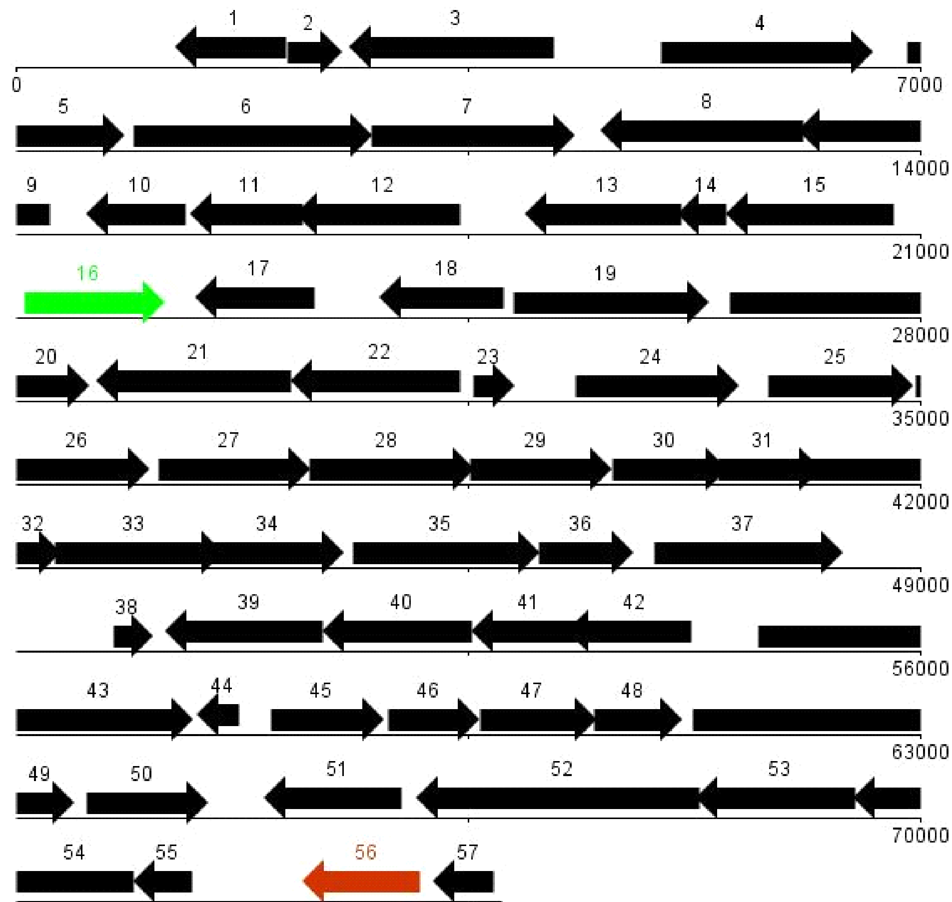


FIG. 5.4 Annotation of Genome Contig 384 of *P. temperata* K122,
Mutant Pm24 occurs in gene 16, *trkA* – potassium uptake protein
Mutant Pm35 occurs in gene 56, *rarD* – transporter protein

1) *hdfR* transcriptional regulator
2) YifE-like protein
3) Putative 2-component regulator YifB
4) *ilvG* acetolactate synthase 2 catalytic subunit
5) *ilvE* branched-chain amino acid transferase
6) *ilvD* dihydroxy-acid dehydratase
7) *ilvA* threonine dehyratase
8) *emrB* Multidrug resistance protein B
9) *emrA* Multidrug resistance protein A
10) *fabG* 3-oxoacyl-(acyl-carrier-protein) reductase
11) Putative acyl dehydratase
12) *fabF* 3-oxoacyl (acyl-carrier-protein) synthase 2
13) Putative cysteine desulferas NifS
14) Putative FeS assembly protein NifU
15) Putative acyl-protein synthetase LuxE
16) *trkA* Potassium uptake protein
17) Putative AraC-family transcriptional regulator
18) *ilvY* DNA-binding LysR-type transcriptional regulator
19) *ilvC* Ketol-acid reductoisomerase
20) *rep* ATP-dependent DNA helicase
21) *gppA* Guanosine pentaphosphate phosphohydrolase
22) *rhlB* ATP-dependent RNA helicase
23) *trxA* Thioredoxin 1 (TRX1)
24) *rho* Transcription termination factor Rho
25) *wecA* undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase
26) *wzzE* Lipopolysaccharide biosynthesis protein
27) *wecB* UDP-N-acetyl-D-acetylglucoasamine 2-epimerase
28) *wecC* UDP-N-acetyl-D-mannosamine dehydrogenase
29) *rffG* dTDP-glucaose 4,6-dehydratase
30) *rffH* Glucoase-1-phosphte thymidyltransferase
31) *rffC* TDP-fucosamine acetyle transferase
32) *wecE* TDP-4-oxo-6-deoxy-D-glucose transaminase
33) *wzxE* Lipopolysaccharide biosynthesis protein
34) *wecF* 4-alpha-L-fucosyl transferase
35) *wzyE* Putative entero common antigen protein
36) *wecG* UDP-N-acetyl-D-mannosaminuronic acid transferase
37) *yifK* Transport protein
38) Hypothetical protein
39) *hemY* Protoheme IX biogenesis protein
40) *hemX* Put. uroporphyrinogen III-c-methyltransferase
41) *hemD* Uroporphyrinogen III synthase
42) *hemC* porophobilinogen deaminase
43) *cyaA* adenylate cyclase
44) *cyaY* Frataxin-like protein
45) *dapF* diaminopimelate epimerase
46) Hypothetical protein
47) *xerC* Site-specific tyrosine recombinase
48) Hypothetical protein (YigB-like)
49) *uvrD* DNA-dependent helicase II
50) *corA* Magnesium/Nickel/Cobalt transporter
51) *dinP* DNA polymerase IV
52) *speF* Ornithine decarboxylase
53) Putative ABC-transporter protein EntS
54) *chuW* Coproporphyrinogen III oxidase
55) *speD* S-adenosylmethionine decarboxylase proenzyme
56) *rarD* transporter protein
57) Putative thioesterase family protein YigI

P. temperata K122 Contig 384	*P. asymbiotica*	*P.luminescens* TTO1
1) *hdfR* transcriptional regulator		plu4688
2) YifE-like protein	pau_04167	plu4687
3) Putative 2-component regulator YifB	pau_04166	plu4686
4) *ilvG* acetolactate synthase 2 catalytic subunit	pau_04165	plu4685
5) *ilvE* branched-chain amino acid transferase	pau_04163	plu4683
6) *ilvD* dihydroxy-acid dehydratase	pau_04162	plu4682
7) *ilvA* threonine dehyratase	pau_04161	plu4681
8) *emrB* Multidrug resistance protein B	pau_04160	plu4680
9) *emrA* Multidrug resistance protein A	pau_04159	plu4679
10) *fabG* 3-oxoacyl-(acyl-carrier-protein) reductase	pau_04158	plu4678
11) Putative acyl dehydratase	pau_04157	plu4677
12) *fabF* 3-oxoacyl (acyl-carrier-protein) synthase 2	pau_04156	plu4676
13) Putative cysteine desulferas NifS	pau_04154	plu4674
14) Putative FeS assembly protein NifU	pau_04153	plu4673
15) Putative acyl-protein synthetase LuxE	pau_04152	plu4672
16) *trkA* Potassium uptake protein	pau_04151	plu4671
17) Putative AraC-family transcriptional regulator	pau_04150	plu4670
18) *ilvY* DNA-binding LysR-type transcriptional regulator	pau_04149	plu4669
19) *ilvC* Ketol-acid reductoisomerase	pau_04148	plu4668
20) *rep* ATP-dependent DNA helicase	pau_04147	plu4667
21) *gppA* Guanosine pentaphosphate phosphohydrolase	pau_04146	plu4666
22) *rhlB* ATP-dependent RNA helicase	pau_04145	plu4665
23) *trxA* Thioredoxin 1 (TRX1)	pau_04144	plu4664
24) *rho* Transcription termination factor Rho	pau_04143	plu4663
25) *wecA* undecaprenyl-phosphate alpha-N-acetylglucosaminyltransferase	pau_04142	plu4662
26) *wzzE* Lipopolysaccharide biosynthesis protein	pau_04141	plu4661
27) *wecB* UDP-N-acetyl-D-acetylglucoasamine 2-epimerase	pau_04140	plu4660
28) *wecC* UDP-N-acetyl-D-mannosamine dehydrogenase	pau_04139	plu4659
29) *rffG* dTDP-glucaose 4,6-dehydratase	pau_04138	plu4658
30) *rffH* Glucoase-1-phosphte thymidyltransferase	pau_04137	plu4657
31) *rffC* TDP-fucosamine acetyle transferase	pau_04136	plu4656
32) *wecE* TDP-4-oxo-6-deoxy-D-glucose transaminase	pau_04135	plu4655
33) *wzxE* Lipopolysaccharide biosynthesis protein	pau_04134	plu4654
34) *wecF* 4-alpha-L-fucosyl transferase	pau_04133	plu4653
35) *wzyE* Putative entero common antigen protein	pau_04132	plu4652
36) *wecG* UDP-N-acetyl-D-mannosaminuronic acid transferase	pau_04131	plu4651
37) *yifK* Transport protein	pau_04130	plu4650
38) Hypothetical protein	pau_04129	plu4648
39) *hemY* Protoheme IX biogenesis protein	pau_04128	plu4647
40) *hemX* Put. uroporphyrinogen III-c-methyltransferase	pau_04127	plu4646
41) *hemD* Uroporphyrinogen III synthase	pau_04126	plu4645
42) *hemC* porophobilinogen deaminase	pau_04125	plu4644
43) *cyaA* adenylate cyclase	pau_04124	plu4643
44) *cyaY* Frataxin-like protein	pau_04123	plu4642
45) *dapF* diaminopimelate epimerase	pau_04121	plu4640
46) Hypothetical protein	pau_04120	plu4639
47) *xerC* Site-specific tyrosine recombinase	pau_04119	plu4638
48) Hypothetical protein (YigB-like)	pau_04118	plu4637
49) *uvrD* DNA-dependent helicase II	pau_04117	plu4636
50) *corA* Magnesium/Nickel/Cobalt transporter	pau_04116	plu4635
51) *dinP* DNA polymerase IV	pau_03222	plu1239
52) *speF* Ornithine decarboxylase		
53) Putative ABC-transporter protein EntS		
54) *chuW* Coproporphyrinogen III oxidase		
55) *speD* S-adenosylmethionine decarboxylase proenzyme		
56) *rarD* transporter protein	pau_04113	plu4632
57) Putative thioesterase family protein YigI	pau_04112	plu4631

Table 5.7 List of identified genes on *P. temperata* K122 contig 384 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

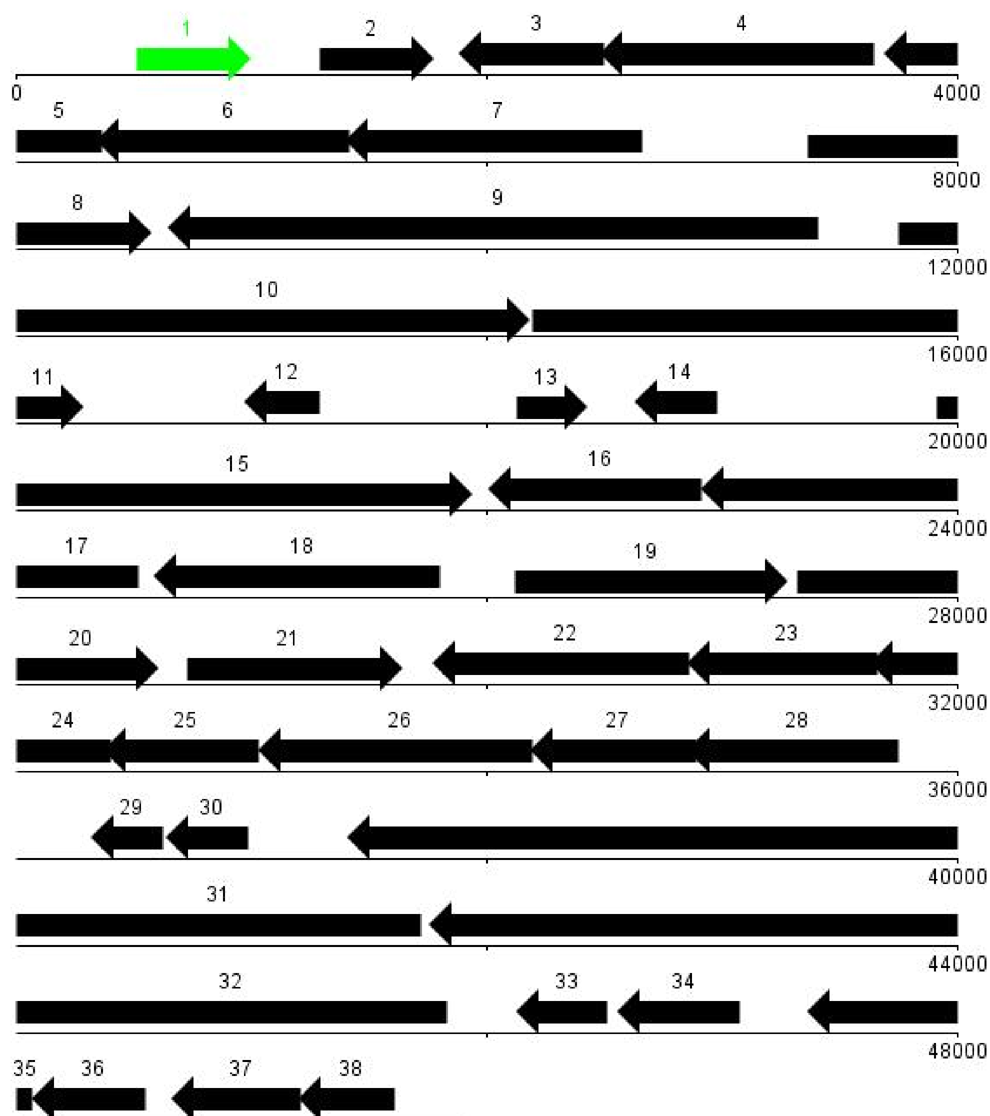


FIG. 5.5 Annotation of Genome Contig 168 of *P. temperata* K122, Mutant Pm25 occurs in gene 1, encoding a hypothetical protein.

1) Hypothetical protein
2) Hypothetical protein
3) Hypothetical protein
4) Putative major facilitator superfamily transporter protein
5) *gph* phosphoglycolate phosphatase-like protein
6) Putative L-iditol 2-dehydrogenase
7) 4-aminobutyrate aminotransferase activity protein
8) Putative Bcr/CflA family drug resitance transporter
9) *malT* transcriptionl regulator
10) *malP* maltodextrin phosphoryase
11) *malQ* 4-alpha-gluconotransferase
12) *yacB* plasmid stabilization protein
13) Putative plasmid stabilization system protein
14) Putative antitoxin YefM
15) Putative phosphoglycerol transferase
16) *malG* maltose transporter permease
17) *malF* maltose transporter membrane protein
18) *malE* maltose ABC transporter periplasmic protein
19) *malK* maltose/maltodextrin transporter ATP-binding protein
20) *lamB* maltoporin
21) *malM* maltose regulon periplasmic protein
22) *rfaB* UDP-D-galactose
23) Hypothetical protein
24) Hypothetical protein
25) Hypothetical protein
26) *csdB2* Putative NifS family aminotransferase
27) Hypothetical protein
28) Hypothetical protein
29) Phage transcriptional regulator
30) Hypothetical protein
31) *rpoC* DNA directed RNA polymerase subunit beta’
32) *rpoB* DNA directed RNA polymerase subunit beta
33) *rplL* 50S ribosomal protein L7/L12
34) *rplJ* 50S ribosomal protein L10
35) *rplA* 50S ribosomal protein L1
36) *rplK* 50S ribosomal protein L11
37) *nusG* Transcription antitermination protein
38) *secE* Preprotein translocase subunit

P. temperata **K122 Contig 168**	***P. asymbiotica***	***P.luminescens*** **TTO1**
1) Hypothetical protein	pau_00387	plu0480
2) Hypothetical protein	pau_00386	plu0479
3) Hypothetical protein	pau_00385	plu0477
4) Putative major facilitator superfamily transporter protein	pau_00384	plu0476
5) *gph* phosphoglycolate phosphatase-like protein	pau_00383	plu0475
6) Putative L-iditol 2-dehydrogenase	pau_00382	plu0474
7) 4-aminobutyrate aminotransferase activity protein	pau_00381	plu0473
8) Putative Bcr/CflA family drug resitance transporter	pau_00380	plu0472
9) *malT* transcriptionl regulator	pau_00379	plu0471
10) *malP* maltodextrin phosphoryase	pau_00377	plu0470
11) *malQ* 4-alpha-gluconotransferase	pau_00376	plu0469
12) *yacB* plasmid stabilization protein	pau_04268	
13) Putative plasmid stabilization system protein	pau_03735	plu4785
14) Putative antitoxin YefM		plu2280
15) Putative phosphoglycerol transferase	pau_00370	plu0461
16) *malG* maltose transporter permease	pau_00369	plu0460
17) *malF* maltose transporter membrane protein	pau_00368	plu0459
18) *malE* maltose ABC transporter periplasmic protein	pau_00367	plu0458
19) *malK* maltose/maltodextrin transporter ATP-binding protein	pau_00366	plu0457
20) *lamB* maltoporin	pau_00365	plu0456
21) *malM* maltose regulon periplasmic protein	pau_00364	plu0455
22) *rfaB* UDP-D-galactose	pau_00363	plu0452
23) Hypothetical protein	pau_00362	plu0451
24) Hypothetical protein	pau_00361	plu0450
25) Hypothetical protein	pau_00360	plu0449
26) *csdB2* Putative NifS family aminotransferase	pau_00359	plu0448
27) Hypothetical protein	pau_00358	plu0447
28) Hypothetical protein	pau_00357	plu0446
29) Phage transcriptional regulator	pau_00353	plu0444
30) Hypothetical protein		
31) *rpoC* DNA directed RNA polymerase subunit beta'	pau_00351	plu0440
32) *rpoB* DNA directed RNA polymerase subunit beta	pau_00350	plu0439
33) *rplL* 50S ribosomal protein L7/L12	pau_00349	plu0438
34) *rplJ* 50S ribosomal protein L10	pau_00348	plu0437
35) *rplA* 50S ribosomal protein L1	pau_00347	plu0436
36) *rplK* 50S ribosomal protein L11	pau_00346	plu0435
37) *nusG* Transcription antitermination protein	pau_00345	plu0434
38) *secE* Preprotein translocase subunit	pau_00344	plu0433

Table 5.8 List of identified genes on *P. temperata* K122 contig 168 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

starved cells by decreasing the synthesis of NADH/FADH$_2$-generating enzymes of the TCA cycle (Nyström *et al*., 1996). ArcA is present on K122 contig 22 (Fig. 4.10, gene 7) with homologues arising in TTO1 (*plu0562*) and *P. asymbiotica* (PAU_0566).

5.8 Mutant Pm39

This mutant, again secondary-like for the pigment phenotype only, was found to be in a hypothetical protein located on K122 contig 776 (Fig. 5.6) With homologues in *P. asymbiotica* (*PAU_03019*) and *P. luminescens* TTO1 (*plu2670*) (Table 5.9). This gene has been identified previously via rapid virulence analysis as being located on a genomic region (RVA6) having a role in the virulence/toxicity of TTO1 (Waterfield *et al.,* 2008). The RVA6 region contains a fimbrial operon and a Pdl-GI-1 island (Pdl-GI-1 = genomic island containing a putative class III lipase *pdl* and the tightly linked *orf*54 gene (Waterfield *et al*., 2004 & 2008)).

5.9 Mutant Pm59

Another mutant secondary-like with respect to pigmentation only, Pm59 is mutated in the DNA polymerase II *dinA/polB* gene found on K122 contig 145 (Fig 5.7). With homologous genes arising in both *P. asymbiotica* (*PAU_00574*) and *P. luminescens* TTO1 (*plu0616*) (Table 5.10), a role has been described for DNA polymerase II in the restart of DNA replication after UV irradiation (Rangarajan *et al*., 1999) and it is regulated by the *lexA* gene which controls the SOS response in *E. coli* (Little & Mount, 1982). The SOS response is induced after bacterial cells experience DNA damage, and consists of the co-ordinated induction of approximately 30 genes by the inactivation of the common repressor LexA (Foster, 2005). This LexA inactivation occurs as a result of a self cleavage induced by a nucleoprotein complex – the RecA filament (Michel, 2005). Other factors which can reduce LexA levels can also result in SOS response induction, for example, alkaline pH levels, aging colonies and cell saturation in rich

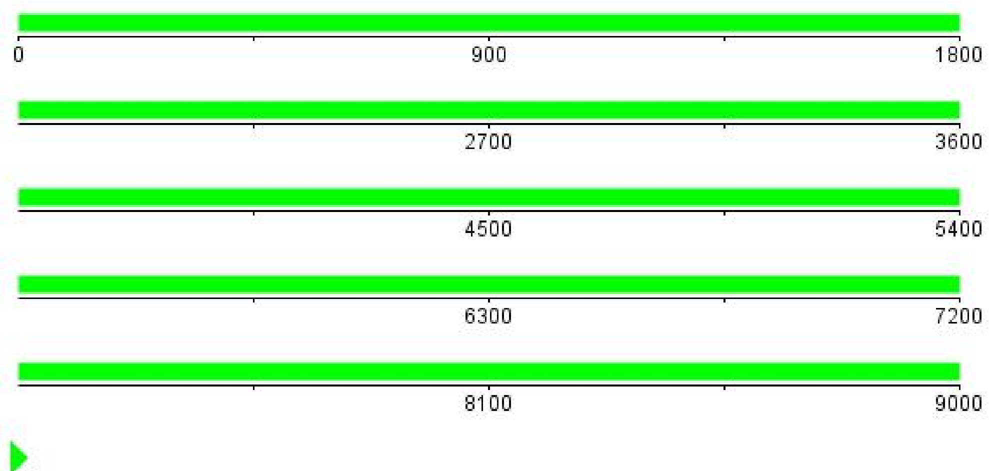


FIG. 5.6 Annotation of Genome Contig 776 of *P. temperata* K122, Consisting of one gene encoding a hypothetical protein, in which Mutant Pm39 occurs.

P. temperata K122 Contig 776	*P. asymbiotica*	*P.luminescens* TTO1
1) Hypothetical protein	pau_03019	plu2670

Table 5.9 List of identified genes on *P. temperata* K122 contig 776 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

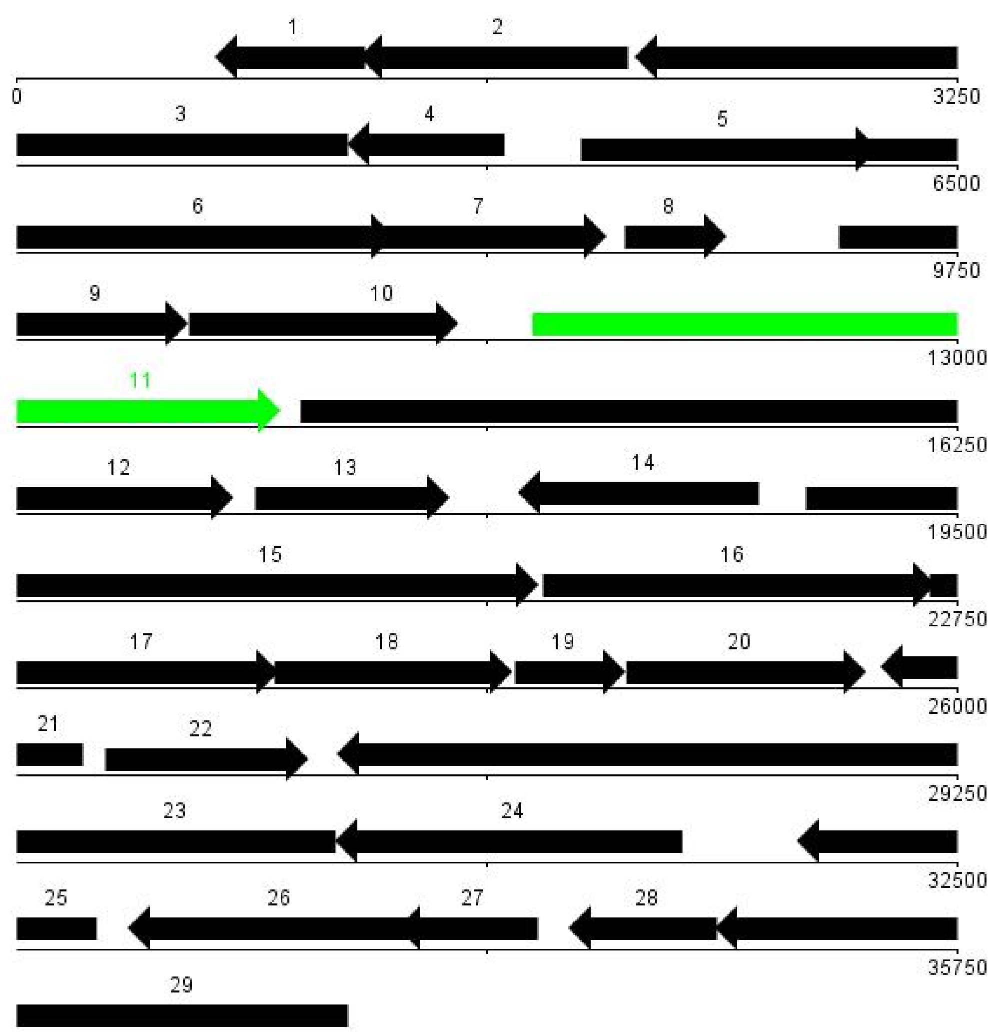


FIG. 5.7 Annotation of Genome Contig 145 of *P. temperata* K122, Mutant Pm59 occurs in gene 11, encoding DNA polymerase II.

1) *thyA* Thymidate synthase
2) *lgt* Prolipoprotein diacylglycerol transferase
3) *ptsP* Fused phosphoenolpyruvate protein phosphotransferase
4) *nudH/rppH* dinucleoside polyphosphate hydrolase
5) *tbpA* Thiamine transporter substrate binding subunit
6) *thiP* Thiamine transporter membrane protein
7) *thiQ* Thiamine transporter ATP-binding subunit
8) Hypothetical protein
9) Hypothetical protein
10) Hypothetical protein
11) *dinA/polB* DNA polymerase II
12) *hepA/rapA* ATP dependent helicase
13) *rluA* Ribosomal large subunit pseudouridine synthase
14) *djlA* DNA-J-like membrane chaperone
15) *imp* Organic solvent tolerance protein
16) *surA* Peptidyl-prolyl cis-trans isomerase
17) *pdxA* 4-hydroxythreonine-4-phosphate dehydrogenase
18) *ksgA* dimethyladenosine transferase
19) *apaG* $Co2^{+}$/$Mg2^{+}$ efflux protein
20) *apaH* Diadenosine tetraphosphate
21) *folA* Dihydrofolate reductase
22) Putative LysE-type translocator
23) *carB* carbamoyl phosphate synthase large subunit
24) *carA* carbamoyl phosphate synthase small subunit
25) *dapB* Dihydrodipicolinate reductase
26) *ispH* 4-hydroxy-3-metylbut-2-enyl diphosphate reductase
27) *fkpB* peptidoprolyl isomerase
28) *lspA* lipoprotein signal peptidase
29) *ileS/lspA* Isoleucyl-tRNA synthetase

P. temperata **K122 Contig 145**	***P. asymbiotica***	***P.luminescens*** **TTO1**
1) *thyA* Thymidate synthase	pau_00582	plu0623
2) *lgt* Prolipoprotein diacylglycerol transferase	pau_00581	plu0622
3) *ptsP* Fused phosphoenolpyruvate protein phosphotransferase	pau_00580	plu0621
4) *nudH/rppH* dinucleoside polyphosphate hydrolase	pau_00579	plu0620
5) *tbpA* Thiamine transporter substrate binding subunit	pau_00578	plu0619
6) *thiP* Thiamine transporter membrane protein	pau_00577c	plu0618
7) *thiQ* Thiamine transporter ATP-binding subunit	pau_00576	plu0617
8) Hypothetical protein		
9) Hypothetical protein		plu3801
10) Hypothetical protein	pau_00575	plu3802
11) *dinA/polB* DNA polymerase II	pau_00574	plu0616
12) *hepA/rapA* ATP dependent helicase	pau_00573	plu0615
13) *rluA* Ribosomal large subunit pseudouridine synthase	pau_00572	plu0614
14) *djlA* DNA-J-like membrane chaperone	pau_00571	plu0613
15) *imp* Organic solvent tolerance protein	pau_00570	plu0612
16) *surA* Peptidyl-prolyl cis-trans isomerase	pau_00569	plu0611
17) *pdxA* 4-hydroxythreonine-4-phosphate dehydrogenase	pau_00568	plu0610
18) *ksgA* dimethyladenosine transferase	pau_00567	plu0609
19) *apaG* $Co2^{+}/Mg2^{+}$ efflux protein	pau_00566	plu0608
20) *apaH* Diadenosine tetraphosphate	pau_00565	plu0607
21) *folA* Dihydrofolate reductase	pau_00564	plu0606
22) Putative LysE-type translocator	pau_00563	plu0605
23) *carB* carbamoyl phosphate synthase large subunit	pau_00562	plu0604
24) *carA* carbamoyl phosphate synthase small subunit	pau_00561	plu0603
25) *dapB* Dihydrodipicolinate reductase	pau_00560	plu0603
26) *ispH* 4-hydroxy-3-metylbut-2-enyl diphosphate reductase	pau_00559	plu0594
27) *fkpB* peptidoprolyl isomerase	pau_00558	plu0593
28) *lspA* lipoprotein signal peptidase	pau_00556	plu0592
29) *ileS/lspA* Isoleucyl-tRNA synthetase	pau_00555	plu0591

Table 5.10 List of identified genes on *P. temperata* K122 contig 145 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

medium can all result in increased LexA inactivation. (Little 1991; Dri & Moreau, 1994; Taddei *et al*., 1995). *polB* is also transcribed during stationary phase in the absence of exogenous DNA damage (Yeiser *et al*., 2002), and this, along with the relatively high basal expression levels of *polB,* combined with its early induction during the SOS response enable a rapid restart to replication after DNA damage (Rangarajan *et al*., 1999). *E. coli* Δ*polB* mutants are delayed in this resumption of replication, but despite the lack of DNA polymerase II, replication does eventually restart, indicating the presence of genetically separate pathways to avoid the effects of DNA damage (Rangarajan *et al*., 1999).
The SOS response and DNA polymerase mediated DNA repair have been implicated in the generation of antibiotic resistant mutants after SOS induction in response to antibiotic induced DNA damage (Cirz *et al*., 2005; Levin, 2004; Miller *et al*., 2004). In addition to this, the SOS response regulated DNA polymerases, including *polB* have been shown to confer a competitive advantage on stationary phase cells, thereby enhancing long-term survival and evolutionary fitness (Yeiser *et al*., 2002).

5.10 Mutant Pm77

Mutant Pm77 is secondary variant like for both pigmentation and antibiotic production with other characters analysed remaining primary-like. Sequencing revealed the site of transposon insertion to be in a gene with homology to the *sdiA* transcriptional activator. Found on K122 contig 487 (Fig. 5.8), homologues to this gene are found on both the *P. asymbiotica* and *P. luminescens* TTO1 genomes (*PAU_04062* & *plu4562* respectively, Table 5.11). The *sdiA* gene encodes a homologue of the quorum sensing regulator LuxR. Quorum sensing is generally defined as the detection of population density (Fuqua *et al*., 1994; Hastings & Greenberg, 1999) and the predominant system of quorum sensing in host-associated gram-negative bacteria appears to be based on the N-acylhomoserine lactone (AHL) signal/corresponding LuxR homologous sensor pathway (Swift *et al*., 1999). Interestingly however, despite the presence of this LuxR homologue in the *Photorhabdus* genome, no proteins with homology to those involved in the formation of AHLs are found, suggesting that

Photorhabdus does not synthesis any AHL derivatives (Derzelle *et al*., 2002). The genera *Escherichia*, *Salmonella,* and *Klebsiella* are similarly devoid of AHL synthases whilst also containing the *sdiA* homologue (Dyszel *et al*., 2010; Smith & Ahmer 2003). In the absence of AHL, other factors must cause the *sdiA* activation of genes, and in *Salmonella*, the lowering of temperature to 30°C has been shown to cause such AHL-independent *sdiA* activation (Smith & Ahmer 2003). Substantial levels of AHL-independent *sdiA* activity have also been observed in *E.coli* at both 37°C and 30°C, with higher levels of activity occurring at the lower temperature (Dyszel *et al*., 2010). The temperatures at which *Photorhabdus* thrive may negate the need for the presence of AHL.

The *P. luminescens* TTO1 homologue *plu4562*, has been shown to positively regulate an operon (*plu4563-4568*) with an apparent role in cell aggregation and this operon is negatively regulated by the previously mentioned HexA transcriptional regulator in a manner likely to be independent of *plu4562* (David Clarke, personal communication). The partial loss of primary phenotype caused by this mutation, allied to the interaction of *plu4562* with genes co-regulated by HexA are strongly suggestive of a role in the phenotypic switch for *sdiA*. Plasmid based *sdiA* expression studies carried out in *E. coli* have indicated that different phenotypes occur when *sdiA* is expressed, including i) suppression of cellular division – a result of the upregulation of the *ftsQAZ* cell division locus (Wang *et al*., 1991) and ii) increased resistance of the bacterial cell suggested to be caused by the *sdiA* mediated upregulation of the *acrAB* multidrug efflux pump locus (Wei *et al*., 2001; Rahmati *et al*., 2002). Counter to this, an *sdiA* null mutant strain was found to have increased sensitivity to fluoroquinolones (Rahmati *et al*., 2002). *E. coli* microarray studies have identified 137 genes whose regulation changed in response to plasmid based *sdiA* expression (Wei *et al*., 2001). In total, 75 genes were upregulated - including genes involved in cell division, amino acid, carbohydrate, carbon and cation transport, DNA degradation, replication repair, restriction and modification, metabolism, drug sensitivity, structural elements and unknowns, and 62 genes were down-regulated – including genes involved in toxin production, mobility, amino acid, carbohydrate and cation transport, metabolism, surface structures and unknowns.

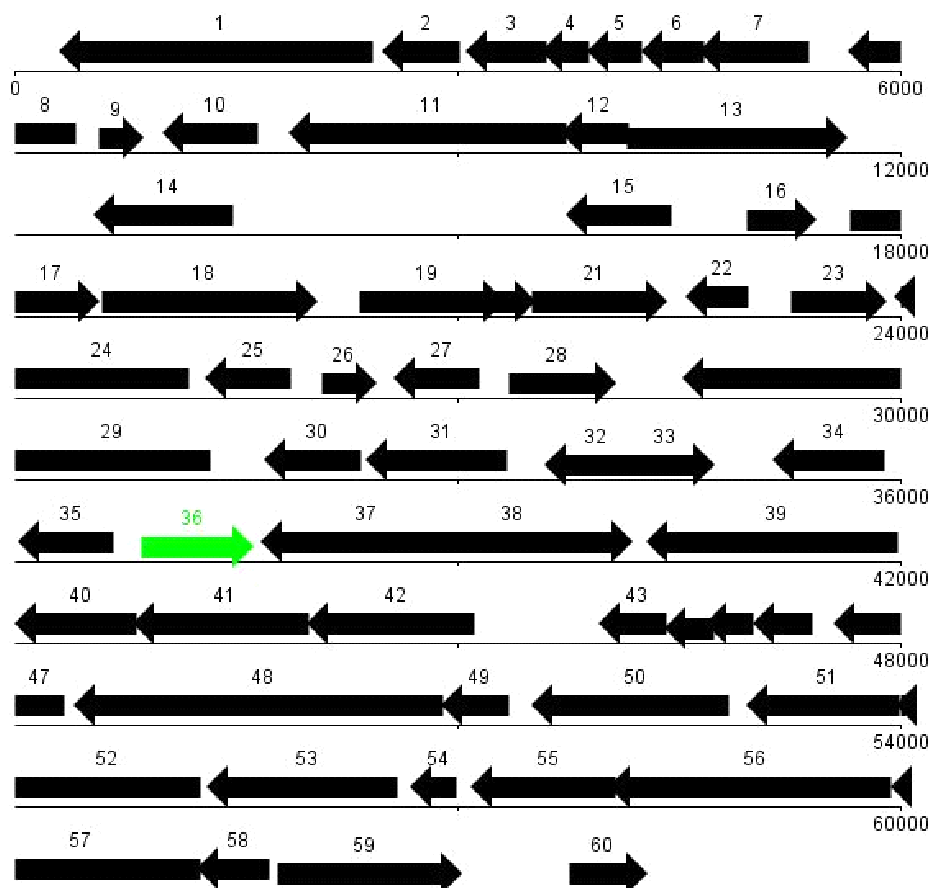


FIG. 5.8 Annotation of Genome Contig 487 of *P. temperata* K122, mutant Pm77 occurs in gene 36, putative transcriptional regulator of *sdiA*.

1) *fusA* Elongation factor G
2) *rpsG* 30S ribosomal protein S7
3) *rpsL* 30S ribosomal protein S12
4) *tusB* tRNA 2-thiouridine synthesizing protein B
5) *tusC* Sulfur relay protein
6) *tusD* Sulfur transfer complex subunit
7) Putative regulator protein YheO domain
8) *fkpA* FKBP-type peptidyl-prolyl isomerase
9) *slyX* putuative SlyX family protein
10) *slyD* FKBP-type peptidyl-prolyl cis-trans isomerase
11) *kefB* Glutathione-regulated potassium-efflux system protein
12) *kefG* Glutathione-regulated potassium efflux system ancillary protein
13) *yheS* ABC transporter ATP-binding protein
14) Putative LysR family transcriptional regulator
15) Hypothetical protein
16) Hypothetical protein
17) *murQ* N-acetylmuramc acid-6-phosphate esterase
18) *murP* PTS system N-acetylmuramic acid transporter subunit EIIBC
19) *yheT* putative hydrolase
20) Hypothetical protein
21) *prkB* Phosphoribulokinase
22) *yhfA* Putative OsmC family protein
23) *crp* cAMP regulatory protein
24) *argD* Bifunctional N-succinyldiaminopimelate aminotransferase
25) *pabA* Para-aminobenzoate synthase component II
26) *ycgJ* Hypothetical protein
27) *ppiA* peptidyl-prolyl cis-trans isomerase (Rotamase A)
28) *engB* Ribosome biogenesis GTP-binding protein
29) *polA* DNA polymerase I
30) *dsbA* Periplasmic protein disulfide isomerase I
31) *rdoA* Serine/threonine protein kinase
32) *mobA* Molybdopterin-guanine dinucleotide biosynthesis protein A
33) *mobB* Molybdopterin-guanine dinucleotide biosynthesis protein B
34) *cysQ* Adenosine-3'(2'),5'-bisphosphate nucleotidase
35) *fklB* Peptidyl-prolyl cis-trans isomerase
36) Putative *sdiA* transcriptional regulator
37) Hypothetical protein
38) Hypothetical protein
39) Putative carbamoyl transferase
40) Hypothetical protein
41) *argG* Argininosuccinae synthase
42) *cysK* Cysteine synthase A
43) *rplI* 50S ribosomal protein L9
44) *rpsR* 30S ribosomal protein S18
45) *priB* primosomal replication protein N
46) *rpsF* 30S ribosomal protein S6
47) *rlmB* 23S rRNA (guanosine 2'-0-)-methyltransferase
48) *rnr* exoribonuclease R (RNase R)
49) *nsrR/yjcB* Transcriptional repressor
50) *purA* Adenylosuccinate synthetase
51) *hflC* FtsH protease regulator
52) *hflK* FtsH protease regulator
53) *hflX* Putative GTPase
54) *hfq* RNA binding protein
55) *miaA* tRNA delta(2) isopentyl pyrophosphate transfera
56) *mutL* DNA mismatch repair protein
57) *amiB* N-acetylmucamoyl-L-alanine amidase
58) *yjeE* Hypothetical protein
59) *yjeS* Hypothetical protein
60) *flhE* Hypothetical protein

P. temperata K122 Contig 487	*P. asymbiotica*	*P.luminescens* TTO1
1) *fusA* Elongation factor G	pau_00341	plu0431
2) *rpsG* 30S ribosomal protein S7	pau_00340	plu0430
3) *rpsL* 30S ribosomal protein S12	pau_00339	plu0429
4) *tusB* tRNA 2-thiouridine synthesizing protein B	pau_00338	plu0428
5) *tusC* Sulfur relay protein	pau_00337	plu0427
6) *tusD* Sulfur transfer complex subunit	pau_00336	plu0426
7) Putative regulator protein YheO domain	pau_00335	plu0425
8) *fkpA* FKBP-type peptidyl-prolyl isomerase	pau_00333	plu0424
9) *slyX* putuative SlyX family protein		plu0423
10) *slyD* FKBP-type peptidyl-prolyl cis-trans isomerase	pau_00330	plu0422
11) *kefB* Glutathione-regulated potassium-efflux system protein	pau_00328	
12) *kefG* Glutathione-regulated potassium efflux system ancillary protein	pau_00327	
13) *yheS* ABC transporter ATP-binding protein	pau_00326	plu0420
14) Putative LysR family transcriptional regulator	pau_00325	plu0410
15) Hypothetical protein		
16) Hypothetical protein	pau_00318	plu0404
17) *murQ* N-acetylmuramc acid-6-phosphate esterase	pau_00315	plu0403
18) *murP* PTS system N-acetylmuramic acid transporter subunit EIIBC	pau_00314	plu0402
19) *yheT* putative hydrolase	pau_00310	plu0399
20) Hypothetical protein	pau_00309	plu0398
21) *prkB* Phosphoribulokinase	pau_00308	plu0397
22) *yhfA* Putative OsmC family protein	pau_00307	plu0396
23) *crp* cAMP regulatory protein	pau_00306	plu0395
24) *argD* Bifunctional N-succinyldiaminopimelate aminotransferase	pau_00305	plu0394
25) *pabA* Para-aminobenzoate synthase component II	pau_00304	plu0393
26) *ycgJ* Hypothetical protein	pau_00303	plu0392
27) *ppiA* peptidyl-prolyl cis-trans isomerase (Rotamase A)	pau_00302	plu0391
28) *engB* Ribosome biogenesis GTP-binding protein	pau_00301	plu0390
29) *polA* DNA polymerase I	pau_00299	plu0386
30) *dsbA* Periplasmic protein disulfide isomerase I	pau_00298	plu0381
31) *rdoA* Serine/threonine protein kinase	pau_00297	plu0380
32) *mobA* Molybdopterin-guanine dinucleotide biosynthesis protein A	pau_00296	plu0379
33) *mobB* Molybdopterin-guanine dinucleotide biosynthesis protein B	pau_00295	plu0378
34) *cysQ* Adenosine-3'(2'),5'-bisphosphate nucleotidase	pau_04060	plu4559
35) *fklB* Peptidyl-prolyl cis-trans isomerase	pau_04061	plu4561
36) Putative *sdiA* transcriptional regulator	pau_04062	plu4562
37) Hypothetical protein	pau_04063	plu4563
38) Hypothetical protein	pau_04064	plu4564
39) Putative carbamoyl transferase	pau_04065	plu4565
40) Hypothetical protein	pau_04066	plu4566
41) *argG* Argininosuccinae synthase	pau_04067	plu4567
42) *cysK* Cysteine synthase A	pau_04068	plu4568
43) *rplI* 50S ribosomal protein L9	pau_04069	plu4570
44) *rpsR* 30S ribosomal protein S18	pau_04070	plu4571
45) *priB* primosomal replication protein N	pau_04071	plu4572
46) *rpsF* 30S ribosomal protein S6	pau_04072	plu4573
47) *rlmB* 23S rRNA (guanosine 2'-0-)-methyltransferase	pau_04073	plu4574
48) *rnr* exoribonuclease R (RNase R)	pau_04074	plu4575
49) *nsrR/yjcB* Transcriptional repressor	pau_04075	plu4576
50) *purA* Adenylosuccinate synthetase	pau_04076	plu4577
51) *hflC* FtsH protease regulator	pau_04077	plu4578
52) *hflK* FtsH protease regulator	pau_04078	plu4579
53) *hflX* Putative GTPase	pau_04079	plu4580
54) *hfq* RNA binding protein	pau_04080	plu4581
55) *miaA* tRNA delta(2) isopentyl pyrophosphate transferase	pau_04081	plu4582
56) *mutL* DNA mismatch repair protein	pau_04082	plu4583
57) *amiB* N-acetylmucamoyl-L-alanine amidase	pau_04083	plu4584
58) *yjeE* Hypothetical protein	pau_04084	plu4585
59) *yjeS* Hypothetical protein	pau_04085	plu4586
60) *flhE* Hypothetical protein	pau_01886	plu4588

Table 5.11 List of identified genes on *P. temperata* K122 contig 487 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

However, more recent studies have shown that the action and targets of chromosomal *sdiA* in the cell is different to that of plasmid expressed *sdiA* (Dyszel *et al*., 2010). It has been suggested that the genes undergoing SdiA regulation may change depending on environmental or metabolic conditions, but that until such conditions, allowing chromosomal *sdiA* activation, are identified then the gene should not be considered part of the *sdiA* regulon. (Dyszel *et al*., 2010).

Deletion of chromosomal *sdiA* has been shown to result in an increased adherence phenotype in enterohaemorrhagic *E. coli* O157:H7 and also resulted in increased motility (Sharma *et al*., 2010). Such mutants have also shown a role for *sdiA* in the repression of the *fliE* and *fliC* flagellar genes (Dyszel *et al*., 2010; Sharma *et al*., 2010).

5.11 Mutant Pm81

Pm81 is not only affected in pigment production, but antibiotic production is absent and lipase activity is strongly reduced. With other analysed phenotypes remaining primary-like, this mutation maps to the *aroQ* gene, coding for 3-dehydroquinate dehydratase. Located on K122 contig 134 (Fig 5.9) homologues arise in *P. asymbiotica* (*PAU_03705*) and in *P. luminescens* TTO1 (*plu4073*) (Table 5.12), with the TTO1 homologue occurring next to a protein (*plu4074*, *accB* - Biotin carboxyl carrier protein of acetyl-CoA carboxylase) identified by proteome analysis as having differential expression between wild type and phenotypic variant strains (Turlin *et al*., 2006).

AroQ (3-dehydroquinate dehydratase) is an enzyme involved in the Shikimate pathway, which links the metabolism of carbohydrates to the biosynthesis of aromatic compounds by the conversion of phosphoenolpyruvate and erythrose-4-phosphate to chorismate – the precursor of the aromatic amino acids and many aromatic secondary metabolites (Fig. 5.10) (Euverink *et al*., 1992; Hermann & Weaver, 1999). Mutations in the aromatic amino acidbiosynthetic pathway have been observed to result in attenuated virulence in several bacterial pathogens,

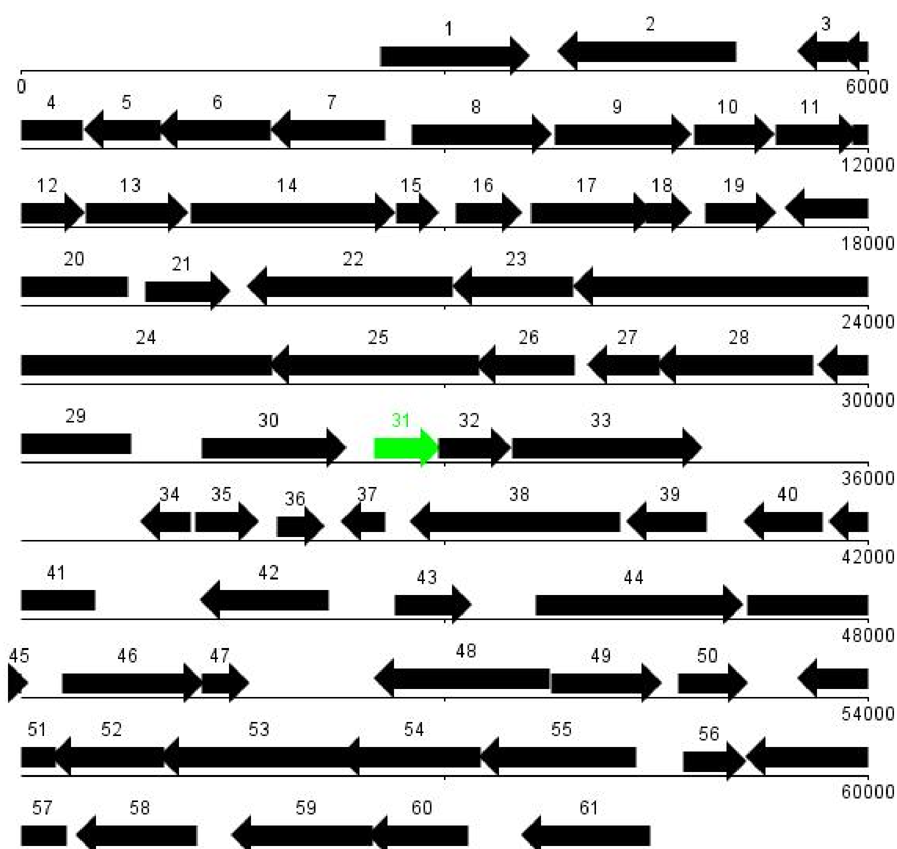


FIG. 5.9 Annotation of Genome Contig 134 of *P. temperata* K122, mutant Pm81 occurs in gene 31, 3-dehydroquinate dehydratase *aroQ.*

1) *degS* Serine endoprotease
2) *murA* UDP-N-acetyl glucoasamine 1-adenyltrasnferase
3) Hypothetical protein
4) Hypothetical protein
5) Hypothetical protein
6) Hypothetical protein
7) *yrbF* Putative ABC transporter ATP-binding protein
8) *yrbG* Putative calcium/sodium transporter
9) *kdsG* D-arabinose 5-phosphate isomerase
10) *kdsC* 3-deoxy-D-mannose-octulosonate 8-phosphate phosphatase
11) Hypothetical protein
12) *lptA* lipopolysaccharide transport periplasmic protein
13) *yhbG* Putative ABC transporter ATP-binding protein
14) *rpoN* RNA polymerase factor sigma 54
15) *yhbH* putative sigma 54 modulation protein
16) *ptsN* PTS system transporter subunit IIA-like nitrogen regulator
17) Putative *glmZ* (sRNA) inactivating NTPase
18) *ptsD* Phosphohistdinoprotein-hexose phosphotransferase
19) *rnk* Nucleoside diphosphate kinase regulator
20) *pmbA* Peptidase
21) *yjgA* Putative transport protein
22) *tldD* Protease (Suppresses inhibitory activity of CsrA)
23) Putative carbon-nitrogen hydrolase
24) Hypothetical protein
25) *cafA* Ribonuclease G (RNase G)
26) *yhdE/maf* Maf-like septum formation protein
27) *mreD* Rod-shape determining protein
28) *mreC* Rod-shape determining protein
29) *mreB* Rod-shape determining protein
30) *yhdH* Putative dehydrogenase
31) *aroQ* 3-dehydroquinate dehydratase
33) *accC* Acetyl-CoA carboxylase biotin carboxylase subunit
34) Hypothetical protein
35) Hypothetical protein
36) Hypothetical protein
37) *insA* IS1 family transposase
38) Hypothetical protein
39) Hypothetical protein
40) ISPlu13C Transposase IS100
41) Hypothetical protein
42) *cynR* Putative LysR family transcriptional regulator
43) *guaD* Putative guanine deaminase
44) *panF* Sodium panthothenate symporter
45) *prmA* Ribosomal protein L11 methyltransferase
46) *dusB* tRNA-dihydrouridine synthase B
47) *fis* DNA-binding protein FIS
48) Putative multidrug resistance protein
49) Putative AraC/XylS family transcriptional regulator
50) Putative DNA-binding protein
51) *livF* Leucine/isoleucine/valine transporter ATP-binding subunit
52) *livG* Leucine/isoleucine/valine transporter ATP-binding subunit
53) *livM* Leucine/isoleucine/valine transporter ATP-binding subunit
54) *livH* Branched-chain amino acid petidase
55) *livK* Leucine-specific binding protein
56) Putative acetyltransferase
57) *garK* Glycerate kinase 2
58) *rpoH* RNA polymerase factor sigma 32
59) *ftsX* Cell division protein

P. temperata **K122 Contig 134**	***P. asymbiotica***	***P.luminescens*** **TTO1**
1) *degS* Serine endoprotease	pau_03658	plu4022
2) *murA* UDP-N-acetyl glucoasamine 1-adenyltrasnferase	pau_03665	plu4028
3) Hypothetical protein	pau_03668	plu4030
4) Hypothetical protein	pau_03669	plu4031
5) Hypothetical protein	pau_03670	plu4032
6) Hypothetical protein	pau_03671	plu4033
7) *yrbF* Putative ABC transporter ATP-binding protein	pau_03672	plu4034
8) *yrbG* Putative calcium/sodium transporter	pau_03673	plu4035
9) *kdsG* D-arabinose 5-phosphate isomerase	pau_03674	plu4036
10) *kdsC* 3-deoxy-D-mannose-octulosonate 8-phosphate phosphatase	pau_03675	plu4037
11) Hypothetical protein	pau_03676	plu4038
12) *lptA* lipopolysaccharide transport periplasmic protein	pau_03677	plu4039
13) *yhbG* Putative ABC transporter ATP-binding protein	pau_03678	plu4040
14) *rpoN* RNA polymerase factor sigma 54	pau_03679	plu4041
15) *yhbH* putative sigma 54 modulation protein	pau_03680	plu4042
16) *ptsN* PTS system transporter subunit IIA-like nitrogen regulator	pau_03681	plu4043
17) Putative *glmZ* (sRNA) inactivating NTPase	pau_03682	plu4044
18) *ptsD* Phosphohistdinoprotein-hexose phosphotransferase	pau_03683	plu4045
19) *rnk* Nucleoside diphosphate kinase regulator	pau_03684	plu4046
20) *pmbA* Peptidase	pau_03692	plu4060
21) *yjgA* Putative transport protein	pau_03693	plu4061
22) *tldD* Protease (Suppresses inhibitory activity of CsrA)	pau_03696	plu4064
23) Putative carbon-nitrogen hydrolase	pau_03697	plu4065
24) Hypothetical protein	pau_03698	plu4066
25) *cafA* Ribonuclease G (RNase G)	pau_03699	plu4067
26) *yhdE/maf* Maf-like septum formation protein	pau_03700	plu4068
27) *mreD* Rod-shape determining protein	pau_03701	plu4069
28) *mreC* Rod-shape determining protein	pau_03702	plu4070
29) *mreB* Rod-shape determining protein	pau_03703	plu4071
30) *yhdH* Putative dehydrogenase	pau_03704	plu4072
31) *aroQ* 3-dehydroquinate dehydratase	pau_03705	plu4073
32) *accB* Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	pau_03706	plu4074
33) *accC* Acetyl-CoA carboxylase biotin carboxylase subunit	pau_03707	plu4075
34) Hypothetical protein	pau_01476	plu3702
35) Hypothetical protein	pau_01479	plu1350
36) Hypothetical protein	pau_01480	plu1351
37) *insA* IS1 family transposase		plu4616
38) Hypothetical protein		plu4615
39) Hypothetical protein		plu4614
40) ISPlu13C Transposase IS100		plu1222
41) Hypothetical protein		plu3709
42) *cynR* Putative LysR family transcriptional regulator		
43) *guaD* Putative guanine deaminase		
44) *panF* Sodium panthothenate symporter	pau_03711	plu4086
45) *prmA* Ribosomal protein L11 methyltransferase	pau_03712	plu4087
46) *dusB* tRNA-dihydrouridine synthase B	pau_03713	plu4088
47) *fis* DNA-binding protein FIS	pau_03714	plu4089
48) Putative multidrug resistance protein	pau_03715	plu4090
49) Putative AraC/XylS family transcriptional regulator	pau_03716	plu4091
50) Putative DNA-binding protein		
51) *livF* Leucine/isoleucine/valine transporter ATP-binding subunit	pau_03719	plu4094
52) *livG* Leucine/isoleucine/valine transporter ATP-binding subunit	pau_03720	plu4095
53) *livM* Leucine/isoleucine/valine transporter ATP-binding subunit	pau_03721	plu4096
54) *livH* Branched-chain amino acid petidase	pau_03722	plu4097
55) *livK* Leucine-specific binding protein	pau_03723	plu4098
56) Putative acetyltransferase	pau_03724	plu4099
57) *garK* Glycerate kinase 2	pau_03725	plu4100
58) *rpoH* RNA polymerase factor sigma 32	pau_03726	plu4101
59) *ftsX* Cell division protein	pau_03727	plu4102
60) *ftsE* Cell division protein	pau_03728	plu4103
61) *ftsY* Cell division protein	pau_03729	plu4104

Table 5.12 List of identified genes on *P. temperata* K122 contig 134 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

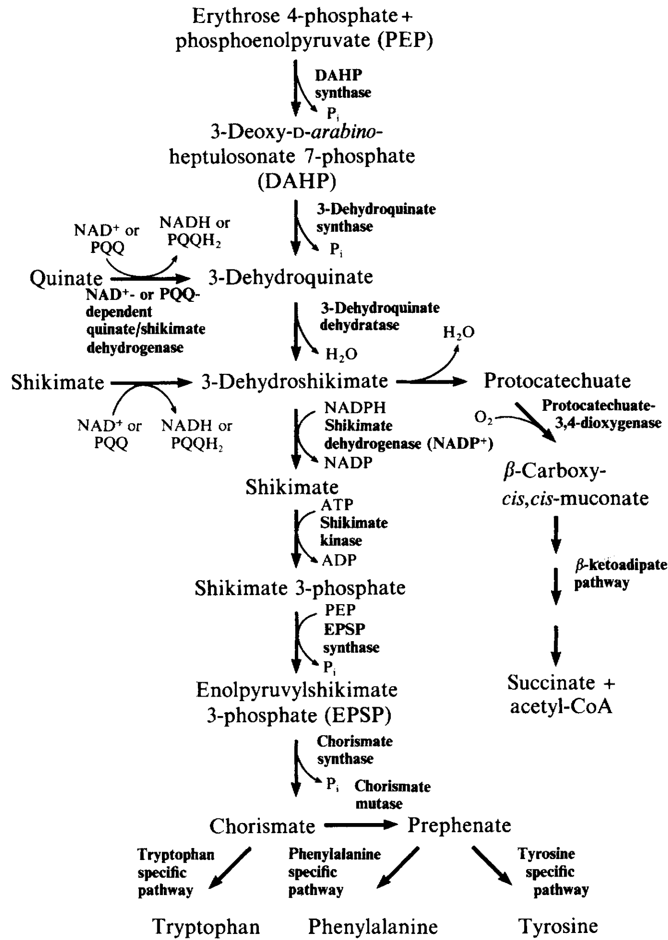


Fig. 5.10. Schematic representation of the biosynthesis of aromatic amino acids via the shikimate pathway and the catabolism of quinate shikimate (From Euverink *et al*., 1992). The aroQ gene product, 3-dehydroquinate dehydratase is involved in the conversion of 3-dehydroquinate to 3-dehydroshikimate.

including *Salmonella* spp., *Yersinia* spp., and the Gram-positive *Corynebacterium pseudotuberculosis* (Simmons *et al*., 1997; D'afonseca *et al*., 2008). *aroQ* mutant strains are not only attenuated in virulence, but also in persistence, with severely restricted *in vivo* growth occurring (Simmons *et al*., 1997). The effects of the *aroQ* mutation observed here in *P. temperata* K122 – lack of pigment and antibiotic production, as well as reduced lipase activity, may be as a result of a reduced bioavailability of the products of the Shikimate pathway.

5.12 Mutant Pm102

Secondary like for pigmentation and lipase activity, the mutation in Pm102 occurs in a putative lipoprotein (LscW/HscY/ExsC) found on K122 contig 162 (Fig. 5.11). Also found in *P. asymbiotica* (*PAU_01067*) and *P. luminescens* TTO1 (*plu3751*) (Table 5.13) this gene was previously identified as part of a genomic island in *P. luminescens* (Waterfield *et a*l., 2002). This genomic island encodes a type III secretion system (TTSS) – a group of proteins that make up a 'molecular syringe' that can deliver bacterial effector proteins across host membranes. The genes of this TTSS are largely conserved across several bacterial species, including *Pseudomonas aeruginosa*, *Yersinia* spp., *Aeromonas hydrophila* and *Aeromonas salmonicida* (Yu *et al*., 2004) however, the gene arrangement in *Yersinia* and *Photorhabdus* spp. differs from that of the other species, with an inversion occurring in the *pscU-exsB* region (Waterfield *et al*., 2002; Yu *et al*., 2004). This TTSS has been assigned a role in pathogenesis and host immune evasion (Hartland *et al*., 1994; Hartland *et al*., 1996; Waterfield *et al*., 2002; Yu *et a*l., 2004; Brugirard-Ricaud *et al*., 2005).
The *Photorhabdus* TTSS is largely conserved across differing strains, with the effectors belonging to a flexible gene pool (Brugirard-Ricaud *et al*., 2004). Interestingly, whereas other *P. temperata* species (Meg & XINach) do not carry an effector gene with their TTSS backbone (Brugirard-Ricaud *et al*., 2004), K122 carries the LopT/YopT seen in *P. luminescens* TTO1 (Fig. 5.11, Table 5.12). In *P. asymbiotica* a LopT/YopT effector is located elsewhere on the genome (Table 5.12).

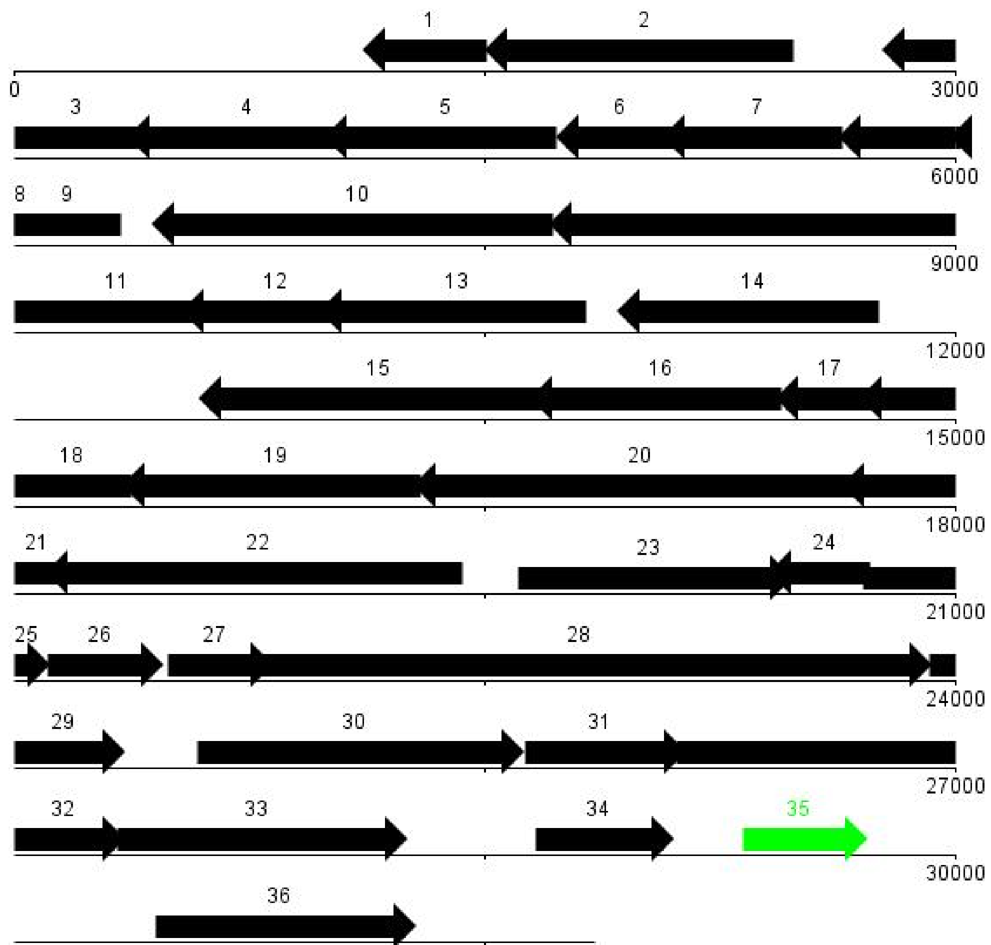


FIG. 5.11 Annotation of Genome Contig 162 of *P. temperata* K122, Mutant Pm102 occurs in gene 35, encoding a putative type III secretion system lipoprotein, LscW.

1) Hypothetical protein
2) *lopT/yopT* Putative Type III secreted effector protein
3) *sctL* Type III secretion system protein LscL/YscL
4) *sctK* Type III secretion system component protein SctK/LscK
5) *sctJ* Type III secretion system component protein SctJ/LscJ
6) *sctI* Type III secretion system component protein SctI/LscI
7) *sctH* Type III secretion system component protein SctH/LscH
8) *sctG* Type III secretion system component protein SctG/LscG
9) *sctF* Type III secretion system component protein SctF/LscF
10) *sctD* Type III secretion system component protein SctD/LscD
11) *sctC* Type III secretion outer membrane protein SctC/LscC
12) *sctB* Type III secretion system component protein SctB/LscB
13) Hypothetical protein
14) *rhaS/lscA* Putative transcriptional activator protein ExsA/LscA/VirF
15) *sctU* Type III secretion system component protein SctU/LscU
16) *sctT* Type III secretion system component protein SctT/LscT
17) *sctS* Type III secretion system component protein SctS/LscS
18) *sctR* Type III secretion system component protein SctR/LscR
19) *sctQ* Type III secretion component SctQ/LscQ
20) *sctP* Type III secretion component SctP/LscP
21) *sctO* Type III secretion component SctO/LscO
22) *sctN* Type III secretion system ATPase SctN/LscO
23) *sctW* Type III secretion control protein SctW
24) Hypothetical protein
25) *lssN* Type III secretion protein
26) *sctX* Type III secretion protein
27) *sctY* Type III secretion protein
28) *sctV* Type III secretion protein SctV/LssD
29) *pcrR* Putative transcriptional regulator
30) *lssV* Putative virulence associated V-antigen
31) *sycD* Type III secretion chaperone
32) Putative LopB/YopB Transmembrane effector
33) Putative outer membrane protein LopD
34) *exsC* Exoprotein synthetase protein C/LscY
35) *exsB* Putative lipoprotein LscW/ExsB
36) *hod* Putative dioxygenase

	***P. temperata* K122 Contig 162**	***P. asymbiotica***	***P.luminescens* TTO1**
1)	Hypothetical protein		plu3789
2)	*lopT/yopT* Putative Type III secreted effector protein	pau_02096	plu3788
3)	*sctL* Type III secretion system protein LscL/YscL	pau_01029	plu3787
4)	*sctK* Type III secretion system component protein SctK/LscK	pau_01030	plu3786
5)	*sctJ* Type III secretion system component protein SctJ/LscJ	pau_01031	plu3785
6)	*sctI* Type III secretion system component protein SctI/LscI	pau_01032	plu3784
7)	*sctH* Type III secretion system component protein SctH/LscH	pau_01033	plu3783
8)	*sctG* Type III secretion system component protein SctG/LscG	pau_01034	plu3782
9)	*sctF* Type III secretion system component protein SctF/LscF	pau_01035	plu3781
10)	*sctD* Type III secretion system component protein SctD/LscD	pau_01036	plu3779
11)	*sctC* Type III secretion outer membrane protein SctC/LscC	pau_01037r	plu3778
12)	*sctB* Type III secretion system component protein SctB/LscB	pau_01038	plu3777
13)	Hypothetical protein	pau_01039	plu3776
14)	*rhaS/lscA* Putative transcriptional activator protein ExsA/LscA/VirF	pau_01040	plu3775
15)	*sctU* Type III secretion system component protein SctU/LscU	pau_01045	plu3774
16)	*sctT* Type III secretion system component protein SctT/LscT	pau_01046	plu3773
17)	*sctS* Type III secretion system component protein SctS/LscS	pau_01047	plu3772
18)	*sctR* Type III secretion system component protein SctR/LscR	pau_01048	plu3771
19)	*sctQ* Type III secretion component SctQ/LscQ	pau_01049	plu3770
20)	*sctP* Type III secretion component SctP/LscP	pau_01050	plu3769
21)	*sctO* Type III secretion component SctO/LscO	pau_01051	plu3768
22)	*sctN* Type III secretion system ATPase SctN/LscO	pau_01052	plu3767
23)	*sctW* Type III secretion control protein SctW	pau_01053	plu3766
24)	Hypothetical protein		plu3765
25)	*lssN* Type III secretion protein	pau_01054	plu3764
26)	*sctX* Type III secretion protein	pau_01055	plu3763
27)	*sctY* Type III secretion protein	pau_01056	plu3762
28)	*sctV* Type III secretion protein SctV/LssD	pau_01057	plu3761
29)	*pcrR* Putative transcriptional regulator	pau_01058	plu3760
30)	*lssV* Putative virulence associated V-antigen	pau_01060	plu3758
31)	*sycD* Type III secretion chaperone	pau_01061	plu3757
32)	Putative LopB/YopB Transmembrane effector	pau_01062	plu3756
33)	Putative outer membrane protein LopD	pau_01063	plu3755
34)	*exsC* Exoprotein synthetase protein C/LscY	pau_01065	plu3753
35)	*exsB* Putative lipoprotein LscW/ExsB	pau_01067	plu3751
36)	*hod* Putative dioxygenase	pau_01068	

Table 5.13 List of identified genes on *P. temperata* K122 contig 162 and homologues in *P. asymbiotica* and *P. luminescens* TTO1.

A role for LscW can be deduced from its *Yersinia* homologue, YscW (formerly VirG) which has been shown to be involved in the stabilisation and localisation of the YscC secretion component of the TTSS machinery, with YscW mutants showing decreased protein secretion (Koster *et al*., 1997; Burghout *et al*., 2004).

5.13 Mutant Pm112

Another pigment and lipase activity deficient mutant, Pm112 is primary-like for all other tested phenotypes. Colocated on K122 contig 58 (Fig. 5.1) with the mutation causing Pm3, the transposon insertion occurred in a gene encoding a product similar to protein gp29 of enterobacteria phage Mu, for which no function has as yet been described in the uniprot database. Homologous to coding unit *plu3441* of *P. luminescens* TTO1 (Table 5.4) and comprising part of the previously mention phage locus (see Section 5.2) described by Wilkinson *et al* (2009), there is no direct homologue in *P. asymbiotica*.

5.14 Summary

The wide variety of genes identified above whose mutation affected various aspects of the *P. temperata* K122 phenotypic switch is suggestive of a complex system comprising many levels of regulation, however the net effect of each mutation may be as a result of either i) a direct effect on phenotype, ii) an indirect effect on phenotype as a result of environmental or stress conditions encountered due to loss of gene function or iii) effects occurring downstream of the mutation, again resulting in activation of stress responses. Whilst the work by Gaudrialt *et al* (2008) in *P. luminescens* TTO1 suggests that the overall primary to secondary phenotypic switch occurs irrespective of global genome architecture, it also observes that some switching of individual phenotypes can occur as a result of localised genomic rearrangements however these switches are generally of a temporary/transient nature unlike the overall phenomenon of phenotypic variation in *Photorhabdus*.
The plethora of genes identified as being involved, and the many aspects of growth and development effected indicate that the true nature of the switch has yet to be elucidated. The partially switched mutants, with several gene knock-outs having similar effects on phenotype seem to support the idea that phenotypic variation occurs as a stress response to growth conditions, with a stepwise progression of switching of phenotypes occurring as a result of environmental sensing – brought about in the cases listed above owing to a lack of preferential growth conditions as a result of gene disruption.

The initial mutant screen used to identify phenotypic mutants resulted in a selective preference for phenotypic mutants lacking pigment. However, studies not selecting for pigment difference have also isolated pigment mutants, showing it to be one of the most frequently identified phenotypically varying characteristics. For example, the *manA* phosphomannose isomerase mutant isolated by Amos *et al* (2011) not only displayed biofilm, motility and pathogenicity defects as a result of extracellular polysaccharide modification, but was also lacking in pigment. Lango & Clarke (2010) isolated a malate

dehydrogenase (*mdh*) mutant that was defective in the TCA cycle and was also non-pigmented. The pigment produced by *Photorhabdus* has been identified as an anthroquinone compound (AQ) (Richardson *et al*., 1988) produced by a type II polyketide synthase (Brachmann *et a*l., 2007). Interestingly, though not used for synthesis of AQ in *Photorhabdus*, a second biosynthetic pathway is used for AQ biosynthesis – that of the aforementioned Shikimate pathway that is also involved in biosynthesis of aromatic amino acids (Velísek *et al*., 2007; Euverink *et al*., 1992; Hermann & Weaver, 1999). The function of the pigmentation of *Photorhabdus* has yet to be clearly elucidated, with possible roles in protecting the insect cadaver from microbial competitors and insect scavengers (Richardson *et al*., 1988; Li *et al*., 1995; Baur *et al*., 1998). In *P. luminescens* TTO1, the genomic region *plu4185-4195* has been identified as being involved in the production of AQ (Brachmann *et al*., 2007) with gene *plu4187* shown to be regulated at the transcriptional level (Lanois *et al*., 2011). Interestingly, a genetic knockout of the *plu4185-4195* region had no effect on the bacterium's pathogenic or mutualistic abilities (Brachmann *et al*., 2007) casting further uncertainty as to why AQ is produced.

Recently, it has been suggested that *Photorhabdus* has evolved to use L-proline as its preferred nutrient source when in the insect haemolymph, with this use of L-proline touted to protect against osmotic stress, cause an enhancement of PMF and regulate the production of pathogenic metabolites (Crawford *et al*., 2010). L-proline is also the preferred energy source of insects (Bursell 1981) and its use by *Photorhabdus* depletes its availability to the insect. AQ production has been observed to be substantially upregulated in response to L-proline (Crawford *et al*., 2010), implying a role for its production during the insect pathogenic phase of the *Photorhabdus* lifecycle. With no defined absolute requirement for AQ, it is perhaps not surprising that it is seemingly a readily switched off phenotype associated with times of genetic or environmental stress, as can occur after mutagenesis or prolonged culturing, with the bacteria eliminating the associated energetic requirement for pigment production.

Chapter 6

6. Discussion

6.1 Introduction

Photorhabdus spp. are Gram negative enterobacteria which have a life cycle involving mutualism with a nematode partner and pathogenicity towards insects. *Photorhabdus* has long been touted as a model organism for the study of bacteria-host interactions and the emergence of human pathogenic species has given the study of *Photorhabdus* increased importance from a medical standpoint, with invertebrate pathogens such as *Photorhabdus* highlighted as potential 'reservoirs' of virulence factors (Waterfield *et al*., 2004). Mutualism and pathogenicity are now recognised as sharing common mechanisms (Hentschel *et al*., 2000) and in *Photorhabdus* have been shown to be coregulated (Joyce & Clarke 2003). Further investigation of the mechanisms of mutualism and pathogenicity will enable the understanding and distinguishing of the molecular processes of beneficial and harmful bacterial interactions (Goodrich-Blair & Clarke 2007). The phenotypic variation which occurs in *Photorhabdus*, resulting in the secondary variant which is deficient for the production pigment, bioluminescence and many extracellular proteins, is of particular interest because of a concurrent loss of mutualistic ability.

The focus of this study was to identify factors involved in the control of phenotypic variation in *P. temperata* K122 with the goal of better understanding the molecular mechanisms of mutualism and pathogenicity employed by the bacterium. To date, two signalling pathways have been identified that play a role in the regulation of phenotypic variation in *Photorhabdus.* Firstly, a gene encoding a LysR type transcriptional regulator – *hexA* – was identified whose elimination in the secondary variant resulted in the derepression of the primary-specific characters and restored mutualistic ability (Joyce & Clarke 2003). HexA is also capable of positively regulating its own expression, an ability which would enable high levels of HexA to be maintained in the secondary variant. This is supported by the observation that overproduction of HexA in the primary variant has also

been shown to induce conditional phenotypic variation (Joyce *et al*., 2006). The BMM215 *hexA* mutant (Joyce & Clarke 2003) also displayed decreased virulence, indicative of an additional requirement for HexA in the regulation of pathogenicity. The conservation of the *hexA* locus across a range of *Photorhabdus* species indicates its importance to the survival of the bacterium and potentially that its role in the regulation of the primary-phase characteristics is conserved. Interestingly, *P. asymbiotica* ATCC 43949, the clinical isolate of the species once thought to be secondary-like in character (Boemare, 2002) does not appear to have increased levels of *hexA* expression, suggesting it may be a primary form that has lost certain primary variant characteristics rather than being a *hexA* expressing, symbiosis repressing secondary variant cell (Joyce & Clarke, 2003). Furthermore, the identification of a *P. asymbiotica* strain with a Heterorhabditis nematode symbiont host (*P. asymbiotica* Kingscliff) further discredits this hypothesis and suggests that for other *P. asymbiotica* strains that there are as yet unidentified symbiotic nematode partners (Gerrard *et al.,* 2006).

The second pathway implicated in phenotypic variation of *Photorhabdus* is the 'adaptation to stationary phase' AstR-AstS two component signal transduction system which has been shown to have a role in the temporal regulation of phenotypic variation (Derzelle *et al*., 2004a). Strains mutated in the *astR* response regulator component of this pathway typically initiated phenotypic variation seven days earlier than the wild type strain. The AstRS pathway was shown to have a role in the positive regulation of UspA – a universal stress protein implicated in stressed cell survival that responds to inhibited growth conditions including oxidative stress (Nyström & Neidhart 1994; Nachin *et al*., 2005) which has led to the suggestion that the AstRS pathway may have a role in protecting the cell from stress and thereby preventing promiscuous or early phenotypic variation (Derzelle *et al*., 2004a). A role for AstRS in the oxygen dependent regulation of motility during phenotypic variation has also been suggested (Joyce *et al*., 2006). Additionally, Joyce *et al* (2006) speculate on a potential link between the AstRS pathway and that regulated by HexA (Fig. 1.6).

Other factors implicated in the regulation of phenotypic variation of *Photorhabdus* include the *ner* gene encoding a putative DNA binding protein whose stationary phase overexpression in the primary variant results in a primary to secondary phenotypic switch, indicative of a role for *ner* in the repression of stationary phase primary variant specific gene expression (O'Neill *et al*., 2002) and the *cipA/cipB* genes encoding the stationary phase crystal proteins of *Photorhabdus*, the inactivation of either of which results in a bacterial cell with many secondary variant like characteristics (Bintrim & Ensign 1998).

6.2 General Conclusion

In total, 24 phenotypic mutants were isolated, identified and characterised during this study (Table 6.1) with a further 106 mutants assessed for phenotypic variability. This study has confirmed the role for HexA in the regulation of symbiosis, virulence and phenotypic variation in *P. temperata* K122 and has identified a role for HexA in regulating the small RNA global regulator *csrB*. In *P. luminescens* TTO1, *csrB* has been linked to the regulation of many genes predicted to be involved in symbiosis, including bioluminescence, antibiotic production and pigment production (Krin *et al.,* 2008). The reduction in expression of *csrB* in the Sm phenotypic mutants identified in this study indicates that, in the secondary variant at least, HexA is a positive regulator of *csrB* expression. This appears to be in contrast to the situation identified in *Erwinia* where HexA negatively regulates the expression of the *csrB* homologue in this bacterium (Fig 3.13) (Mukherjee *et al.,* 2000). However, role for HexA in the regulation of *csrB* is consistent with the model for the regulation of pathogenicity and mutualism proposed by Joyce *et al.* (2006) (Fig. 1.6). Another gene identified in this study, *sdiA*, encoding a LuxR type regulator, has been identified as a positive regulator of an operon (*plu4563-4568*) implicated in cell aggregation (D. Clarke, personal communication). This operon is negatively regulated by HexA, suggesting a possible role for *sdiA* in phenotypic variation in conjunction with HexA, with SdiA activity prevented by the presence of HexA in the secondary

Mutant	*P. temperata* K122 Gene/Protein Identified	*P. asymbiotica*	*P. luminescens* TTO1	Relevant *Photorhabdus* literature
Sm11	*hipB*	*pau_00435*	*plu4901*	n/a
Sm13	*hexA*	*pau_01518*	*plu3090*	Joyce & Clarke (2003)
Sm15	*hexA*	*pau_01518*	*plu3090*	Joyce & Clarke (2003)
Pm3	DNA N-6-adenine methyl transferase (DNA Methylase)	*n/a*	*plu3449*	Wilkinson *et al*., (2009) Gaudrialt *et al*., (2008)
Pm6	*galK* (galactokinase)	*pau_00469*	*plu0576*	Gaudrialt *et al*., (2006)
Pm7	RtxA toxin	*pau_02098*	*plu2400*	Waterfield *et al*., (2004) Münch *et al*., (2008)
Pm9	*sciC*	*pau_02278*	*plu2297*	n/a
Pm20	*sciC*	*pau_02278*	*plu2297*	n/a
Pm24	*trkA* (Potassium uptake)	*pau_04151*	*plu4671*	n/a
Pm25	Hypothetical protein	*pau_00387*	*plu0480*	Turlin *et al*., (2006) Lanois *et al*., (2011)
Pm35	*rarD*	*pau_04113*	*plu4632*	n/a
Pm39	Hypothetical protein	*pau_03019*	*plu2670*	Waterfield *et al*., (2004 & 2008)

Table 6.1. Summary of the *P. temperata* K122 phenotypic mutants isolated, identified and characterised during this study.

Listed alongside are the corresponding genes in *P. asymbiotica* and *P. luminescens* TTO1, along with relevant/related published on the gene identified (Continued overleaf).

Mutant	*P. temperata* K122 Gene/Protein Identified	*P. asymbiotica*	*P. luminescens* TTO1	Relevant *Photorhabdus* literature
Pm53	*sciB*	*pau_02278*	*plu2297*	n/a
Pm55	*nuoA*	*pau_01520*	*plu3089*	Amos *et al*., (2011)
Pm58	*serB*	*pau_00520*	*plu0551*	Easom *et al*., (2010)
Pm59	*dinA/polB*	*pau_00574*	*plu0616*	n/a
Pm76	*lysR* type	*n/a*	*plu4133*	n/a
Pm77	Transcriptional regulator *sdiA*	*pau_04062*	*plu4562*	Positive regulator of TTO1 operon *plu4563-4568* D. Clarke, (pesonal communication)
Pm81	*aroQ* – 3 dehydroquinate dehydratase	*pau_03705*	*plu4073*	n/a
Pm85	*flhB*	*pau_02666*	*plu1895*	n/a
Pm92	*flhB*	*pau_02666*	*plu1895*	n/a
Pm100	*flhB*	*pau_02666*	*plu1895*	n/a
Pm102	Putative lipoprotein LscW/HscY/ExsC	*pau_01067*	*plu3751*	Waterfield *et al*., (2002) Yu *et al*., (2004) Brugirard-Ricaurd *et al*., (2004) & (2005)
Pm112	Similar to protein gp29 of Enterobacteria phage Mu	*n/a*	*plu3441*	Wilkinson *et al*., (2009) Gaudrialt *et al*., (2008)

Table 6.1 (contd.) Summary of the *P. temperata* K122 phenotypic mutants isolated, identified and characterised during this study.
Listed alongside are the corresponding genes in *P. asymbiotica* and *P. luminescens* TTO1, along with relevant/related published on the gene identified.

variant. Despite the fact that *E. coli*, like *Photorhabdus*, does not produce any AHL, SdiA has been implicated in the regulation of multiple phenotypes (Wang *et al*., 1991; Wei *et al*., 2001; Rahmati *et al*., 2002), with Dyzel *et al.* (2010) suggesting that the SdiA regulon may change in response to environmental or metabolic conditions. Given the normal environmental conditions of *Photorhabdus*, it seems probable that significant levels of *sdiA* activity can occur despite the lack of the AHL quorum sensing signal. Therefore, it would seem this gene may have an important role to play in the sensing and response to stress and any subsequent phenotypic variation.

Phenotypic variation in *Photorhabdus* has been observed to occur after prolonged growth in stationary phase and/or under osmotically stressful conditions (Akhurst 1980; Boemare and Akhurst 1988; Krasomil-Osterfeld 1995). The reason for the phenotypic switch has yet to be elucidated, but the unidirectional nature of the switch and its observed occurrence under stress conditions have lead to phenotypic variation in *Photorhabdus* being described as an extreme reaction to ensure survival (Boemare *et al.,* 1997; ffrench-Constant *et al.,* 2003). This study has isolated various genes that have links to known bacterial stress response pathways or mechanisms of persistence. The knockout of an orphan *hipB* antitoxin homologue has been shown to influence the secondary to primary phenotypic switch. With *hipB* having been shown to be directly required for development of a 'persistent' state (Correroa *et al.,* 2006), the identification of *hipB* in this study further supports the hypothesis that variation in *Photorhabdus* is a survival reaction. Although the occurrence of an orphan *hipB* is unusual, given the apparent unidirectional nature of the phenotypic switch in *Photorhabdus*, it may be a method of ensuring the switch only occurs under extreme conditions. Another gene identified in this study that has been directly linked with stress responses is the *dinA/polB* encoding DNA polymerase II, which is induced early in the SOS stress response and is involved in the reinitiation of DNA replication after UV irradiation. Indeed, *polB*, along with other SOS response regulated DNA polymerases, has been shown to confer a competitive advantage on stationary phase cells, resulting in enhanced long-term survival and evolutionary fitness in *E. coli* (Yeiser *et al*., 2002).

The remaining genes identified by this study, whilst not readily linked to stress responses, are involved in a widely diverse range of processes including cellular metabolism, ion transport, protein secretion and regulation of transcription. Therefore, it is possible the observed phenotypes of these mutations may arise indirectly. For example, in mutants Pm3 and Pm112, the disruption of phage genes may result in a loss of phage-imparted niche adaptations as described by Brüssow *et al*., (2004) resulting in a loss of fitness and therefore a stressed bacterial cell. The *trkA* mutant (Pm24) identified by this study may be deficient in K^+ uptake resulting in osmotically stressful conditions which have been shown to result in phenotypic variation (Krasomil-Osterfeld 1995). The GroEL molecular chaperone, shown to be upregulated in the secondary variant (Derzelle *et al.,* 2004a) and known to be involved in the bacterial response to K^+ induced salt stress (Kilstrup *et al*., 1997) may therefore have an involvement in the phenotypic changes observed in this Pm24. Other genes identified by this study may all result in a deficiency of metabolites, a loss of normal transcriptional regulation, altered cell surface properties or a build up of unsecreted proteins in the cell, all of which could result in an environmentally or physiologically stressed cell, with a subsequent phenotypic shift occurring as a response to such stresses. The wide variety of genes identified as having an effect on phenotype may be as a result of investment by *Photorhabdus* in maintaining a mutualistic relationship with the *Heterorhabditis* nematodes, which may have resulted in the development of specific environmental requirements for an optimal mutualistic partnership. The absence of such mutualistic factors may then result in the activation of multiple stress responses to ensure survival of the bacteria in the absence of the nematode partner.

It has already been shown by Lango and Clarke (2010) that differences in metabolism result in different lifestyles for the bacterium – they have shown a requirement for functioning secondary metabolism to maintain a successful mutualism with the nematode by identifying a mutant that retained its pathogenic ability but was non-mutualistic and unable to produce the anthraquinone pigment (AQ) and the stilbene based antibiotic 3,5-dihdroxy-4-isopropylstilbene (ST), both of which have previously been identified as factors produced in the post-exponential phase of growth which are required for mutualism (Joyce & Clarke,

2003; Brachmann *et al*., 2007; Joyce *et al*., 2008). This mutation arose in the *mdh* gene, encoding malate dehydrogenase, an enzyme involved in the tricarboxylic acid (TCA) cycle. With this mutant unable to undergo the transition from pathogen to mutualist, as occurs in wild type *Photorhabdus* strains, a clear link has been established between bacterial metabolism and the adaptation of *Photorhabdus* to different environments. Mutants such as those isolated in this study, if affected in metabolism, may well be affected in adaptation and as a result undergo stress responses leading to the occurrence of a phenotypic switch.

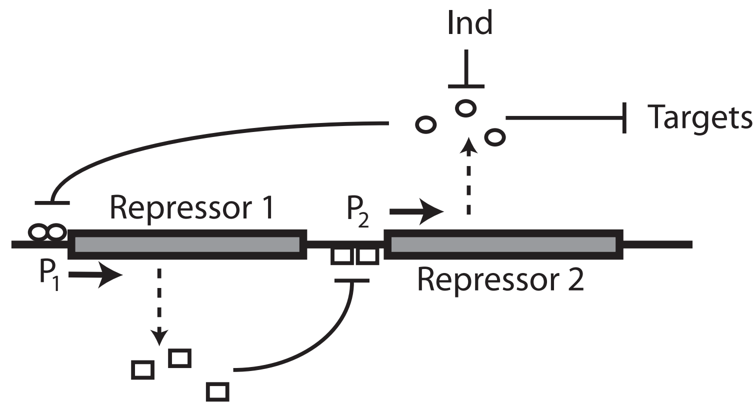
With loss of mutualistic ability being one of the major defining features of a *Photorhabdus* secondary phenotypic variant, it is not surprising that the integrity of the bacterial cell surface has also been shown to play a role in the establishment of the bacterial-nematode partnership. Easom *et al*, (2010) through the use of green fluorescent protein (*gfp*) labelling, identified 6 classes of mutants which displayed reduced nematode colonisation ability. These mutants were all affected in the assembly/maintenance of LPS or in other cell-surface associated factors. Whilst the work of Easom *et al*, (2010) was directed at investigating mutualistic colonisation of the nematode rather than at phenotypic variation, given that secondary type phenotypic variants lack mutualistic ability, the genetic mechanisms involved may be linked. Interestingly, Easom *et al* (2010) isolated a *galE* mutant that was affected in transmission to the infective juvenile. Mutant Pm6, identified in this study, occurs in *galK*, coding for an enzyme whose activity has been shown to complement an *E. coli galE* mutant in the presence of exogenous galactose (Nnalue & Stocker, 1986) however, with *Photorhabdus* shown to lack galactose catabolic activity (Easom *et al*., 2010) the relationship between GalK and GalE in *Photorhabdus* may be of a different nature. Surprisingly, none of the factors identified by Easom *et al* (2010) were identified during this study of phenotypic variation, however this may be as a result of the differences in screening method employed (loss/gain of pigment as opposed to *gfp* studies of bacterial transmission) and the different phenotypic aspects being studied, with Easom *et al* (2010) examining mutants with reduced colonisation ability rather than an absolute presence/absence of mutualism.

Cumulative evidence indicates that the secondary variant occurs as a result of stress induction, and this, along with the observation that the phenotypic switch in *Photorhabdus* occurs during stationary phase growth, suggests it falls into the 'Type I' persistence category (Balaban *et al.,* 2004). However, unlike other Type I persisters, *Photorhabdus* does not readily undergo phenotypic reversion upon inoculation into fresh medium after stationary phase. This lack of an observed phenotypic reversion may be as a result of a similar situation to the Type III phenotypic variant of the pathogenic soil and water bacterium *Burkholderei pseudomallei* where it has been suggested that such variants occur under harsh environmental conditions in the absence of a challenge/signal from host factors (Chantratita *et al*., 2007). The absence of such a signal may be the reason for the apparently 'locked-in' status of the *Photorhabdus* secondary variant. A genetic model for such a locked switch has been described by Gardner *et al* (2000) who suggest the presence of mutually repressing repressors which, once repression is activated, remain activated, maintaining their own activation until a further signal is received (see Fig. 6.1). In *Photorhabdus*, phenotypic variation may involve such a switch that is activated in response to stress signals and remain activated even with the removal of such stress signals, requiring a specific, as yet unidentified, environmental or nematode factor to relieve repression and allow reversion of the phenotypic switch. Another function of such a system may be its use to ensure bacterial fitness as a mutualistic partner of the nematode, with the phenotypic switch occurring to ensure the shut out of bacteria that are unfit to maintain a successful mutualistic relationship with the nematode.

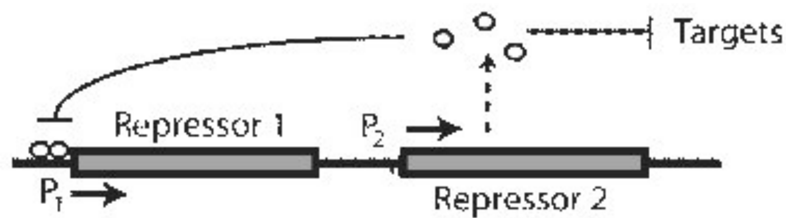
This study has further clarified the nature of HexA activity in the phenotypic switch undergone by *Photorhabdus*, identified other potential participants in the switch and also provided insight into the reasons for the occurrence of phenotypic variation in *P. temperata* K122. Further investigation of the phenotypic switch – for example determination of the interactions between *hexA* and *csrB, hexA* and *sdiA,* and investigation of the role of the orphan *hipB* identified in this study – will provide extra insight into the nature of the phenotypic switch and thereby the symbiotic and pathogenic interactions undergone by the bacterium as a part of its tripartite life cycle. Such information is of increasing value for developing improved commercial production of nematodes, a process affected by the switch to

a non-symbiotic phenotype (Ehlers, 2001) and more importantly to increase our overall understanding of bacteria-host interactions in the context of new and emerging pathogens.

A.



B.



C.

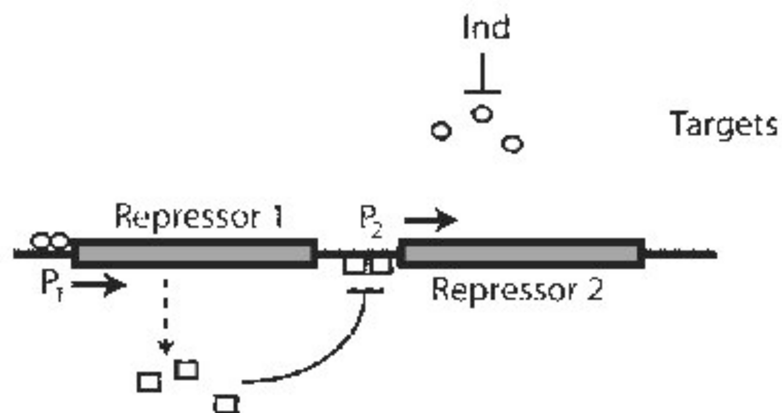


Fig 6.1 Toggle switch design (Dubnau & Losick 2006; Gardner *et al*., 2000).
A) Entire switch illustrated, B) Switch in absence of inducer, C) Switch with Inducer present
If Repressor 2 is inactivated (perhaps because an Inducer, (Ind) is added) then Repressor 1 is produced, halting synthesis of Repressor 2. This also results in an equivalent to positive autoregulation, because the increase in Repressor 1 results in even more Repressor 1 production. If Repressor 2 represses a set of downstream genes, these will now be expressed. If Ind is withdrawn, the system will tend to remain in the state in which Repressor 1 is ON and Repressor 2 is OFF. To toggle the switch, an extra factor may be required to sequester Repressor 1 and thereby restore the activity of repressor 2.

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