The social ecology of *Serratia*:

Pathogen of the New Zealand grass grub, *Costelytra giveni*

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The New Zealand grass grub (*Costelytra giveni*) is an endemic New Zealand pest species which feeds on the roots of common pastoral plants. Grass grub populations in the soil can be managed using biopesticides, such as those based on *Serratia entomophila*, a host-specific pathogen which causes a chronic disease in *C. giveni* larvae called amber disease. Following ingestion of this bacterium by *C. giveni*, the larvae cease feeding, and a clearance of the gut results in amber discolouration, with the infection leading to death after two-to-three months. The amber disease virulence determinants are encoded by the *Serratia* amber disease associated plasmid (pADAP), which is also found in pathogenic *S. proteamaculans* strains. Non-pathogenic plasmid-free strains of *S. entomophila* and *S. proteamaculans* have also been isolated from pasture soils. The interactions between pathogenic and non-pathogenic *Serratia* spp. within the soil are not well defined. Therefore, increasing knowledge on the conditions and outcome of intra-species competition between pathogenic and non-pathogenic *Serratia* spp. will provide important information regarding both the ecology of the strains in the field, and how competition can impact pathogenic efficacy. The results generated will assist in predicting the outcome of field applications and improving biopesticide efficacy.

The interactions between pathogenic and non-pathogenic *Serratia* strains were explored through *in vitro* and *in vivo* competition assays using constructed strains containing antibiotic markers positioned in intergenic regions of the chromosome and the pADAP backbone. This allowed for the selective recovery of strains during mixed inoculation assays in liquid media, soil microcosms, and within the larvae. The assays were conducted by competing the pathogenic *S. entomophila* strain A1MO2 against an isogenic non-pathogenic plasmid-cured variant, 5.6. Additional experiments were conducted using 477, a naturally occurring plasmid-free *S. entomophila* soil isolate, and AGR96X, a hyperpathogenic strain of *S. proteamaculans* associated with increased virulence, causing mortality in challenged larvae

within five to ten days. Due to this rapid kill time, AGR96X has also been identified as a potential biopesticide agent of *C. giveni* larvae.

The results obtained demonstrated an increased competitive fitness for 5.6 in all mixed inoculation experiments with A1MO2, including a faster doubling time than A1MO2 during the exponential growth phase in *in vitro* assessments, which was validated with Lotka/Volterra-style competition models. Comparative transcriptomics between 5.6+pADAP (an A1MO2 proxy strain) and 5.6 cultured to the mid-exponential growth phase revealed an increased expression of operons relating to carbohydrate metabolism and transport in 5.6 relative to 5.6+pADAP, including operons related to maltose and trehalose transport. This finding was validated when 5.6 outcompeted A1MO2 during mixed inoculation in M9 medium with a maltose carbon source. However, during mixed inoculation in liquid media A1MO2 outcompeted the natural plasmid-free *S. entomophila* isolate 477. In soil microcosms and within the larvae 5.6 was also able to outcompete A1MO2, showing a higher persistence in microcosms in the presence of healthy *C. giveni* larvae. Using the antibiotic markers, the occurrence of pADAP horizontal transfer between A1MO2 and other non-pathogenic *Serratia* spp. was observed at a low rate in challenged *C. giveni* larvae during *in vivo* assessments.

A key result obtained from *in vivo* assessments was that pre-inoculation of larvae with non-pathogenic strains, such as 5.6 or 477, led to a reduction in amber disease occurrence when challenged with A1MO2. Furthermore, mixed inoculation of larvae with both A1MO2 and 5.6, or A1MO2 and 477 led to a delay in amber disease onset compared to larvae challenged only with A1MO2. These findings were validated with a pot trial, where the pre-inoculation of soil with 5.6 or 477 led to a reduction in amber disease rates in larvae challenged with A1MO2, with amber disease reduced from 75 to 14 %. A pot trial was also conducted with AGR96X, a hyperpathogenic *S. proteamaculans* strain, where the pre-inoculation of soil with 5.6 and 477 reduced mortality rates in larvae challenged with AGR96X from 62 to 25 %. These results demonstrate that the presence of non-pathogenic *Serratia* spp. in the soil can detrimentally impact the efficacy of pathogenic *Serratia* strains such as A1MO2 and AGR96X, limiting their effectiveness against grass grub larvae. These results will have important implications in both the improvement of *Serratia*-based biopesticides and predicting the field efficacy of the applied *Serratia* strains.

Keywords: Microbial ecology, biological control, entomopathogen, pathogen and non-pathogen interactions, bacterial competition, pathogen efficacy

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List of Common Abbreviations

_k	Strain tagged with kanamycin
_s	Strain tagged with spectinomycin
_sk	Strain tagged with spectinomycin and kanamycin
_t	Strain tagged with tetracycline
_tk	Strain tagged with tetracycline and kanamycin
_gfp	Strain tagged with green fluorescence protein
_rfp	Strain tagged with red fluorescence protein
Amp^R	Ampicillin resistance
Cm^R	Chloramphenicol resistance
Kan^R	Kanamycin resistance
Sp^R	Spectinomycin resistance
Tc^R	Tetracycline resistance
a.u.	Arbitrary unit
ALA	Aminolaevulinic acid
BLAST	Basic local alignment search tool
BlastN	BLAST nucleotide query
BlastP	BLAST protein query
Bt	*Bacillus thuringiensis*
CFU	Colony forming units
COG	Cluster of orthologous groups
CTA	Caprylate thallous agar
DNA	Deoxyribonucleic acid
dpi	Days post-inoculation
g	Grams
HGT	Horizontal gene transfer
hpi	Hours post-inoculation
Kan	Kanamycin
kb	Kilobases
LB agar / broth	Luria Bertani agar / Luria Bertani liquid medium
RE	Restriction enzyme
mL	Millilitres
µL	Microlitres
OD	Optical density
pADAP	Amber disease associated plasmid
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	Whole transcriptome shotgun sequencing
rpm	Revolutions per minute
Spec	Spectinomycin
Tet	Tetracycline
tRNA	Transfer ribonucleic acid
WT	Wild-type
wpi	Weeks post-inoculation
x *g*	G-force / relative centrifugal force

Chapter 1

Introduction

1.1 *Costelytra giveni*: The New Zealand grass grub

The New Zealand grass grub, *Costelytra giveni* (formerly *Costelytra zealandica*) (Coleoptera: Scarabaeidae) (Coca-Abia and Romero-Samper, 2016), is a prevalent and persistent endemic pest species commonly found throughout Aotearoa New Zealand's grasslands and pastures. Grass grub larvae typically emerge late in the summer (late February to early March), where they feed on plants commonly found within pastures, such as grasses and clovers, causing damage to the plant roots and impacting the productivity of grazing pastures. If the grass grubs are left unchecked and the pastures remain untreated, populations will begin to increase steadily over a period of several years, with larger infestations potentially exceeding 400 grubs per m^2 of soil (Stucki et al., 1984). Pasture damage can be multi-faceted; the damage to the plant roots leads to an overall reduction in yield, which has an impact on meat and dairy farms that rely on the pastures to feed their livestock. The resultant damage can also allow for the invasion and establishment of weeds, which can either prevent or limit the rejuvenation of the pasture over time, whilst also requiring additional expenditure to remove unwanted plants and maintain a high-quality pasture. In total, the damage and losses in production associated with grass grubs can incur costs of between $225M to $585M per year ($140-380M p.a. for dairy, $75 - 205M p.a. for sheep & beef farms) (Ferguson et al., 2018). As the total estimated cost of damage caused by invertebrate pests within New Zealand pastures ranges between $1.7B to $2.3B p.a., measures are being taken to manage and prevent the occurrence of invertebrate pest infestations, including the use of traditional chemical-based pesticides, as well as the development of biological pesticides.

1.2 Biopesticides

Synthetic pesticides have long been used for the management of agricultural pests, however, due to the adverse effects associated with some synthetic pesticides, such as organophosphates and neonicotinoids, and resultant changes in regulatory legislation, their usage is declining (Chandler et al., 2011; Derbalah et al., 2019). This provides an opportunity and a need for research and development of biological-based pesticides to be used in their place. This can involve the use of insecticidal microorganisms or plant extracts active against multiple insect pest species. *Bacillus thuringiensis* (Bt) is an example of an insecticidal bacterium which has been successfully used in biopesticides against a variety of pest species (Roh et al., 2007). Bt produces crystal and cytolytic pore-forming toxins which

are activated following ingestion, resulting in the disruption of midgut cells and insect death (Palma et al., 2014). The use of biopesticides provides several advantages over synthetic pesticides. Biopesticides can be tailored towards a specific pest species, and they typically have greatly reduced non-target toxicity (Shao and Zhang, 2017). In addition, as biopesticides are made from naturally occurring organisms and/or their associated products they carry a reduced risk of the toxic contamination of both the environment and agricultural produce, in comparison with synthetic pesticides (Mitra et al., 2011; Carvalho, 2017). Furthermore, the development of biopesticides allows for a potential replacement for synthetic organophosphate pesticides, which either have, or are in the process of, being phased out.

Many synthetic pesticides have been used previously to treat grass grubs, such as diazinon, chlorpyrifos, terbufos, and phorate, and neonicotinoids such as clothianidin. Whilst these synthetic pesticides are sometimes effective at managing grass grub populations, their toxicity can lead to adverse effects. For instance, the use of diazinon has been linked to an increased risk for lung cancer and leukaemia (Beane Freeman et al., 2005) and was found to be genotoxic towards human nasal mucosal cells (Tisch et al., 2002). These findings, amongst others, resulted in diazinon no longer being approved for use in the European Union (European Commission, 2009). Similarly, neither terbufos nor phorate are approved for use in the European Union, with phorate also being banned in New Zealand. Whilst clothianidin was found to be effective at managing grass grub populations (Mansfield et al., 2017) low soil-binding capability and high levels of water solubility can lead to contamination of waterways via runoff (Miles et al., 2017). This contamination can also cause harm to aquatic life and was found to have degenerative effects in rainbow trout (*Oncorhynchus mykiss*) tissue cells (Dogan et al., 2021). These findings, amongst others, led to clothianidin being classified as toxic to aquatic life with long lasting effects, and as such its approval for use in the European Union was not extended past 2019, leading to it being phased out (European Commission, 2019). The phasing out of these synthetic pesticides through various initiatives (Pesticide Action Network, 2013; EPA 2021) is promoting the advancement of biopesticides as alternative tools for the management of agricultural pests.

1.3 Naturally occurring insect pathogens

Alongside bacterial species such as *B. thuringiensis*, there are various other entomopathogenic organisms that exist naturally within the soil and on the plants themselves, including other microorganisms such as fungal and protozoan species, and larger organisms such as nematodes (Glare et al., 1993a). Each of these species can fill specific roles or niches within the soil ecosystem, such as the promotion of plant growth, decomposition, nitrogen fixation, or they can assist in the management of the many invertebrate and vertebrate pest species that feed on and cause damage to plants. This

can be facilitated in a variety of ways, such as by a microbial induced systemic resistance response in the plant, triggered by symbiotic rhizobacterial species which can ward off certain insect pest species via the production of toxins within plant tissues upon activation of a phytohormone signal (Van Oosten et al., 2008). For example, the enhancement of an induced response utilising jasmonic acid in the plant species *Arabidopsis* is linked to the presence of the bacterium *Pseudomonas fluorescens* strain WCS417 near the roots, leading to reduced predation from the beet armyworm, a generalist lepidopteran insect species (*Spodoptera exigua*) (Chung et al., 2008).

Viruses can also have insecticidal effects towards herbivorous pest species. Baculoviridae, a group of large DNA viruses (80 - 180 kbp) have been used as biological control agents, although they have very narrow host ranges, primarily infecting caterpillars, with one virus (*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)) being able to infect up to 30 species from several genera of Lepidoptera (Clem and Passarelli, 2013). These viral particles are typically taken up by insects feeding on infected leaves after which enveloped virions are released into the intestines of the host, allowing the infection to spread between host cells. These viral infections usually manifest as alterations to host phenotype and behaviour, including the inhibition of larval development. In the instance of the lepidopteran gypsy moth (*Lymantria dispar*) larvae, an infection by a baculovirus has been shown to induce larval climbing behaviour (Hoover et al., 2011). Typically, these caterpillars live in the soil and only emerge at night to avoid predation, but infected larvae were found to climb to high branches before dying and liquefying, allowing for the 'raining down' of viral particles on to the surrounding vegetation.

Another avenue for biological control is through the use of nematodes, invertebrate species found in a wide range of ecological niches that can be insecticidal to a vast array of pest species. Nematodes can be found as parasitic organisms free-living in the soil, where they can act either as help or as a hinderance towards plant species; beneficial nematodes are termed as predatory as they can prey upon common invertebrate pest species. Some nematode species, such as *Neoaplectana glaseri* have previously shown efficacy against grass grubs (Kain et al., 1982). In the main genera exploited for biological control, nematodes can exert insecticidal actions through symbiotic bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus* (for *Steinernema* and *Heterorhabditis* nematodes, respectively) (Askary, 2009). When in the soil, juvenile nematodes infected with these bacteria will be taken up by insect species, either through ingestion from the soil or penetration, where the nematode will travel to the host haemocoel and expel the bacterial cells from its own intestines. The bacteria will then feed on the nutrient-rich haemolymph, multiplying rapidly and ultimately causing the death of the host within 48 hours (Sahayaraj and Atwa, 2014). The nematode must also feed on the host to reach its adult stage, after which it reproduces to create more juvenile nematodes, which are in turn infected by the symbiotic bacteria, before beginning the cycle anew. Due to their resistance to

environmental stresses, ease of mass production, and the wide host range of *Steinernema* and *Heterorhabditis* nematodes, they have been developed into biopesticide products where they are often applied in tandem with other biocontrol methods (Lacey and Georgis, 2012).

B. thuringiensis is not the only member of the *Bacillus* genus to have been used in the development of biopesticides for insect control. Some strains of *B. subtilis* are pathogenic towards various species of mosquitos, using lipopeptides to exert their insecticidal activity (Assié et al., 2002). Additionally, *B. subtilis* biosurfactants were also found to be effective in controlling populations of olive moths (*Prays oleae*) and the Egyptian cotton leafworm (*Spodoptera littoralis*), indicating a broad host range (Ghribi et al., 2012). *B. sphaericus* is also active against a wide range of insect species, producing toxins specific towards certain insect species, such as the binary toxins A and B, and Mtx1 and Mtx2 toxins, all of which are specific towards mosquito larvae (Rungrod et al., 2008). Insecticidal activity against larvae can also be found amongst members of the genus *Pseudomonas*, acting via the production of exotoxins and rhamnolipids to manage populations of the yellow fever mosquito (*Aedes aegypti*) by *P. fluorescens* and *P. aeruginosa* (Silva et al., 2015).

Insecticidal activity is also exhibited by some members of the bacterial genus *Serratia*, with *S. marcescens* able to establish disease within apple maggot flies (*Rhagoletis pomonella*) via the use of toxic compounds (Lauzon et al., 2003). However, the use of this strain as a biopesticide is not possible due to the pathogenicity of some strains towards vertebrate species, in particular as an opportunistic pathogen of humans (Jones et al., 2000). Other *Serratia* species, such as *S. entomophila* and *S. proteamaculans*, have been shown to be effective in controlling populations of *C. giveni* larvae through the plasmid-mediated production of toxic compounds which result in a chronic non-feeding disease (described later in Section 1.5). However, many more naturally occurring entomopathogens of *C. giveni* exist, and have been previously used to manage *C. giveni* larvae, including various viral, nematode, fungal, and bacterial species (Glare et al., 1993a). Despite having a wide pool of candidates to draw from when selecting organisms that could act as potential biopesticides of grass grubs, many of them have their own disadvantages that prevent or limit their usage. For example, some of the species listed by Glare at al. (1993a), such as *Bacillus (Paenibacillus) popilliae* and some *Rickettsia* species were unable to induce the high levels of disease within the larvae necessary to significantly reduce population size (Moore et al., 1974; Jackson, 1990).

1.4 The *Serratia* genus

The members of the *Serratia* genus (Yersiniaceae) (Adeolu et al., 2016) are Gram-negative, non-spore forming, facultative anaerobic bacteria. There are currently 19 recognised and published species within the genus as of 2020: *S. aquatilis*, *S. entomophila*, *S. ficaria*, *S. fonticola*, *S. grimesii*, *S. liquefaciens*, *S. marcescens*, *S. microhaemolytica*, *S. myotis*, *S. nematodiphilia*, *S. ororiferae*, *S. oryzae*, *S. plymuthica*, *S. proteamaculans*, *S. quinivorans corrig*, *S. rubidaea*, *S. symbiotica*, *S. urelytica*, and *S. vespertilionis*. *Serratia* spp. are found in a diverse range of environments and ecological niches across the globe, including being present in soil, water, and on plant surfaces (Grimont and Grimont, 2015). In addition, they can also be found within various host organisms, such as insects and humans (Petersen and Tisa, 2013). Their prevalence in this wide variety of habitats has been attributed to the secretion of various secondary metabolites with antimicrobial and proapoptotic properties, such as prodigiosin (produced by *S. marcescens*) (Darshan and Manonmani, 2015), and sodorifen (produced by *S. plymuthica*) (Schmidt et al., 2017), allowing for their survival when competing for resources within a polymicrobial environment. *Serratia* species generally act as opportunistic pathogens towards their hosts, and approximately one to two percent of nosocomial human infections have been associated with *S. marcescens* in immunocompromised and intensive care patients (Khanna, 2013).

1.5 The use of *Serratia* as a biopesticide for grass grubs

The genus *Serratia* was first noted as containing pathogens of grass grub larvae in 1981 (Trought et al., 1982), when bacteria initially classified as *Hafnia alvei* were isolated from diseased 2nd and 3rd instar grass grub larvae in the South Canterbury region of New Zealand. These larvae showed signs of feeding cessation, with the gut sections of the larvae being described as “clear of ingested soil and organic matter, constricted, and translucent-honey coloured” (Trought et al., 1982), leading to disease initially being named as honey disease. The bacterium *H. alvei* was reclassified as a member of the *Serratia* genus several years later following subsequent analysis of the diseased larval cadavers. Two strains were isolated as causative agents of the disease, and were named *S. marinorubra* and *S. liquefaciens*, and later reclassified as *S. entomophila* and *S. proteamaculans*, respectively (Grimont et al., 1988), and the disease associated with these characteristic symptoms became referred to as amber disease (Tan et al., 2006). During early field trials these bacteria were found to be effective at reducing grass grub population densities by up to 59 % over a three-month period within the soil, with the highest rates of disease occurring one month after application of the bacteria (Jackson et al., 1986). These results, alongside the ease of culturing of the strains within a laboratory highlighted the potential for their use as an effective biological control method and an alternative to synthetic pesticides for control of grass grubs. Another advantage for the development of *S. entomophila* as a biopesticide is that it has a high

degree of specificity towards grass grub larvae. This host specificity is a likely result of a co-evolutionary relationship, initiated 85 million years ago following the separation of New Zealand from Gondwana (Jackson, 2007). As a result of this close relationship, *S. entomophila* has not been found to cause disease in any other species, both vertebrate and invertebrate, indicating that there is a very low risk of non-target toxicity for other species present in a pasture (Jackson, 2003).

Following field trials (Jackson et al., 1992), *S. entomophila* was used as a basis for the commercial development of a biopesticide for grass grub larvae. The product was released in 1992 under the name Invade®, where it was applied to pastures as a liquid drench. However, there were several limitations with this product which restricted its usage. The product had to be applied using large volumes of water (up to 100 litres per hectare) with modified seed drills to ensure that the bacteria were placed beneath the surface of the soil, and the product also required refrigerated storage at 4 °C during storage and transport to prevent cell death (Johnson et al., 2001). As a result of this, other formulations of biopesticides using *S. entomophila* have been developed, such as the granule-based Bioshield™. This product is effective at treating grass grub populations of up to 500 grubs per m^2 of soil (Jackson et al., 2017), and field trials with an early prototype found a 20% increase in the number of diseased larvae when compared to control sites (Townsend et al., 2004). These results, together with the prolonged stability of the bacteria in a granular form under a variety of storage conditions, allowed for an effective method of treatment for grass grub populations (Fig. 1.1). Another comparative study assessed the effectiveness of Bioshield against the synthetic pesticide diazinon (Zydenbos et al., 2016). Whilst both treatment methods were found to be effective at reducing grass grub populations within the soil over a two-year period, populations treated with diazinon began to increase during the third year, whereas the areas treated with Bioshield maintained low numbers of healthy larvae, indicating the persistence of the applied *S. entomophila* in the soil over the three-year time period of the trial.

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Figure 1.1. Amber disease dynamics following application of Bioshield^{TM}; bacteria were applied to feeding larvae in February. Disease levels rose to approximately 25 % during the first year, with a level of 10^3 to 10^4 pathogenic bacteria per gram of soil. Whilst remaining healthy larvae pupated and laid eggs, the second generation of grass grub in year 2 encounters the pathogenic bacteria in soil and drops to a low population level whilst suffering from a higher level of disease. Reproduced from Jackson et al. (2017).

More recently, a highly virulent *S. proteamaculans* strain, AGR96X, was isolated from diseased grass grub larvae (Hurst et al., 2018). This strain killed larvae within five to 10 days (compared to three to four months for *S. entomophila*) and was also found to be effective against the manuka beetle larvae (*Pyronota* spp.; Coleoptera: Scarabaeidae), an emerging invertebrate pest species in New Zealand. As a result of this rapid onset of mortality following ingestion, and the capability for targeting more than one invertebrate pest species, AGR96X presents itself as a viable fast-acting alternative to *S. entomophila* based biopesticides.

1.6 The mechanisms of action for amber disease-causing *Serratia* spp.

The characteristic symptoms associated with grass grub larvae infected with amber disease are the cessation of feeding and the clearance of material from the gut which changes from a regular dark grey/brown colour seen in healthy larvae to an amber colour, visualised in Figure 1.2.

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Figure 1.2. A comparison between a healthy grass grub larva (left) and a larva infected with amber disease (right). Due to the clearance of the dark gut material from the larval midgut the infected grub takes on an amber colouration. From Popay (2008).

The initiation of amber disease occurs following ingestion of pathogenic *S. entomophila* or *S. proteamaculans*, where the symptoms, such as feeding cessation and gut clearance appear after approximately three days (Jackson et al., 1993). The genes responsible for encoding the virulence determinants required for the causation of amber disease and its associated symptoms are located in two separate clusters on the *Serratia* amber disease associated plasmid (pADAP), a 153 kbp single-copy mega-plasmid which is carried by all pathogenic strains of *S. entomophila* and *S. proteamaculans* (Glare et al., 1993b, Hurst et al., 2011). These key determinants are the anti-feeding prophage (Hurst et al., 2004) and the *Serratia entomophila* pathogenicity toxin complex (sepTc) (Hurst et al., 2000) (Fig. 1.3). The 17 kbp *sep*Tc gene cluster region of pADAP is comprised of three sequential genes (*sep*A, *sep*B, and *sep*C, respectively) which work together to produce insecticidal proteins which facilitate the expulsion of gut material, leading to the development of the characteristic amber discolouration within the infected larvae (Hurst et al., 2000; Hurst et al., 2007). Further characterisation of the *sep*Tc region revealed similarities to the toxin cluster genes of other entomopathogenic bacteria, such as those seen in the chromosomes of *Photorhabdus luminescens* and *Xenorhabdus nematophilus*, which also use multiple subunit protein complexes to kill their hosts (Bowen et al., 1998; Hurst et al., 2000). The similarity between these toxin complexes and those found in the pathogenic *Serratia* species has led to the speculation that these virulence regions are mobile and can move between species via plasmid transfer (Dodd et al., 2006). In addition, the insertion of transposons into each of the individual *sep* genes was found to prevent the onset of amber disease by inhibiting both gut clearance and reducing feeding activity, indicating that all three of the subunits are essential in the causation of the disease (Hurst et al., 2000).

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Figure 1.3. A colour coded sequence annotation of the *Serratia* amber disease associated plasmid. The regions encoding the anti-feeding prophage (Afp) and *Serratia entomophila* pathogenicity toxin complex (SepTc) regions are highlighted in red and orange (respectively). Reproduced from Hurst et al., 2011.

The ~ 30 kbp anti-feeding prophage (Afp) gene cluster encodes for a R-type pyocin phage-like structure encoded by 18 open reading frames (ORFs), the translated product of which forms a virus-like structure, which facilitates the delivery of toxins used to cause the cessation of feeding within infected larvae (Desfosses et al., 2019). There are noted similarities between the structure and function of the *Serratia* Afp particle and of toxin-delivery particles found in *Photorhabdus* species (*Photorhabdus* virulence cassettes) and other bacterial type IV secretion systems (Hurst et al., 2004; Heymann et al., 2013). A unique variant of Afp, termed AfpX, was identified in *S. proteamaculans* strain AGR96X, and was found to confer a higher level of virulence towards the grass grub and manuka beetle larvae (Hurst et al., 2018). This 'hypervirulent' phenotype is thought to result from the over-production of AfpX following ingestion by the grass grub larvae and the ability of AGR96X to breach the insect's intestine.

S. entomophila-mediated amber disease can also interfere with the actions of digestive endopeptidases such as trypsin, chymotrypsin, and elastase, which are used to break down proteins. Following infection with a pathogenic *S. entomophila* strain, a significant reduction in proteolytic activity was observed when compared to healthy larvae following the cessation of feeding (Jackson et al., 2004), signifying a potential link between the onset of amber disease and digestive enzyme synthesis.

pADAP is carried by all pathogenic *S. entomophila* and S. *proteamaculans* strains; the presence of the plasmid carrying both virulence determinants are prerequisites for the initiation of disease, and the loss of the plasmid results in a loss of virulence (Glare et al., 1993b). The loss of pADAP was demonstrated through heat-curing by Glare et al. (1993b) using agar plates inoculated with *S. entomophila* strain A1MO2 and maintained at 40 to 42 °C for seven days. At this time the absence of pADAP was determined with a plasmid visualisation method and a bioassay with *C. giveni* larvae, with seven out of 74 recovered isolates showing both a loss of pADAP and a loss of virulence. Alongside this, many plasmid-free *Serratia* strains have been isolated from soil samples across New Zealand, indicating that either some strains never contained the plasmid or the potential natural loss of pADAP. Between 40 to 60 % of *Serratia* strains free-living within the soil are plasmid-free (Jackson et al., 1991). It has also been shown that the direct injection of some *Serratia* species into the larval haemocoel resulted in the onset of disease distinct from amber disease, indicating the role of a general toxin which causes disease independent of pADAP (Tan et al., 2006). In addition to the lack of, or natural loss of, the plasmid, it is also possible for pADAP, and the ability to cause amber disease, to be transferred between some *Serratia* species through horizontal gene transfer (HGT). This has been previously demonstrated by Glare et al., in 1996, who used an *in vitro* liquid culture method to follow the movement of pADAP between pathogenic and non-pathogenic *S. entomophila* and *S. proteamaculans* strains, alongside other species (Glare et al., 1996).

1.7 The role of plasmids in disease

Plasmids play an important role in other entomopathogenic bacterial species, and often encode for various types of toxins and virulence determinants required to establish infections within their host species. In addition, plasmids also encode for genes which can affect the general functionality of the cell, for instance, a loss of six out of seven plasmids in *B. thuringiensis* strain H14 during fermentation resulted in detrimental changes to morphology and aggregation ability when compared to a mother strain containing all seven plasmids (Sarrafzadeh et al., 2007). Further traits conferred by plasmids upon their host cell to enhance cell survivability are depicted in Figure 1.4. Additionally, the diversity of plasmid sizes, ranging from as little as 300 bp up to 2,400 kbp, show how they may have evolved over time to meet the needs of the individual host bacterium, with the number of plasmids and their copy numbers also varying between strains, though plasmids are also thought to be parasitic in nature. An example of plasmid-mediated enhanced survival is through the acquisition of antibiotic resistance genes. This has been noted previously in the bacterium *Paenibacillus larvae*, which causes American Foulbrood, a disease of honeybees. The acquisition of a novel plasmid, named pMA67, containing a gene encoding for tetracycline resistance (*tetL*), allows *P. larvae* to cause disease despite the application of oxytetracycline (Murray and Aronstein, 2006).

Figure 1.4. Schematic of four fundamental classes of plasmid traits conferred on bacteria: resistance, energy metabolism, virulence & pathogenicity, and dissemination & perpetuation. Subclasses are also displayed beneath each of the classes. Reproduced from Kado (1998).

Due to its long-term use as a biopesticide, the plasmids of *B. thuringiensis* are well characterised, with some of the genes encoding for the production of toxic crystal proteins (Cry) isolated from a 75 kb plasmid in the early 1980s (Adang et al., 1985). These toxins have a broad insecticidal range, able to kill insect species within the orders of Lepidoptera, Diptera, Coleoptera, and Hymenoptera (de Maagd et al., 2001), and also a small number of non-insect species such as nematodes (Marroquin et al., 2000). One of the key Cry toxins produced by *B. thuringiensis* is encoded by the gene *cry1Ba*, contained in the thuringiensin synthesis cluster on the 110 kb endogenous plasmid pBMB0558 (Liu et al., 2010). This toxin is notable as it is active against larvae of multiple orders, encompassing Lepidoptera, Diptera, and Coleoptera (Zhong et al., 2000). Also present on this plasmid were genes encoding for a type IV secretion system and other mobile genetic elements (Liu et al., 2010).

Genes encoded on the plasmid may also be detrimental to the fitness of the bacterium. A comparison between *B. thuringiensis* strains bearing plasmids against plasmid-cured strains, showed that the strains which still contained the plasmids, or contained multiple plasmids, were more sensitive to ultra-violet (UV) light (Benoit et al., 1990). Furthermore, the conjugation of *B. thuringiensis* plasmids into *B. cereus* resulted in a corresponding increase in UV sensitivity. Conversely, the loss of plasmids may also be beneficial to cell survivability; the loss of plasmids encoding for *cry1Aa*, *cry2Aa*, and *cry2Ab* in *B. thuringiensis* subsp. *kurstaki* resulted in the production of melanin, which increased the resistance of the strain to UV light (Saxena et al., 2002). Alongside this, this UV-mutated melanin-producing strain also had higher toxicity towards the lepidopteran cotton bollworm (*Helicoverpa armigera*) than the parent strain, with Cry1Ac being the only polypeptide toxin produced by the strain.

1.8 The metabolic burden of plasmid carriage

Whilst plasmids confer bacteria with the ability to cause disease and resist antibiotics and heavy metals (Maclean and San Millan, 2015), the carriage of plasmids has often been linked to a marked reduction in fitness for the bacterium (San Millan et al., 2018). Furthermore, the acquisition of plasmids can also lead to alterations in chromosomal gene expression. This was demonstrated with *P. aeruginosa* strain PAO1 by San Millan et al. (2018), where conjugation with pABML1 and pAMBL2 preferentially altered the expression of chromosomal metabolic genes. The conjugation of additional plasmids and subsequent transcriptomics with wild-type *P. aeruginosa* strains established a link between plasmid conjugation in PAO1 and an overexpression of genes linked to metabolism, leading to significant alterations in metabolic profiles of the bacterium. Further reviews of the metabolic burdens associated with plasmid carriage show that the fitness costs are relative to the disruption caused by plasmid acquisition, with the costs varying depending on the genes found at the plasmid insertion site and the resultant expression of genes by the plasmid (Baltrus, 2013). Due to these associated costs, it is

expected that plasmids which do not confer a selective advantage are likely to go extinct, as plasmid-free strains can often outcompete plasmid-carrying strains (De Gelder et al., 2007). This is referred to as the 'plasmid paradox' (Harrison and Brockhurst, 2012), as the resultant fitness costs of the plasmid, and some of the unnecessary genes encoded, would actively select against plasmid presence. However, if this is not the case, it would lead to the plasmid becoming a parasite.

Despite this, plasmids can be maintained even when their carriage incurs a metabolic cost. For instance, pQBR103 in *P. fluorescens* strain SBW25 contains a mercury resistance operon encoded on a mega-plasmid (pQBR103) which allows for the bacterium to survive in mercury-contaminated environments (Lilley and Bailey, 1997; Trett et al., 2007). However, when the bacterium grows in the absence of mercury, the genes expressed by pQBR103 incur a large fitness cost, and so plasmid extinction could be expected in these cases. Compensatory evolution by the strain was found by Harrison et al. (2015) to ameliorate these costs, with evolution often targeting the *gacA*/*gacS* regulatory system whilst leaving the plasmid intact. Both the mutation and deletion of this regulatory system was found to ease the burdens for carriage of pQBR103, providing a way through which compensatory evolution can allow the maintenance of metabolically costly plasmids even when they may not be immediately beneficial to the cell.

1.9 The social ecology of bacteria

It is estimated that between 40 to 60 % of *S. entomophila* and *S. proteamaculans* strains free-living within the soil do not carry pADAP, and thus are non-pathogenic (Jackson et al., 1991). When pADAP-free variant strains were ingested by grass grub larvae they were readily excreted from the larval gut, as they lacked the ability to proliferate within the larvae (Jackson et al., 1993). However, whilst pADAP-free strains were unable to proliferate within the larvae, they were found to persist within larvae that were already infected with pathogenic *Serratia* strains. Where both pathogenic and non-pathogenic *Serratia* strains are present together, such as by co-existing in diseased larvae, it is not yet clear whether the strains compete, and what effect, if any, their co-existence within the larvae may have on the progress and outcome of the disease.

It is possible that both strains may co-exist within the diseased host and share the available resources and nutrients in order to fuel continued cell replication before their eventual release from the larval cadaver into the soil. It is also possible that the opposite may occur, resulting in a scenario where the non-pathogenic strains utilise the majority of available nutrients, effectively 'outcompeting' the pathogenic strain. In this instance, competition may detrimentally impact the replication rates of the pathogenic strain, hindering the progression of amber disease or preventing it entirely if both strains

are ingested before the disease process can be initiated and established. This was explored with a model of competition between pathogenic and non-pathogenic *Serratia* spp. in the field by Godfray et al. (1999). This model describes how the host threshold density in grass grub larvae, the minimum number of larvae required to maintain disease within the population, is reduced when only the pathogenic *Serratia* species are present due to a lack of competition for resources with non-pathogenic strains. In order to gain a greater understanding of how competition between pathogenic and non-pathogenic *Serratia* strains in the soil and larvae may influence the onset and outcomes of amber disease, comparisons can be made to other well-documented bacterial species, such as *B. thuringiensis*. This bacterium serves as a useful comparison because it has been successfully used in biopesticides against numerous pest species (Rosas-Garcia, 2009). Furthermore, like *Serratia*, *B. thuringiensis* often utilises plasmid-encoded virulence determinants to elicit insecticidal effects.

The main mode of action for *B. thuringiensis* is its insecticidal pore-forming crystal (Cry) toxins, which are δ-endotoxin proteins produced during sporulation and are effective against the larval stages of various insect pest species (Raymond et al., 2007). Ingestion of Cry toxins causes a disruption of the larval midgut tissues via the creation of pores following the solubilisation of the Cry proteins, resulting in eventual death via septicaemia caused by other gut bacteria (Broderick et al., 2009). The mortality may be due to, or enhanced by, additional effects exerted by other opportunistic pathogens that may also be present in the larval midgut after being ingested from the soil. This has been seen during other studies which have found that co-infection with pathogens with differing replication rates and competition between different bacterial species for nutrients within the host can be positively linked to increased virulence (Garbutt et al., 2011). Furthermore, it is also possible that increased virulence from mixed infections is only one potential outcome, and there are many more variables which may influence virulence both positively and negatively.

The production of *B. thuringiensis* Cry toxins is an effective way of assessing cooperation and the effects of competition during an infection. This is due to the high metabolic cost associated with producing the toxins and their secretion into the larval gut leading to it being considered as a 'public good' (Raymond et al., 2010). It is termed as a public good because the pore-forming effects of Cry can be used by any proximal microbial species, both toxin-producing and non-toxin-producing, allowing them to obtain nutrients from the host in order to fuel replication (Raymond et al., 2007). As such, *B. thuringiensis* is considered as a 'cooperative' bacterium because, whilst the toxin is costly to produce, it can be used to benefit other microbial species in addition to itself. A detrimental side-effect of this altruistic behaviour is that it can be exploited by avirulent bacteria, which are able to take advantage of the Cry toxins effects to obtain nutrients without contributing anything in return; these avirulent bacteria are termed 'cheats' (Sandoz et al., 2007; Diggle et al., 2007; Raymond et al., 2012).

The interactions between co-operators and cheats can often be predicted and interpreted using the concepts of "game theory" and "the tragedy of the commons" (Hardin, 1968). The use of game theory can provide a mathematical framework which can be applied to the interactions between two or more individuals, with the benefits obtained from interactions changing depending on the behaviour of the individuals. A simple example matrix based on this concept is displayed in Table 1.1. In relation to biological interactions, it can be assumed that cooperative behaviour which benefits the entire microbial population, such as the production of Cry toxins by *B. thuringiensis*, is disadvantageous to *B. thuringiensis* when compared to the selfish behaviour associated with cheat bacteria.

Table 1.1. A pay-off matrix of the outcomes of competition between cooperative and cheat species of bacteria, with the outcome changing depending on the strategy of the bacteria. *C*: cooperates, *A*: advantage, *D*: disadvantage, *P*: punished (A > C > P > D). Adapted from Lambert et al. (2014).

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Evidence has shown that it is possible for non-pathogenic species of bacteria to impede the onset of disease by pathogenic bacterial species within the same genus. The non-pathogenic *Rickettsia* species *R. peacockii* has been previously isolated from Rocky Mountain Wood ticks (*Dermacentor andersoni*) and was isolated from ticks on the eastern side of the Bitterroot Valley (Montana, United States of America). It was found that the ticks already carrying the non-pathogenic *R. peacockii* were prevalent on the eastern side of the valley, whilst the majority of cases of Rocky Mountain spotted fever, caused by *R. rickettsii*, were occurring on the western side of the valley (Niebylski et al., 1997; Uchiyama, 2012). The authors assumed that the presence of *R. peacockii* in the tick limited transovarial transmission of *R. rickettsia* from occurring either by conferring a reproductive advantage, as *R. rickettsia* is lethal towards the tick, or by limiting the spread of the disease to these ticks pre-exposed to the non-pathogenic strain.

The effects of competition can also be seen in other systems, such as with viruses (Dennehy and Turner, 2004). Mier et al. (2020) found the absence of the functional replicase protein Δ1764 in the MS2 virus led to the generation of cheat viruses which were unable to replicate in a cell in the absence of a wild-type genome virus. However, these cheat viruses displayed an increased fitness over wild-type viral particles, replicating at a faster rate. Despite this increased fitness, an equilibrium with the wild-type strain is eventually reached as the mutant cannot survive separately from the wild-type MS2 virus. However, the emergence of multiple cheat species led to competition between cheats, where one of the cheats would eventually disappear from the population. A similar effect was noted in other

RNA viruses through the continued passage of the vesicular stomatitis virus, where the growth of viruses of equal fitness under competition resulted in continuous fitness increases for the clones as they continually evolved. However, eventually one clonal genotype suddenly outcompeted the other, preventing the growth of other strains (Solé et al., 1999). This has been observed with the foot-and-mouth virus, where continued passage led to the clonal population diversifying either into competitors or colonisers. Whilst the competitors had an intracellular competitive advantage, the mutant coloniser genotype displayed a higher fitness and increased capability to cause cell death (Ojosnegros et al., 2010). However, coinfection with both resulted in reduced cell death and the competitor outcompeting the coloniser, with selection being density dependent.

The concept of cheat bacteria has been explored using *Escherichia coli* in competition experiments with wild-type and mutant *E. coli* strains able to maintain a growth advantage during stationary phase, outcompeting the wild-type *E. coli* strains under starvation conditions in media (Lambert et al., 2014). A pay-off matrix can be used to describe the predicted results of competition and cheating behaviour (Table 1.1). Using these predictions, the optimal strategy in co-inoculation conditions was found to be the defection of a cooperative bacterium to a cheat, as this leads to the greatest increase in fitness where the mutants can survive in conditions of reduced nutrients and elevated stress (Vulić and Kolter, 2002). However, if too many cheat bacteria arise, it can lead to a situation described as the “tragedy of the commons”.

The tragedy of the commons was first defined as a scenario where an individual existing in a shared resource environment acts solely in its own self-interest in an uncooperative manner, thereby leading to the rapid depletion of the shared resources to the detriment of other species that were acting cooperatively (Hardin, 1968; de Roode et al., 2005). This concept is depicted in Figure 1.5. It is also possible to observe the effects of this concept within bacterial interactions. Quorum sensing is a method of cellular communication that is used by bacteria such as *Pseudomonas aeruginosa* to regulate the production of public good materials, such as extracellular proteases and growth substrates, in relation to population density (Dandekar et al., 2012). It is possible for mutant *P. aeruginosa* cheat strains to arise which can use these public goods without incurring the cost of producing them. If enough of the population defects to this cheating behaviour, there will not be enough public goods avaliable to maintain the population, and it will collapse.

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Figure 1.5. A representation of the progression of the tragedy of the commons, where the fitness benefits associated with selfish cheat behaviour leads to the incentivised defection of co-operators to cheats. As the density of cheats (-) increases the remaining co-operators (+) are no longer able to produce sufficient quantities of public goods used for bacterial maintenance, leading to population collapse. Adapted from Rankin et al. (2007).

Another factor which may lead to increased incentive for cheat behaviour, the metabolic costs associated with the maintenance and function of apparatus required to initiate and maintain plasmid-borne infections, is usually found on plasmids possessed by co-operative bacteria. When a plasmid is acquired by the bacterium by horizontal transfer it can confer a variety of beneficial effects facilitating bacterial evolution, such as providing resistance to certain antibiotics, heavy metal tolerance, or the ability to cause disease (Carroll and Wong, 2018). However, despite these beneficial effects there is an expected initial 'up-front' cost for plasmid acquisition which may have an adverse impact on the fitness of the bacterial host, with these fitness costs becoming more apparent when nutrients and resources are restricted or diminished (Platt et al., 2011). These costs often stem from the replication of genes found on the plasmid, which diverts the resources set aside for host cell processes, leading to potential periods of cellular stress (Carroll and Wong, 2018). In this way, it would be advantageous for the bacterium to shed the plasmid and take advantage of the effects when they are produced by other bacterial species, such as non-pathogenic bacterial species taking advantage of the *Bacillus thuringiensis* extracellular Cry toxins.

Following a process of natural selection, it could be assumed that if the acquisition of a plasmid was to have a detrimental effect on host survival, then it would be lost via selection with any beneficial genes being integrated into the host chromosome. This has been demonstrated in minimal media cultures using *E. coli* cells with pBluescript-derived plasmids of varying sizes, with findings indicating a reduced level of fitness in strains containing larger plasmids, and the rate of plasmid loss also correlating positively with plasmid size (Smith and Bidochka, 1998). However, it is possible for these costs to be overcome through methods such as compensatory and adaptive evolution, and through cooperative communal actions (Harrison and Brockhurst, 2012). Horizontal transfer of plasmids is one such method through which cooperation can be achieved; this can occur by forcing cheat bacteria to contribute to the production of public goods via the overexpression and subsequent horizontal transfer of the genes required to produce virulence factors (Smith, 2001).

In addition to the occurrence of horizontal plasmid transfer between bacteria in a growth medium, it can also occur naturally in the environment in both the soil and within host species, such as insects. There are several factors that can influence the frequency of horizontal plasmid transfer within the soil, including the content and movement of moisture within the soil, as well the pH and soil type (van Elsas et al., 2003). These factors can increase the probability of HGT occurring by increasing the metabolic activity of bacteria within the soil, notably in the rhizosphere due to the abundance of various bacterial species and root exudates, leading to increased cell densities within the soil. Additionally, biological factors, such as the presence of plant species or decomposing organic matter in the soil (both animal and plant) can increase the likelihood of HGT rates due to the release of nutrients favouring bacterial growth. The mixing of bacteria within the gut of soil-inhabiting animals also provides a favourable environment for HGT to occur (Sørensen et al., 2005).

For efficient transmission of plasmids between cells to occur within the soil, either from an introduced donor strain or from strains already existing within the soil, cell-to-cell contact must be established and maintained. This may be hampered by spatial distribution, where the donor bacteria are separated by barriers such as soil, which prevents plasmid transfer from occurring efficiently (Dechesne et al., 2005), and limitations on cell-to-cell contact inherent in different bacterial strains. In addition to spatial separation, previous work by Dodd (2003) with *Serratia* spp. found that temporal separation may also prevent plasmid transfer from occurring. In natural soil samples where a donor strain was added following the recipient, a time difference of 24 hours between application led to no instances of plasmid transfer occurring when compared to a gap of zero and three hours between the addition of the donor and recipient strains. It is also possible for some broad-range plasmids to be transferred between a wide variety of soil-borne bacterial species. This was demonstrated by Klümper et. al. (2014) with the prevalence of IncP-1 and PromA type plasmids and their transfer in the soil from three donor strains to recipient bacteria strains from 11 different phyla. Some of the genes encoded on these plasmids related to ecological fitness and enhancing plasmid transfer in soil (Zhang et al., 2014). With regards to *Serratia*, using a modified pADAP Grkovic et al. (1995) demonstrated that the plasmid could be conjugated to several different genera of soil bacteria, including *S. marcescens*, *Enterobacter agglomerans*, and *Klebsiella* spp.

Plasmid transfer has also been reported to occur within insect hosts, occurring in sites such as the gastrointestinal tract. In one example, the transfer of antimicrobial resistance plasmids between two bacterial strains associated with food-borne illnesses (*Salmonella enterica* and *E. coli*) was measured inside the gut of the lesser mealworm beetle (*Alphitobius diaperinus*) as a means of identifying a potential reservoir for new multidrug-resistant bacterial strains (Poole and Crippen, 2009). The horizontal transfer of antimicrobial resistance genes occurred in the guts of beetles simultaneously exposed to the *E. coli* donor and *S. enterica* recipient. Additional studies corroborated these findings

(Kado, 2009), with the horizontal transfer of antimicrobial resistance plasmids occurring between *B. thuringiensis* subspecies *kurstaki* and *tenebrionis* (donor and recipient, respectively) within lepidopteran larvae (*Lancanobia oleracea*) (Thomas et al., 2000). In conjunction with the transfer of antimicrobial resistance, the transfer of plasmids containing genes related to toxicity has also been shown to take place within the gut of insects. House flies (*Musca domestica*) were used to demonstrate the horizontal and phage transduction-mediated transfer of both antimicrobial resistance genes and the bacteriophage-borne Shiga toxin gene *stx1* from donor *E. coli* strains to recipient *E. coli* strains lacking these genes (Petridis et al., 2006). These studies indicate that it is possible for plasmid transfer to occur within the larval gut, with plasmid acquisition potentially increasing bacterial fitness (Russell and Moran, 2005).

The horizontal transfer of pADAP within soil and the larval gut has been studied previously by Dodd (2003) using a variety of *Serratia* spp. to characterise the limitations of plasmid transfer between members of the *Serratia* genus. This study found that the intra- and inter-species transfer of pADAP occurred in both sterile and non-sterile soil samples, and *in vivo* within the larvae, with the larval gut being the most conducive environment for plasmid transfer. Additionally, it was found that plasmid transfer only occurred in non-sterile soil samples when the larvae were present, although the onset of disease was not required for HGT to occur. It was also found that pADAP was transferred from both *S. entomophila* and *S. proteamaculans* into *S. marcescens*, *S. ficaria*, and *S. liquefaciens*, with all transconjugant strains able to cause amber disease. However, Dodd (2003) used *Serratia* strains selected for resistance to nalidixic acid, which can result in mutations in important enzymes such as DNA gyrase and DNA topoisomerase IV (Hooper and Jacoby, 2015), enzymes important to the replication of DNA, confounded by spontaneous nalidixic acid mutants. To avoid this issue, an alternative approach was used in this study; antibiotic markers were inserted into a specific intergenic region on the chromosome between two open reading frames, and into an intergenic section on pADAP. This will prevent the insertion of the markers from negatively impacting the fitness of the cell.

1.10 Research relevance

The aim of this project is to characterise and define the interactions of pathogenic and non-pathogenic *Serratia* spp. through culturing of strains in a variety of environments, including *in vitro* in nutrient-rich growth media and in soil samples, as well as *in vivo* within the larval gut. The work is focussed primarily on the interactions between plasmid-bearing and plasmid-free strains of *S. entomophila* and *S. proteamaculans,* and whether the differences between these strains due to the presence of pADAP is either detrimental or beneficial to the fitness and competitive abilities of the pathogenic *Serratia* strains. This also allowed for comparisons to be made between *Serratia* and the other entomopathogenic bacterial species used as biopesticides, such as *B. thuringiensis*. This project also sought to expand upon previous knowledge on the horizontal transfer of pADAP between pathogenic and non-pathogenic *Serratia* strains, detailing the rate at which it occurs and the conditions that are most favourable to plasmid transmission.

The implications of this research relate to the optimisation of *Serratia* as a biopesticide for managing field populations of grass grub, where, as stated previously, between 40 to 60 % of *Serratia entomophila* and *Serratia proteamaculans* strains free-living within the soil are plasmid-free (Jackson et al., 1991). Currently there exists a gap in knowledge regarding the occurrence of competition between pathogenic and non-pathogenic *Serratia* in the field. By studying these interactions, and the potential role that competition may play in pathogenic efficacy, it will be possible to predict the outcome of pathogen and non-pathogenic interactions within the field, whilst also finding methods of improving pathogen efficacy.

1.11 Objectives and hypotheses

i. Characterising the outcome of mixed inoculation with pathogenic and non-pathogenic *Serratia* spp. in liquid culture

The first objective of this study was to characterise the growth dynamics for pathogenic and non-pathogenic *Serratia* spp. when inoculated in a variety of liquid growth media. This included both separate and mixed inoculation with antibiotic-tagged *Serratia* strains to determine how the growth of the strains differed when grown separately compared to being grown together due to the initial cell density in mixed inoculation under optimal laboratory conditions. These experiments determined whether any competitive difference existed between the strains, and how they might confer a competitive advantage. Results are presented in Chapter 3.

Hypothesis: Plasmid-free *Serratia* strains will show increased competitive capabilities when compared to plasmid-bearing *Serratia* strains when cultured *in vitro* due to metabolic burden associated with plasmid carriage.

ii. Identifying chromosomal gene expression differences between plasmid-bearing and plasmid-free *Serratia* strains

The second objective of this study aimed to determine whether the carriage of pADAP by pathogenic *Serratia* strains, or lack thereof in the non-pathogenic strains, resulted in any differences in chromosomal gene expression between the strains. Differences in chromosomal gene expression could provide insights into variability in fitness between the strains. Results are presented in Chapter 4.

Hypothesis: The carriage of pADAP by pathogenic *Serratia* spp. will result in chromosomal gene expression differences between pathogenic and non-pathogenic strains.

iii. Characterising the outcome of mixed inoculation of *Serratia* spp. in the soil

The third objective of this project was to characterise the dynamics for pathogenic and non-pathogenic *Serratia* spp. when inoculated into natural non-sterile soil samples both separately and in mixed ratios to determine the persistence and interactions between the strains over a longer period of time. The use of natural soil samples also allowed for the first steps towards a better understanding of the strain dynamics in a field environment. Results are presented in Chapter 5.

Hypothesis: When inoculated into natural soil microcosms the non-pathogenic *Serratia* strains will show a higher persistence relative to the pathogenic strains.

iv. Assessing the impact of mixed inoculation with pathogenic and non-pathogenic *Serratia* spp. on amber disease occurrence in larvae

The fourth objective of this project was to determine how pre-challenging of grass grub larvae with both pathogenic and non-pathogenic *Serratia* spp. can alter the efficacy of the subsequently applied pathogenic strain. Assessments were conducted within larvae (*in vivo*) where disease phenotypes in the larvae were assessed in bioassays. Results are presented in Chapter 6.

Hypothesis: Non-pathogenic *Serratia* spp. will display a greater fitness within the larvae, reducing the efficacy of the pathogenic strain when used to subsequently challenge the larvae.

v. Assessing the outcome of mixed inoculation with pathogenic and non-pathogenic *Serratia* spp. in a pot trial

The final objective of this project was to determine whether the pre-inoculation of soil with non-pathogenic *Serratia* spp. would alter the efficacy of a pathogenic *Serratia* spp. when subsequently applied as a liquid drench. Pots containing natural non-sterile soil samples, larvae, and plants allowed for the interactions between the strains to be assessed under conditions that more closely approximated the field environment, simulating how these interactions might occur within the pasture. This was one of the key objectives of the project, and results are presented in Chapter 7.

Hypothesis: The pre-inoculation of soil with non-pathogenic *Serratia* spp. will reduce the efficacy of an applied pathogenic strain, reducing the occurrence of amber disease in larvae.

Chapter 2

Methods and Materials

2.1 Growth media and bacterial strains used

2.1.1 Media

The bacterial strains used in this study were grown primarily on a solid Luria-Bertani (LB) agar medium. All strains grown on LB agar were incubated overnight, with *Serratia* strains incubated at 30 °C, and *Escherichia coli* strains at 37 °C. Following overnight incubation agar plates were stored at 4 °C. Caprylate thallous agar (CTA) medium was used for the selective growth of *Serratia* spp. (Starr et al., 1976), which required incubation at 30 °C for three to five days. A combination of DNase-toluidine blue, adonitol, and itaconate agars were used for differentiation and typing of *Serratia* spp. (O'Callaghan and Jackson, 1993). The components of the media used are provided in Appendix A.1. Media were sterilised in an autoclave at 120 °C for 20 minutes in glass Schott bottles (Duran). Agar plates were poured within 12 hours of sterilisation, and plates were stored at an ambient room temperature of ~ 22 °C prior to use. Media and solution components are listed in Appendix A.

2.1.2 Preparation of bacterial overnight cultures

Liquid bacterial cultures were prepared using 3 mL of LB broth contained in a 30 mL glass universal tubes which were inoculated using a sterile toothpick from either a freshly grown bacterial colony or from stock samples maintained in Microbank cryotubes (Pro Lab Diagnostics, Inc) at – 80 °C. Overnight liquid cultures were incubated in a Ratek OM11 orbital shaker at the designated temperature at 200 rpm. Antibiotics and growth supplements were added to the cultures at the specified concentrations (Section 2.1.4).

2.1.3 Serial dilution of bacteria

Bacterial samples were serially diluted 1:10 in sterile 0.01 M phosphate-buffered saline (PBS) (Sigma), produced by dissolving one PBS tablet in 200 mL of Milli-Q water via autoclaving. Unless stated otherwise, 100 µL of sample was diluted in 900 µL of sterile PBS.

2.1.4 Antibiotics and growth supplements

Antibiotics and growth supplements were stored at - 20 °C and were thawed at room temperature (~ 22 °C) prior to use (Table 2.1). When required these substrates were added to both solid and liquid growth media at the following concentrations:

Table 2.1. The concentration of antibiotics and growth supplements used in liquid media.

Substrate	Abbreviation	*Serratia* (µg/mL)	*E. coli* (µg/mL)
Ampicillin	Amp	400	100
Tetracycline	Tet	30	30
Spectinomycin	Spec	100	100
Kanamycin	Kan	100	50
Gentamicin	Gent	60	30
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-gal	100	100
δ-aminolevulinic acid	ALA	-	50

2.1.5 Bacterial strains and plasmids used

All stock samples of bacterial strains and plasmids used in this study were maintained in the AgResearch Insect Pathogen culture collection (AgResearch, Lincoln, New Zealand) at - 80 °C in cryotubes. The strains and plasmids used are presented in Tables 2.2 and 2.3, respectively.

Table 2.2. The bacterial strains used in this study.

Species	Strain	Description	Reference
S. entomophila	A1MO2	Wild-type chronic pathogen	Grimont et al. (1998)
S. entomophila	A1MO2_t	A1MO2 with a chromosomal Tet cassette	This study
S. entomophila	A1MO2_tk	A1MO2 with a chromosomal Tet cassette and Kan plasmid cassette	This study
S. entomophila	A1MO2_gfp	A1MO2 with green fluorescence transposon	This study
S. entomophila	A1MO2_rfp	A1MO2 with red fluorescence transposon	This study
S. entomophila	5.6	Non-pathogenic, heat-cured plasmid-free isogenic form of A1MO2	Glare et al. (1993)
S. entomophila	5.6_s	5.6 with a chromosomal Spec cassette	This study
S. entomophila	5.6+pADAP	Variant of 5.6 containing conjugated pADAP used in RNA sequencing and transcriptomics analysis	This study
S. entomophila	5.6_gfp	5.6 with green fluorescence transposon	This study

S. entomophila	5.6_rfp	5.6 with red fluorescence transposon	This study
S. entomophila	IDIA	Wild-type pathogenic isolate from Lincoln, Canterbury	AgResearch culture collection
S. entomophila	IDIA pADAP-	Non-pathogenic pADAP cured IDIA	This study
S. entomophila	IDIA_s	IDIA with a chromosomal Spec cassette	This study
S. entomophila	219	Wild-type pathogenic isolate from Reefton, West Coast	AgResearch culture collection
S. entomophila	219_s	219 with a chromosomal Spec cassette	This study
S. entomophila	329	Wild-type non-pathogenic isolate	Glare et al. (1993b)
S. entomophila	329_s	329 with a chromosomal Spec cassette	This study
S. entomophila	477	Wild-type non-pathogenic isolate from Wakanui, Canterbury	Claus et al. (1995)
S. entomophila	477_s	477 with a chromosomal Spec cassette	This study
S. proteamaculans	143	Wild-type pathogenic isolate	Glare et al. (1993b)
S. proteamaculans	AGR96X	Wild-type hyper pathogen	Hurst et al. (2018)
S. proteamaculans	AGR96X_s	AGR96X with a chromosomal Spec cassette	This study
S. proteamaculans	Tukino	Wild-type non-pathogenic isolate	Glare et al. (1993b)
S. proteamaculans	Tukino_s	Tukino with a chromosomal Spec cassette	This study
E. coli	DH10b	Used for the propagation of insert DNA	Durfee et al. (2008)
E. coli	GM2929	Unmethylated (Dam-) *E. coli* strain	*E. coli* genetic stock centre
E. coli	EC100D	Electrocompetent cells	Epicentre
E. coli	ST18	Conjugation donor strain	Thoma et al. (2009)

Table 2.3. The plasmids used in this study.

Plasmid name	Description	Reference
pHP45Ω	Vector containing a Spec cassette (Amp^R, Sp^R)	Prentki and Krisch (1984)
pACYC184	Vector containing a Tet cassette (Tc^R, Cm^R)	Chang and Cohen (1978)
pUC19	High copy cloning vector (Amp^R)	Yanisch et al. (1985)
pRegA	RegionA amplicon in pGEM vector	This study
pJP5603	Plasmid used as a suicide vector (Kan^R)	Riedel et al. (2013)
pS_Con_1	Chromosomal Spec tag in pJP5603 suicide vector	This study
pT_Con_1	Chromosomal Tet tag in pJP5603 suicide vector	This study
pJS_Con_2	Chromosomal Spec tag in ST18 donor strain	This study
pJT_Con_2	Chromosomal Tet tag in ST18 donor strain	This study
pGEM®-T Easy Vector	Plasmid used for construct sequencing	Promega
pJP5608_AFP_Kan	Kanamycin pADAP backbone tag in ST18 donor strain (Amp^R)	Sitter (2020)
pMRE-Tn7-145	Red fluorescence transposon in ST18 donor strain	Schlechter et al., 2018
pMRE-Tn7-147	Green fluorescence transposon in ST18 donor strain	Schlechter et al., 2018

2.2 General molecular methods

2.2.1 Isolation of genomic DNA

The preparation of genomic DNA samples was performed using an Isolate II Genomic DNA kit (Bioline) following the manufacturer's instructions. Following preparation genomic DNA samples were quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher) and stored at - 20 °C. DNA sample were also validated with BOX repeat-based PCR (Section 2.2.12).

2.2.2 Mini-preparation of plasmid DNA

Samples were prepared from 3 mL overnight cultures, from which either 1 mL or 500 µL, for *E. coli* and *Serratia*, respectively, was pelleted via centrifugation at 15,700 *g* for 30 seconds. The samples were aspirated and resuspended in 150 µL of solution I and 200 µL of solution II (Appendix A.2) and incubated at 37 °C for four minutes. An additional 150 µL of 3 M pH 5.2 sodium acetate was added, and the samples were vigorously mixed and placed on ice for five minutes. The microcentrifuge tubes were then pelleted via centrifugation at 15,700 *g* for ten minutes, with the supernatant being tipped into 1 mL of absolute ethanol, and gently mixed by inversion, and centrifuged at 15,700 *g* for a further five minutes. The samples were aspirated, briefly centrifuged for six seconds at 15,700 *g* and then re-

aspirated before being dried for 20 minutes at 37 °C. The resultant pellet was resuspended in 30 µL of Milli-Q water and stored at - 20 °C until used.

2.2.3 Restriction digestion of DNA samples

Restriction digestions were performed with restriction enzymes (New England BioLabs) following the manufacturer's instructions. Restriction enzymes were stored at - 20 °C and were kept chilled in ice blocks during use. The CutSmart buffer (New England Biolabs) was stored at - 20 °C and thawed prior to use.

2.2.4 Ethanol precipitation of DNA

A one-tenth volume of 3 M sodium acetate was added to the sample and mixed briefly, after which a 2 X total volume of absolute ethanol was added. The samples were mixed via vortex. The samples were incubated for two minutes at room temperature (~ 22 °C) and centrifuged for five minutes at 15,700 *g*. The samples were aspirated and spun briefly again for ten seconds to remove any remaining supernatant, before being dried for five to ten minutes in a 37 °C incubator. The dried pellet was resuspended in 10 µL of Milli-Q water.

2.2.5 Ligation of DNA samples

Ligations of DNA samples were performed using either a T4 DNA Ligase (New England BioLabs) or pGEM®-T Easy Vector (Promega) using the manufacturer's recommended protocol with precipitated insert and vector DNA samples, using a 1:3 ratio of vector to insert DNA ratio, respectively. Samples were incubated at room temperature overnight (~ 22 °C) and stored at - 80 °C until transformation.

2.2.6 Preparation of chemically competent cells

A 3 mL overnight culture was prepared using fresh *E. coli* DH10b cells from an agar plate and incubated overnight at 37 °C. From the culture 1 mL was removed with a pipette and used to inoculate 50 mL of LB broth, which was incubated for a further two hours at 37 °C in a Ratek OM11 orbital shaker at 200 rpm until an optical density (OD) of 0.4 at a wavelength of 600 nm was achieved (OD_{600}). Following incubation, the culture was placed on ice for 20 minutes. The culture was pelleted via centrifugation (Eppendorf 5810 R centrifuge) for ten minutes at 2,189 *g* at 4 °C. The supernatant was discarded, and the pellet was resuspended in 25 mL of chilled 50 mM $CaCl_2$ + 10 mM Tris pH 8.0. The cells were pelleted again with the same parameters, with the pellet being resuspended in 1 mL of 50 mM $CaCl_2$ + 10 mM Tris pH 8.0. The resultant solution was kept on ice for a further hour prior to transformation of DNA (Section 2.2.8).

2.2.7 Preparation of electro-competent cells

A 3 mL overnight culture was prepared using fresh *E. coli* GM2929 or EC100D cells incubated overnight at 37 °C. From the culture 1 mL was removed via pipette and used to inoculate 50 mL of LB broth, which was incubated at 37 °C in a Ratek OM11 orbital shaker at 200 rpm until the culture reached an undiluted OD_{600} of ~ 0.4 (BioRad SmartSpec). The culture was pelleted via centrifugation (Eppendorf 5810 R centrifuge) for ten minutes at 2,513 *g*. The supernatant was removed, and the cells were resuspended in 50 mL of Milli-Q water prior to centrifugation at 3,102 *g* for ten minutes. This step was repeated with 40 mL of Milli-Q water and a speed of 3,754 *g*. The pellet was resuspended in 30 mL of Milli-Q water containing 10 % glycerol before centrifugation at 4,032 *g* for ten minutes. This step was repeated with 20 mL of Milli-Q water with glycerol with a final centrifugation at 4,468 *g*. All centrifugation steps were conducted at room temperature (~ 22 °C). The majority of the supernatant was removed, leaving 500 µL in which the pelleted cells were resuspended. Forty µL of the suspension was aliquoted into 1.5 mL microcentrifuge tubes and frozen at - 80 °C prior to use in transformation (Section 2.2.9).

2.2.8 Transformation using chemically competent cells

Three µL of a ligation was pipetted into a 1.5 mL microcentrifuge tube and placed on ice for five minutes prior to the addition of 100 µL of fresh chemically competent DH10b cells, which were mixed via light pipetting. The samples were heat shocked for 30 seconds in a waterbath heated to 42 °C before being placed back on ice for a further 30 seconds. One mL of LB broth was added to the sample before being placed horizontally for one hour in a 37 °C incubator. The samples were pelleted via centrifugation at 5,900 *g* for three minutes after which ~ 875 µL of supernatant was removed, and the pellet was resuspended in the remaining 100 µL. The cells were divided amongst two LB agar plates supplemented with X-gal alongside any appropriate antibiotics and incubated overnight at 37 °C.

2.2.9 Transformation using electro-competent cells

Electro-competent cells were defrosted and pipetted into chilled 0.2 cm electro cuvettes (Invitrogen) and kept on ice alongside 1 µL of plasmid DNA, which was also placed on ice for five minutes in a 1.5 mL microcentrifuge tube. The electroporation process was performed using an *E. coli* pulser (Bio-Rad) at 2.5 kV. The chilled electro-competent cells were added to the plasmid DNA samples, mixed, and pipetted back into the electro cuvette and electroporated. 1 mL of LB broth was added to the cuvette immediately after and mixed via pipetting, and the sample was poured back into a clean 1.5 mL microcentrifuge tube before incubation for one hour at 37 °C. The samples were pelleted via centrifugation at 5,900 *g* for three minutes. The supernatant was removed, leaving approximately 100 µL of supernatant, which was used to resuspend the pellets. This suspension was spread across LB agar plates containing the appropriate antibiotics, which were incubated overnight at 37 °C.

2.2.10 Conjugation

Three mL overnight cultures were prepared using both the donor suicide constructs and recipient strains alongside the required antibiotics. One mL was taken from these cultures and pelleted via centrifugation at 5,900 *g* for three minutes. The samples were aspirated and resuspended in 1 mL of LB broth and spun for a further five minutes at the same speed, after which they were aspirated and resuspended in 500 µL of LB broth with 0.5 µL ALA. One hundred µL of the suspension was removed via pipette for both the donor and recipient suspensions and pipetted onto LB agar plates with ALA, which were gently mixed, with the plates incubated for six hours at 30 °C. The overnight cultures of both donor and recipient strains were also incubated at 30 °C for the same duration. After incubation 1 mL of LB broth was pipetted over the top of the cells and mixed using a sterile plate spreader, which was then spread across three LB agar plates containing the appropriate antibiotics. Control plates were also made using the initial overnight cultures, with 10 µL being independently spread across LB agar plates containing the same selectable markers as the prospective transconjugants. These plates were then incubated overnight at 30 °C.

2.2.11 Primers

A list of the primers used in this study are listed in Table 2.4. The primers used were designed using Geneious Prime (version 2019.1.1) to a maximum length of 20 bp prior to additions of poly-A tails and restriction sites, a GC% content of approximately 55 %, and a melting temperature of between 55 to 60 °C. In addition, primers chosen from results generated in Geneious lacked both self-dimerisation and hairpin formation qualities.

Table 2.4. The primers used in this study. Underlined text denotes restriction enzyme cut site.

Primer name	Sequence (5' to 3')	Description
BacF	AAA**GGGCCC**AGGAGTTAGTCTTGAAGT	Amplification of chromosome insertion region (RegionA)
BacR	AAA**GAATTC**TGGAAGTGCATATCCATA	
TcF	AAA**GGGCCC**AGGAGTTAGTCTTGAAGT	Amplification of Tet cassette in pACYC184
TcR	AAA**GGGCCC**GGTGAATCCGTTAGCGAG	
SpF	AAA**GGGCCC**GCAGTTGCAAACCCTCAC	Amplification of Spec cassette in pHP45Ω
SpR	AAA**GGGCCC**AATTATGTGCTTAGTGCA	
AFP_Kan_F	GCCCCTAACATTACGCTGAGTA	Amplification of Kan cassette in pADAP
AFP_Kan_R	CCCCTGGTTAATCTCGTCTTGTG	
BacConF	CTCCTTTGAAGCAGCCTGGT	Validation primers for RegionA insertion
BacConR	GCCATCCTGTCACGTCTGAT	
BOX A1R	CTACGGCAAGGCGACGCTGACG	BOX-PCR primer
RepA_1	TGCAGGGGAACGATCTTCTTGAGG	

RepA_2	GCCCCACTTTCTTGTACCATCCAG	Amplification of *repA* gene on pADAP
FWD_Tn5-7_gt	ATGGTGAGCAAGGGCGAG	Multiplex primers used in validation of fluorescence reporter transconjugants
FWD_Tn7_gt	ACATAACGGACTAAGAAAAACACTACAC	
Tn7_gt	GAATTACAACAGTACTGCGATGAG	
REV_Tn7_gt	GATCAACTCTATTTCTCGCGGG	
REV_Tn5-7_gt	CAACAGGAGTCCAAGCTCAG	
M13F	GTAAAACGACGGCCAGT	Macrogen pGEM-T Easy primer set
M13R	GCGGATAACAATTTCACACAGG	

2.2.12 Polymerase chain reactions (PCR)

PCR samples were prepared in 0.2 mL thin-walled polypropylene tubes and, prior to loading into the PCR machine, were spun for three seconds in a mini centrifuge. Reactions were performed using a C1000 Touch Thermal Cycler (BioRad). Standard reactions were performed using PlatniumTaq (Invitrogen), 2X ReddyMix Master Mix (ThermoFisher), and 2X DreamTaq (ThermoFisher) polymerases using the manufacturer's recommended instructions and optimisation processes; the PCR parameters used are listed in Appendix A.3. DNA samples used in the reaction were prepared either from fresh bacterial colonies resuspended in 500 µL of Milli-Q water (ReddyMix and DreamTaq) or from a 1:10 diluted sample of genomic DNA (PlatniumTaq). Plasmid DNA and primer samples were diluted 1:1000. The genomic DNA of wild-type strains was assessed using a BOX PCR fingerprint typing analysis with the A1R primer, based on dispersed-repeat motifs.

2.2.13 Purification of PCR products

PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche) following the manufacturer's included instructions. The concentration of the purified samples was measured using a NanoDrop 2000 spectrophotometer (ThermoFisher).

2.2.14 DNA sequence validation

Prior to DNA sequencing PCR amplicons were ligated into the pGEM®-T Easy vector. Samples were then prepared for sequencing using a High Pure Plasmid Isolation Kit (Roche) following the manufacturer's included instructions. DNA sequencing was performed by Macrogen (South Korea) using their Standard-Seq service and M13 primer set for pGEM®-T Easy to validate the insertion of PCR amplified regions.

2.2.15 Agarose gel electrophoresis

Electrophoresis of DNA was conducted using a 1 % agarose gel containing 0.1 % RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology) unless stated. Three µL of PCR sample was mixed with 3 µL

of purple loading dye (New England Biolabs) and added to each well, with an additional well containing 3 µL of a 1 kb ladder (ThermoFisher). The gels were run at a current of 95 V for 40 minutes using a BRl Model 500 power supply (Life Technologies, Inc.), and then visualised using an HD5 transilluminator (Uvidoc).

2.2.16 Identification of a *Serratia* chromosome region for insertion of antibiotic markers

Tagged *Serratia* strains (listed in Table 2.2) were constructed to allow co-culturing of more than one strain during *in vitro* and *in vivo* competition experiments; this was achieved through the insertion of antibiotic cassettes into the strains. The antibiotic insertion site was decided based on genome comparisons between *S. entomophila* and *S. proteamaculans*, in which a conserved ~ 4.2 kb DNA region was identified between the strains. This site, named RegionA, contained a unique ApaI restriction enzyme cut site (5'-GGGCCC-3') located between two inward facing open reading frames (Fig. 2.1). Due to the intergenic position, insertion of antibiotic cassettes into RegionA would not impact promotor regions, and thus should not impact cell fitness, whilst the downwards transcription from the cassettes would not interfere with the opposing gene. Upstream of the insertion site was *obg*, a gene encoding for GTPase Obg, a protein essential for bacterial survival (Bonventre et al., 2016), and downstream of the insertion site was *basS*, a gene encoding for Sensor protein BasS, a histidine kinase involved in signal transduction (Yamamoto et al., 2005). The full sequence of RegionA is available in Appendix A.4.

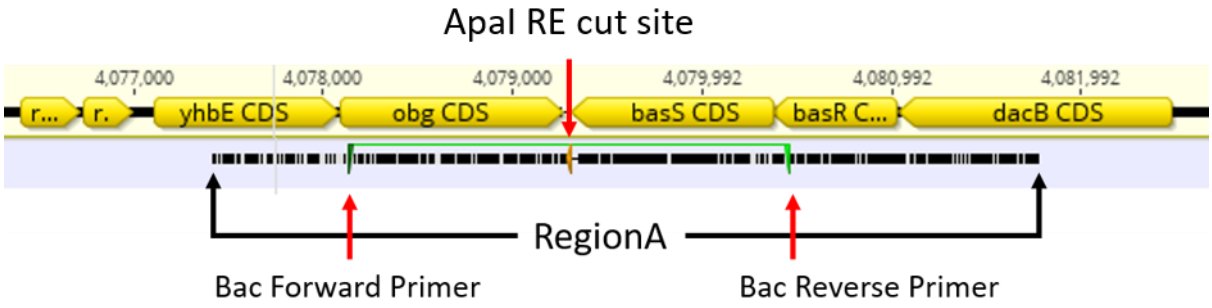


Figure 2.1. The RegionA chromosomal marker insertion site mapped to the *S. entomophila* 626 genome. The region was PCR amplified with the BacF/R primer set, with Tet and Spec antibiotic markers being inserted in the ApaI RE cut site. The black bar denotes shared nucleotide identity between *S. entomophila* and *S. proteamaculans*.

2.2.17 Amplification of RegionA and insertion of antibiotic markers

The genomic DNA for selected *Serratia* strains was isolated and prepared, with the RegionA insertion site being amplified via PCR, producing a ~ 2.3 kb amplicon, which was purified. The RegionA amplicon was ligated into the pGEM-T Easy vector. The ligated product was then transformed into chemically competent DH10b cells. Prospective clones (white colonies on X-gal agar) were miniprepped and digested with the EcoR1-HF restriction enzyme, where clones that released the correct band size were prepped and subject to DNA sequencing. The sequence validated plasmid (pRegA1) was transformed into chemically competent *dam*- GM2929 cells, with the resultant transformant being miniprepped and digested with the DAM sensitive ApaI restriction enzyme. Plasmid DNA was prepared from pAYCY184 and pHP45Ω, and the respective Tet and Spec antibiotic cassettes, which was PCR amplified, with the purified products being digested with ApaI and ligated with pRegA1. Resultant antibiotic-tagged constructs were transformed into chemically competent DH10b cells, miniprepped, and digested with EcoR1-HF, and ligated into a digested pJP5603 suicide vector. The resultant ligations were transformed into electrocompetent EC100D cells and plated on agar containing the antibiotics respective to the cassettes and X-gal. The colonies were assessed for Amp sensitivity, with any Amp sensitive colonies being miniprepped. Clones with the correct restriction enzyme profiles were electroporated into the ST18 donor strain. The ST18 constructs (pJS_Con_2 for Spec and pJT_Con_2 for Tet) were conjugated into the appropriate *Serratia* strains. Successful insertion of the cassettes resulted in a ~ 4kb band following amplification using PCR and the BacConF/R confirmation and BOX primer set (Table 2.4 and Fig. 2.2), after which the amplicon was sequenced.

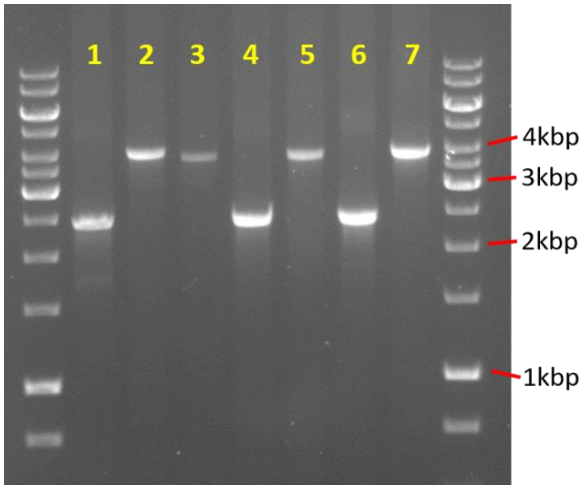


Figure 2.2. Agarose gel showing band size for wild-type *Serratia* strains and chromosomally tagged *Serratia* strains using BacConF/R primer set and a 10 kb ladder reference maker on a 1 % agarose gel. 1 = wild-type A1MO2, 2 = A1MO2_t, 3 = A1MO2_tk, 4 = wild-type 5.6, 5 = 5.6_s, 6 = wild-type AGR96X, 7 = AGR96X_s.

2.2.18 Visualisation of large plasmid DNA

Visualisation of large plasmid DNA was performed using the method of Kado and Liu (1981). Overnight cultures were prepared as described previously in Section 2.1.2. Three hundred µL from the overnight cultures were pelleted via centrifugation for two minutes at 15,700 *g* and resuspended in 100 µL 2 X E-buffer and 200 µL lysis buffer (Appendix A.5). The samples were incubated in a water bath for one hour at 55 °C. A further 300 µL of phenol-chloroform was added to the samples, and the samples were gently mixed and centrifuged for a further 15 minutes at 15,700 *g*. Sixty µL of the aqueous phase was extracted via pipette and loaded onto a 0.7 % agarose gel alongside 6 µL of loading dye and a λ DNA HindIII digest ladder (New England Biolabs) and ran for two hours at 80 V. The gels were stained by being submerged in ethidium bromide for 25 minutes and de-stained in water for ten minutes before visualisation using a Uvidoc HD5 transilluminator (Fig. 2.3).

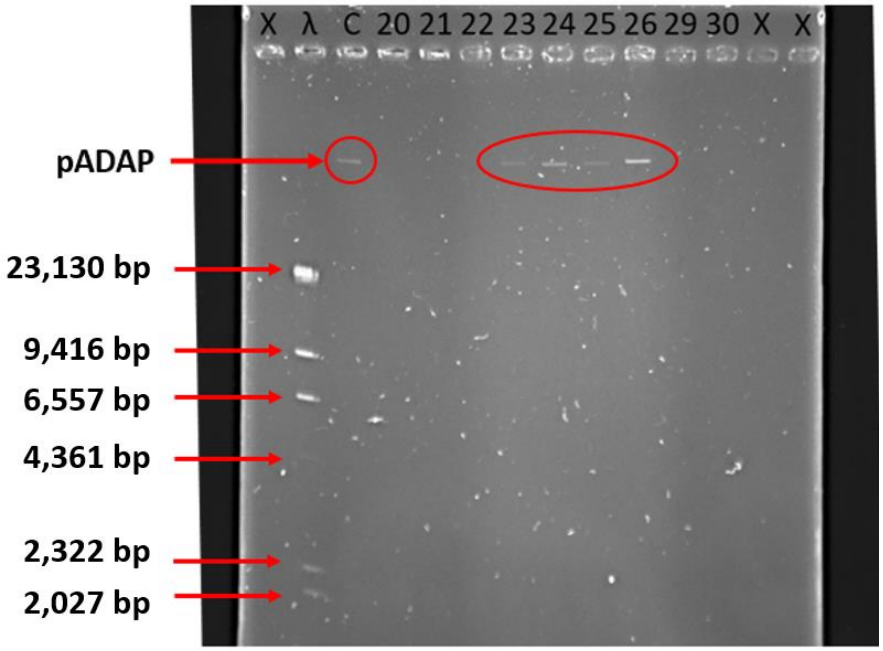


Figure 2.3. Agarose gel showing band size for *Serratia* pADAP (~ 153 kb) following visualisation using Kado and Liu (1981) method using a λ DNA HindIII digest ladder and a 1 % agarose gel. C = wild-type IDIA, 20 – 22, 29 - 30 = plasmid cured IDIA isolates, 23 - 26 = plasmid bearing IDIA isolates. X = empty wells. Samples with pADAP present are circled in red.

2.2.19 Heat curing of plasmids

Strains were cured of plasmids following the method of Morrison et al. (1983) and Glare et al. (1993b), in which 100 µL of an overnight culture was spread across LB agar plates and incubated at 42 °C for up to seven days. The plates were then moved to incubation at 30 °C for overnight incubation with any resultant colonies being assessed using a PCR with the 2 X ReddyMix polymerase and a large plasmid visualisation method (Section 2.2.18) and BOX PCR (Section 2.2.12).

2.3 Methods for measuring competition between *Serratia* spp. *in vitro*

2.3.1 M9 Minimal salts medium

In addition to LB agar and broth, M9 minimal salts media (M9) were used with supplemental carbon sources during *in vitro* competition assays to simulate growth in nutrient-poor conditions. Casamino acids, glucose, maltose, trehalose, and glycine betaine (all prepared as 0.5 M stock solutions based on molecular weight and diluted to 25 µM concentration in media (1/20)) were used as the supplemental carbon sources. For every 50 mL of M9 medium used an additional 50 µL of 1 M magnesium sulphate ($MgSO_4$) was added. The M9 medium was prepared using the following recipe:

5X salts stock	Mass (g)
$Na_2HPO_4 \bullet 7H_2O$	32
KH_2PO_4	1.5
NaCl	1.25
NH_4Cl	2.5
Milli-Q water	500 (mL)

M9 medium	Volume (mL)
5X salts stock	200
0.5 M Carbon source	20
0.1 M $CaCl_2$	1
Milli-Q water	780

2.3.2 Preparation of mixed ratio tagged *Serratia* inocula for competition assays

Prior to the conduction of *in vitro* assays, 1 mL of overnight culture for the selected strains was centrifuged in 1.5 mL microcentrifuge tubes for 30 seconds at 15,700 *g*. The supernatant was aspirated, and the pellets were resuspended in 1 mL of 0.01 M PBS. The samples were serially diluted further at a 1:10 ratio with PBS (Section 2.1.3) and the OD_{600} values were recorded using a spectrophotometer (BioRad SmartSpec). The samples were adjusted with additional PBS until OD_{600} measurements of 0.5 (± 0.1) were reached. These adjusted samples were used in the production of 500 µL inocula used in various growth assays. Single and mixed ratio inocula for competition assays were produced by combining differing volumes of the adjusted samples (Table 2.5).

Table 2.5. The volumes of adjusted overnight culture samples combined to produce 500 µL single and mixed ratio inocula for competition assays conducted in liquid media, soil, and within the larvae.

Treatment	Desired ratio	Strain A (µL)	Strain B (µL)
1	100:0	500	0
2	0:100	0	500
3	90:10	450	50
4	70:30	350	150
5	50:50	250	250
6	30:70	150	350
7	10:90	50	450

2.3.3 Bacteria growth curves

Growth curves were performed using sterile 250 mL glass flasks containing 50 mL of either LB broth or M9 medium with the appropriate carbon source. The flasks were inoculated with a 500 µL inoculant of an appropriate competition ratio (Section 2.3.2) and incubated in an orbital shaker (Infors) at 30 °C rotating at 200 rpm for the duration of the experiment. To determine the number of colony forming units (CFUs) in the culture, 100 µL of culture was removed from the flask via pipette and serially diluted in PBS, with 50 µL of diluted culture from a specific dilution being spread across LB agar plates containing the appropriate antibiotics. The plates were incubated overnight at 30 °C. The cultures were assessed after 0-, 1-, 2-, 4-, 6-, 8-, 10-, 12-, 16-, 20-, 24-, 36-, and 48-hours post-inoculation (hpi) using the following dilutions: 10^{-4} (0 - 2 hpi), 10^{-5} (4 - 6 hpi), and 10^{-7} (8 - 48 hpi). The diluted culture samples were independently spread across three plates during each assessment time to produce triplicate CFU counts.

2.3.4 Competition growth curves

Competition growth curves were performed using the same method detailed in Section 2.3.3 with an inoculant comprised of an equal proportioned ratio (50:50) of the competing strains (Table 2.5).

2.3.5 Seven-day shake flask assays

Mixed inoculant assays conducted over seven days were performed in sterile 250 mL glass flasks containing 50 mL of either LB broth or M9 medium with the appropriate carbon source. The flasks were inoculated with 500 µL mixed strain inocula (Section 2.3.2) and were incubated orbital shaker (Infors) at 30 °C rotating at 200 rpm for the duration of the experiment. To determine the number of CFUs in the culture, 100 µL of culture was removed from the flask via pipette and serially diluted in PBS, with 50 µL of diluted culture from a specific dilution being spread across LB agar plates containing the appropriate antibiotics. The plates were incubated overnight at 30 °C. The cultures were assessed after 1-, 2-, 3-, 4-, 5-, 6-, and 7-days post-inoculation (dpi) using the following dilutions for LB broth:

10^{-7} (1 - 3 dpi), 10^{-6} (4 - 5 dpi), and 10^{-5} (6 - 7 dpi), and the following dilutions for M9 medium: 10^{-7} (1 dpi), and 10^{-6} (2 - 6 dpi). The diluted culture samples were spread across three plates during each assessment time to produce triplicate CFU counts.

2.3.6 Re-seeding of fresh medium with established mixed ratio inocula

These assays were performed following the same method as detailed in Section 2.3.5, however, following the dilution and plating of culture samples after one dpi, 500 µL of the culture for each mixed ratio treatment was removed from the flask via pipette and used to inoculate a 250 mL flask containing 50 mL of fresh LB broth. This process was repeated during each subsequent assessment of the cultures, and the cultures were assessed using a dilution of 10^{-7} on each day. The diluted culture samples were spread across three plates during each assessment time to produce triplicate CFU counts.

2.3.7 Live/dead staining of *Serratia* cells

The cultures used to produce samples for Live/Dead staining were prepared following the same method detailed in Section 2.3.5. Flasks (250 mL) containing either 50 mL of LB broth or an M9 20 % casamino acids medium inoculated with a 500 µL 1:10 dilution of wild-type A1MO2, 5.6, or AGR96X adjusted with PBS to an OD_{600} of 0.5 (± 0.1), as detailed in Section 2.3.2. The cultures were assessed using the same time scale and dilutions as the seven-day shake flask assays to generate CFU counts during the assay to assess the number of viable cells within the culture (Section 2.3.5). An additional 50 µL of culture was removed via pipette, from which 3 µL was mixed with 3 µL of BacLight Bacterial Stain (ThermoFisher) on a clean glass microscope slide, which was incubated in darkness at ambient room temperature (~ 22 °C) for 15 minutes. The slides were examined using an Olympus BX53 fluorescence microscope using the 10 x and 40 x objective lenses, using the PH and DAPI imaging settings to capture three randomly selected areas that were representative of the cells on the slide. When not in use the staining solution was stored at - 20 °C.

The captured DAPI images were assessed using ImageJ (https://imagej.nih.gov/ij/) (Schneider et al., 2012) using the following process: the threshold colour of the image was adjusted to encompass either a green or red hue, to isolate living and dead cells, respectively (Image -> Adjust -> Color Threshold). Following selection, the isolated cells were converted to a binary image to convert the image to black and white (Process -> Binary -> Make Binary). The number of cells were then counted by counting the number of cells with a size between 1 – 15 $pixels^2$ and a circularity between 0.0 and 1.0 (Analyze -> Analyze Particles). The total number of cells in an image was calculated using the total number of living and dead cells, from which percentages for each were generated.

2.3.8 Conjugation and validation of mScarlet-I and mClover3 fluorescence transposons

The red mScarlet-I and green mClover3 fluorescence transposons used were contained in ST18 donor plasmids pMRE-Tn7-145 and pMRE-Tn7-147 (Schlechter et al., 2018) and conjugated into recipient *Serratia* strains A1MO2 and 5.6, following the conjugation method detailed in Section 2.2.10. Transconjugant cells were then patched onto Amp-400 LB agar plates, as all transconjugant cells were expected to still contain the donor plasmid, which confers resistance to ampicillin. This resistance would not be transposed to the chromosome. The agar plates were incubated overnight at 30 °C. Transconjugant cells were validated using two methods. A multiplex PCR with validation primers (FWD_Tn5-7_gt, FWD_Tn7_gt, Tn7_gt, REV_Tn7_gt, and REV_Tn5-7_gt) (Table 2.4) would identify the fluorescence gene with a band of ~ 700 bp, with any larger bands (~ 1000 bp) being derived from the pMRE-Tn7-145 or pMRE-Tn7-147 plasmid backbone. As all transconjugants still retained the plasmid, both bands would be required for validation. Following PCR, the transconjugants were validated with fluorescence microscopy using GFP and PHYCO imaging settings. Putative correct colonies were cultured overnight, pelleted via centrifugation at 6000 *g* for three minutes, resuspended in 300 µL of LB broth and assessed using an Olympus BX53 fluorescence microscope using the RFP (for pMRE-Tn7-145 recombinants) and GFP (for pMRE-Tn7-147 recombinants) imaging settings.

Putative colonies validated using the multiplex PCR (Table 2.4) and fluorescence microscopy were cultured in 3 mL of LB broth overnight at a temperature of 37 °C with no antibiotics to heat-cure the transconjugants of the plasmid. The cultures were then plated on LB agar containing no antibiotics and incubated overnight at 30 °C. Isolated colonies were patched onto Gent-60 and Amp-400 LB agar plates, selecting for the inserts and vector, respectively. Any transconjugants resistant to Gent and sensitive to Amp were re-assessed using the previously described multiplex PCR with previously described validation primers. Successful heat-cured transconjugants would only produce a single ~ 700 bp band for the fluorescence gene. The heat-cured transconjugants were also re-assessed for fluorescence using fluorescence microscopy and the same microscope image settings described above.

2.3.9 Preparation of overnight cultures for fluorescence intensity assays

Overnight cultures of wild-type A1MO2 and 5.6, and *Serratia* strains containing either the mScarlet-1 (A1MO2_rfp, 5.6_rfp) or mClover3 (A1MO2_gfp, 5.6_gfp) were prepared in 15 mL plastic centrifuge tubes containing 3 mL of LB broth, and incubated overnight in an Infors orbital shaker at 200 rpm and 30 °C. The cultures were pelleted via centrifugation at 4000 *g* for three minutes and resuspended in PBS and were adjusted with additional PBS until a 1:10 serially diluted OD_{600} of 0.4 (± 0.02) was reached.

2.3.10 Fluorescence intensity competition assays

The fluorescence intensity assays were performed in a flat-bottomed 96-well plate (Costar) using a FLUOstar Omega plate reader (BMG Labtech). Each well contained 160 µL of either LB broth or M9 medium with the appropriate carbon source with no antibiotics, to which a 40 µL inoculant was added, prepared from overnight cultures described in Section 2.3.9. For mixed ratio inocula the volume of culture to be added was divided proportionally between the two strains (Table 2.6). The plates were covered with a self-adhesive film to prevent evaporation and condensation build-up during the assay. Multichromatic filters were used for excitation (584 nm) and emission (620 nm, 10 nm spectral band pass) specific to mScarlet-I, and excitation (485 nm, 12 nm spectral bandpass) and emission (520 nm) specific to mClover3, with a gain of 1,750. Orbital averaging of the wells was set to a diameter of 3 mm to create an average from the intensity measurements of the well on a defined orbit. A total of 288 measurement cycles were performed over 48 hours, with ten flashes per cycle, and each cycle lasting for 600 seconds (ten minutes). The 96-well plate was incubated at 30 °C and shaken at 250 rpm for the duration of the assay.

Table 2.6. The volumes of adjusted overnight culture samples combined to produce 40 µL single and mixed ratio inocula for fluorescence intensity assays.

Ratio	Strain A (µL)	Strain B (µL)
100:0	40	0
0:100	0	40
75:25	30	10
50:50	20	20
25:75	10	30

2.3.11 Preparation of samples for RNA sequencing

RNA extractions on 5.6 and pADAP conjugated into 5.6 (+pADAP) were performed using an RNeasy Mini Kit (Qiagen), and prior to beginning all bench areas and equipment used during the process were cleaned using RNAseZAP spray (ThermoFisher). Pipetting was performed using filter tips, and two pairs of gloves were worn, with gloves also being cleaned by the spray. Five hundred µL of a 1:10 diluted overnight culture was used to inoculate 50 mL of LB broth, which was incubated on an orbital shaker at 37 °C and 200 rpm for up to nine hours. One mL of the culture was extracted and combined with 2 mL of RNAprotect and mixed briefly using a vortex. The samples were incubated for five minutes at room temperature (~ 22 °C) and then pelleted via centrifugation at 5,000 *g* for ten minutes. The supernatant was removed, and the pellets were left upside-down to dry. These samples were stored at - 80 °C prior to extraction.

2.3.12 Extraction and secondary RNaseI treatment of RNA samples

The extraction method from the RNeasy Kit (Qiagen) was used alongside additional steps. The prepared RNA samples were thawed at room temperature (~ 22 °C) and resuspended in a 100 µL mixture of TE (Tris and EDTA), lysozyme, and proteinase K, and incubated at room temperature for ten minutes. During the incubation process the samples were vortexed for ten seconds every two minutes. Seven hundred µL of RLT cell lysis buffer (included in the kit) and β-Mercaptoethanol (10 µL of β-Mercaptoethanol and 1 mL of buffer RLT) were mixed with the samples, to which a further 500 µL of absolute ethanol was added. Seven hundred µL of the lysate was transferred to a spin column and spun at 8,000 *g* for 15 seconds at 4 °C. The flow-through was discarded and the centrifugation was repeated with any remaining lysate. Three hundred and fifty µL of RW1 washing buffer (included in kit) was added to the column, and spun at 8,000 *g* for 15 seconds at 4 °C. An 80 µL mixture of DNaseI (10 µL) and RDD binding buffer (included in kit) (70 µL) was added to the column and incubated for 15 minutes at room temperature. An additional 350 µL was added to the column and incubated for five minutes at room temperature, after which the columns were spun at 8,000 *g* for 15 seconds at 4 °C, with the flow-through being discarded. Five hundred µL of RPE washing buffer (included in kit) was added to the column, with centrifugation at 8,000 *g* for 15 seconds initially, and then two minutes after the flow-through had discarded, with both centrifugation steps occurring at 4 °C. The column was transferred to a 1.5 mL microcentrifuge tube, 47.5 µL of RNase-free H_2O was added, and spun at 8,000 *g* for one minute at 4 °C. An additional 40 µL of the RNase-free H_2O was added, and the sample was spun again.

A further 10 µL of RDD buffer and 2.5 µL of DNaseI were added to the samples and they were incubated at room temperature (~ 22 °C) for ten minutes. Three hundred and fifty µL of RLT buffer and 250 µL of absolute ethanol were added, with the samples being mixed in between. The mixture was added to a spin column and spun for 8,000 *g* for 15 seconds at 4 °C, with the flow-through being discarded. Two further centrifugations were performed following the addition of 500 µL of RPE buffer at the same speed and temperature for 15 seconds and for two minutes. The column was placed in a new 1.5 mL microcentrifuge tube, to which 90 µL RNase-free H_2O was added, spun at 8,000 *g* for one minute at 4 °C, and then repeated with an additional 90 µL of RNase-free H_2O.

2.3.13 Isopropanol precipitation of RNA samples

To the samples 18 µL of 3 M sodium acetate and 600 µL of chilled 100 % isopropanol was added, and they were incubated overnight at - 20 °C. The samples were pelleted via centrifugation at 10,000 *g* for 30 minutes at 4 °C, and the supernatant discarded. Two additional centrifugation steps were conducted in which 500 µL of 75 % ethanol was added and mixed gently before centrifugation at 10,000 *g* for five minutes at 4 °C. A final brief spin was conducted to remove any remaining

supernatant. The pellet was air-dried at 37 °C for approximately ten minutes and resuspended in 40 µL RNase-free H_2O. The RNA concentration and purity were assessed using a Nanodrop and Qubit 4 fluorometer (ThermoFisher), and by visualisation on agarose gel.

2.3.14 Visualisation of RNA samples

All equipment used for agarose gel electrophoresis was cleaned with RNAseZAP (ThermoFisher) prior to use. Fresh 1 X TAE buffer was also use alongside a loading dye with no added RNase. The gel was prepared in the same way as detailed in Section 2.2.15, however no RedSafe was added. Samples were loaded using filter tips, with 1 µL of RNA, 4 µL of RNase-free H_2O, and 1 µL of loading dye added to each well. The gels were stained via submersion in ethidium bromide for 30 minutes, and de-stained in RNase-free H_2O. Similar to the visualisation of DNA samples, the gels were visualised using a Uvidoc HD5 transilluminator.

2.3.15 BLAST of hypothetical protein sequences

The basic local alignment search tool (BLAST) in Geneious Prime was used to perform nucleotide BLAST analysis of unidentified contigs with an attached hypothetic protein. Searches were made using the NCBI reference protein database (https://www.ncbi.nlm.nih.gov/refseq/) and the BlastN program, with a maximum of ten hits per search. Protein BLASTS were made using the NCBI BlastP tool (https://blast .ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

2.3.16 Protein structure prediction of hypothetical products using the Phyre^2 engine

Any genes which remained unidentified following initial BLAST analysis (Section 2.3.15) were re-assessed using the protein homology/analogy recognition engine (Phyre^2) for protein modelling, prediction, and analysis (Kelley et al., 2015). The gene was extracted in Geneious Prime and translated into an amino acid sequence, which was input into Phyre^2. The output from the engine provided information on the matched template and coverage of the template, a 3D predicted model of the protein, the confidence in homology between the sequence and template, and the template information. The hypothetical protein information generated in the Phyre^2 engine was cross referenced with Uniprot (https://www.uniprot.org/) to gain further information on the potential protein match regarding the species of origin.

2.3.17 Inoculation of an aged 20 % maltose M9 medium with fresh inocula

To determine persistence of A1MO2 and 5.6 in aged cultures 250 mL flasks containing 50 mL of fresh M9 20 % maltose medium were inoculated with both single and mixed ratio cultures of both strains (Table 2.7), prepared using the methods described in Sections 2.3.2 and 2.3.5, which were then

incubated at 30 °C in an Infors orbital shaker at 200 rpm for six days. After six days the cultures were centrifuged for ten minutes at 12,410 *g* to pellet the cells. The liquid supernatant was decanted and filter sterilised using a 0.2 µm filter. The filtered supernatant was added to sterile 250 mL flasks and re-inoculated with fresh inocula for the corresponding treatments in Table 2.7, which were also produced using the method in Section 2.3.2.

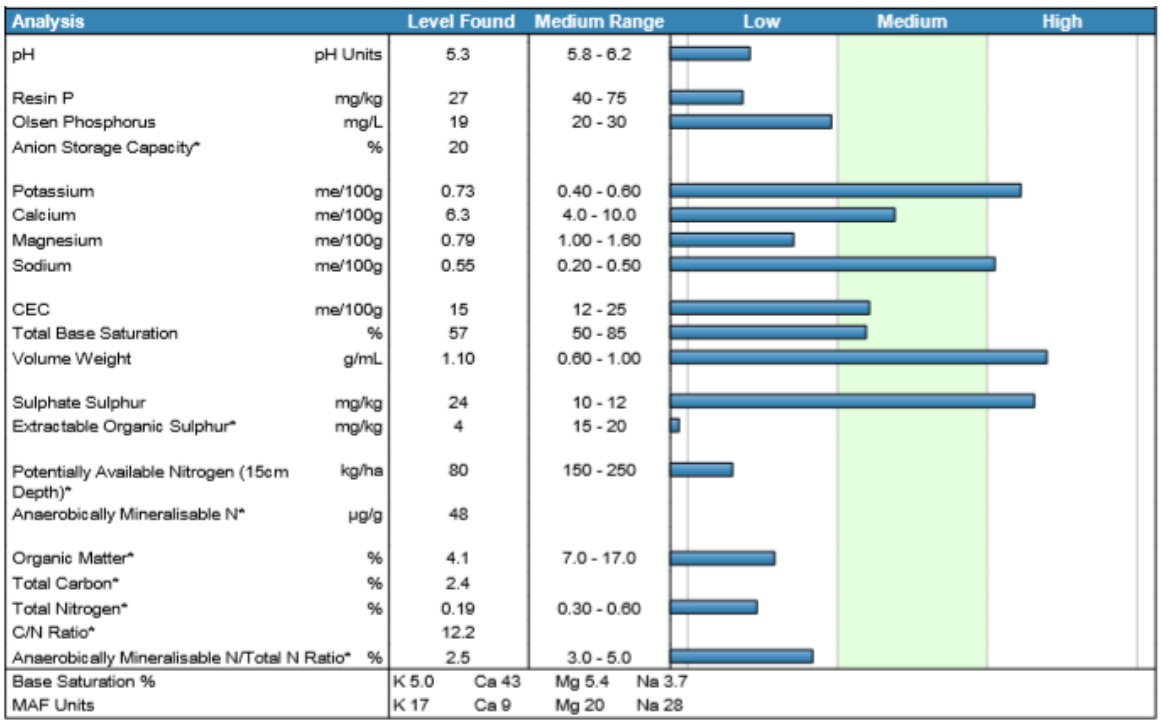
Table 2.7. The volumes of adjusted overnight culture samples combined to produce 500 µL single and mixed ratio inocula for competition assays in aged M9 20 % maltose medium.

Treatment	Ratio	Strain A (µL)	Strain B (µL)
1	100:0	500	0
2	0:100	0	500
3	90:10	450	50
4	70:30	350	150
5	50:50	250	250

2.4 Assessment of competition between *Serratia* spp. in soil microcosms

2.4.1 Analysis of soil nutrient content

All soil-based experiments conducted during this study used a *Serratia*-free non-sterile Wakanui silt loam. When not in use the soil was stored in a separated outdoor storage bin at AgResearch Lincoln. An overview of the nutrients contained within, as determined by Hill Laboratories, is presented in Figure 2.4 (R. J. Hill Laboratories Limited, Canterbury, New Zealand). The full soil analysis output is displayed in Appendix A.6. Prior to use in experiments the soil was checked for the presence of *Serratia* using an enumeration method (Section 2.4.5) and plating on a CTA medium, this ensured that no free-living background *Serratia* spp. would be included during microcosm experiments and the pot trial. Unless otherwise stated, all soil-based experiments were kept in double containment storage at 15°C, meeting PC2 requirements.



Analysis		Level Found	Medium Range	Low	Medium	High
pH	pH Units	5.3	5.8 - 6.2			
Resin P	mg/kg	27	40 - 75			
Olsen Phosphorus	mg/L	19	20 - 30			
Anion Storage Capacity*	%	20				
Potassium	me/100g	0.73	0.40 - 0.60			
Calcium	me/100g	6.3	4.0 - 10.0			
Magnesium	me/100g	0.79	1.00 - 1.60			
Sodium	me/100g	0.55	0.20 - 0.50			
CEC	me/100g	15	12 - 25			
Total Base Saturation	%	57	50 - 85			
Volume Weight	g/mL	1.10	0.60 - 1.00			
Sulphate Sulphur	mg/kg	24	10 - 12			
Extractable Organic Sulphur*	mg/kg	4	15 - 20			
Potentially Available Nitrogen (15cm Depth)*	kg/ha	80	150 - 250			
Anaerobically Mineralisable N*	µg/g	48				
Organic Matter*	%	4.1	7.0 - 17.0			
Total Carbon*	%	2.4				
Total Nitrogen*	%	0.19	0.30 - 0.60			
C/N Ratio*		12.2				
Anaerobically Mineralisable N/Total N Ratio*	%	2.5	3.0 - 5.0			
Base Saturation %		K 5.0	Ca 43	Mg 5.4	Na 3.7	
MAF Units		K 17	Ca 9	Mg 20	Na 28	

Figure 2.4. Output from Hill Laboratories highlighting the levels of various nutrients contained within the Wakanui silt loam soil when compared to a baseline interpretation reference soil.

2.4.2 Preparation of soil for soil microcosms and pot trial

Prior to use in microcosm and pot trial experiments the soil was passed through 4 mm and 2 mm sieves to remove any foreign matter. Prior to any adjustments the soil contained a gravimetric water content varying between 7 and 9 % w/v. The gravimetric water content of the soil was determined using Equation 1 after the soil had been incubated for 24 hours at 80 °C:

(1)

$$Water\ content\ \% = \left(\frac{wet\ weight\ (g) - dried\ weight\ (g)}{dried\ weight\ (g)}\right) * 100$$

Soil was adjusted to a moisture content of 20 % w/v prior to the commencement of microcosm experiments, with a portion of the moisture added being made of liquid bacterial inocula (Section 2.4.3). A total of 20 g (± 0.5 g) of moisture adjusted soil was added to 35 mL plastic tubes and secured with a screw-on cap. Additional sacrificial soil samples were prepared and used to monitor moisture content during assays, with moisture adjustments being made using Equation 1 to ensure the soil moisture content was maintained at 20 %. Unless specified all microcosm assays used three replicates per treatment.

Soil used in the pot trial was adjusted to a moisture content of 20 % w/v prior to the commencement of the trial, with a portion of the moisture added being made of liquid bacterial inocula (Section 2.4.3). A total of 125 g (± 3 g) of moisture adjusted soil was added to rectangular plastic pots (6 x 6 x 7 cm). The pot containing the soil was placed inside another pot containing an absorbent liner to prevent larvae from passing through the holes in the bottom of the pot. The pots were kept in an outdoor mesh-walled shadehouse at AgResearch, Lincoln, and were watered every one-to-two days (weather dependent) with a hose on a low-pressure misting setting. The pot trial was conducted during April of 2021, with average monthly temperatures between 18 and 7 °C. The pot trial assay used ten replicates per treatment, with each replicate containing a single healthy grass grub larvae and three Perennial ryegrass (*Lolium perenne*) seeds (Grasslands Samson (Nil Endophyte; Line A1104)).

2.4.3 Inoculation of soil with liquid bacterial cultures

Overnight cultures were used to inoculate soil samples following an adapted method from Hurst et al. (2020) in which the 20 % adjusted moisture content included a small proportion of liquid inoculant (typically 300 µL in a 20 g soil microcosm). Using Equation 2, bacteria were added to the soil at a rate of 5 x 10^5 CFU/g for wild-type and mixed inocula assays.

(2)

$$\frac{X\ mL}{Desired\ soil\ CFU} = \frac{1\ mL}{Culture\ CFU}$$

The moistened soil was mixed thoroughly with the liquid inocula in large plastic bags prior to use enabling better distribution of the inoculant throughout the soil.

2.4.4 Inoculation of soil used in the pot trial with liquid inocula

The soil used in the pot trial assay was inoculated using the same method detailed in Section 2.4.3, however non-pathogenic *Serratia* strains 5.6 and 477 were also added to the soil at rates of 5 x 10^4, 10^5, and 10^6 CFU/g (Table 2.8). Following inoculation of soil with non-pathogenic strains, the pots were filled with the designated amount (125 g), into which three perennial ryegrass (*L. perenne*) seeds were sown at a depth of approximately 1 cm. A single healthy pre-screened grass grub larva exhibiting active feeding behaviour (Chapter 2.5.2) was added to each pot and left for 15 minutes to burrow into the soil; any larvae which did not burrow were replaced. After three days the pathogenic strains of A1MO2 or AGR96X were added via liquid drench of 2.3 mL via pipetting at a rate of 5 x 10^5 CFU/g. The pots

were watered after inoculation with the pathogenic strains to assist in the dispersal of the bacteria through the soil.

Table 2.8. The rates of pathogenic and non-pathogenic *Serratia* strains added to soil during pot trial.

Treatment	Name	Non-pathogen	CFU/g	Pathogen	CFU/g
1	Blank	-	-	-	-
2	A1MO2 ctrl	-	-	A1MO2	5×10^5
3	5.6 ctrl	5.6	5×10^5	-	-
4	477 ctrl	477	5×10^5	-	-
5	AGR96X ctrl		5×10^5		
6	5.6 (5×10^4)	5.6	5×10^4	A1MO2	5×10^5
7	5.6 (5×10^5)		5×10^5		
8	5.6 (5×10^6)		5×10^6		
9	477 (5×10^4)	477	5×10^4		
10	477 (5×10^5)		5×10^5		
11	477 (5×10^6)		5×10^6		
12	* 5.6 (5×10^5)	5.6	5×10^5	AGR96X	5×10^5
13	* 477 (5×10^5)	477	5×10^5		

- denotes no strain added, * denotes larvae were challenged with AGR96X instead of A1MO2.

2.4.5 Enumeration of bacteria from soil samples

The method for enumerating bacteria from the soil was based on a previously published method by O'Callaghan and Jackson (1993). Twenty g of soil was added to a flask and brought to a 1:10 dilution with a SpecM-tween buffer (1 mL tween 80, 1 g tetrasodium pyrophosphate, 1 L water) and sonicated in a water bath for three minutes. One hundred µL of the aqueous phase was extracted from approximately 3 cm below the meniscus and serially diluted to a factor of 10^{-4} in 0.1 % bacto-peptone solution (1 g bacto-peptone in 1 L Milli-Q water). The samples were spread across LB agar and CTA plates and incubated overnight at 30 °C for up to three to five days.

2.4.6 Retrieval of bacteria from aged soil samples

To determine whether the inoculated bacteria contained in aged and desiccated soil samples were able to be cultured two retrieval methods were assessed. In the first method 5 g of soil from the microcosms was added to a 50 mL plastic centrifuge tube, into which 5 mL of either 50 % or 10 % of LB broth, or an M9 20 % casamino acids medium was added. The samples were mixed and stored at 4 °C. After three days 100 µL was removed via pipette and serially diluted to a dilution of 10^{-3} in 0.1 % bacto-peptone solution, with 50 µL being plated in LB agar and CTA plates. After three days 1 mL of 20 %

itaconate broth was added to the tubes, with the tubes being sampled after 14 days post-addition of the itaconate broth using the same dilution method. The second revival method used was detailed previously by Zhao et al. (2021) in which 5 g of the soil was wetted to 30 % of its holding capacity with water (1.7 mL) and incubated at 28 °C for two weeks. The soil was serially diluted to a dilution of 10^{-3} in 0.1 % bacto-peptone, with 50 µL being plated in LB agar and CTA plates.

2.5 Assessment of competition between *Serratia* spp. within grass grub larvae

2.5.1 Collection of grass grub larvae for use in bioassays

The 3rd instar grass grub larvae used in this project were collected from various pasture sites in Canterbury (AgResearch Lincoln campus, Lincoln University Research Dairy Farm, Foundation for Arable Research test paddocks in Lincoln and Rakaia) and the West Coast (Cape Foulwind). The larvae were collected from a soil depth of up to 10 cm, with any 2nd instar larvae or larvae showing symptoms of amber disease being discarded. Upon collection the larvae were stored either in ice-cube trays or pots containing soil. The collected larvae were maintained at 4 °C prior to their use in experiments.

2.5.2 Preparation of bioassays and pre-feeding of larvae

Bioassays were performed in ice-cube trays using a standard bioassay method described previously by Jackson and Saville (2000). Each individual well in the trays had a volume of approximately 3.38 cm^3 (Fig. 2.5). Three days prior to the commencement of any experiments, larvae were pre-screened for both feeding behaviour with carrot pieces cut into cubes of approximately 2 to 3 mm^3 and an assessment of phenotypic characteristics. The larvae which retained a healthy phenotype and feeding behaviour after three days were used in experiments. The ice-cube trays containing larvae were kept within sealed plastic bags alongside a moistened paper towel and maintained at 15 °C in a Contherm 1000C incubator.

Figure 2.5. Photograph of grass grub larvae contained within an ice-cube tray during a bioassay.

During bioassay experiments the larvae used in the various treatments were split between multiple trays, with the placement of the larvae being different between each tray to prevent edge effects, such as differences in airflow on the end well as opposed to the central wells. The larvae were also separated with a row between them, as depicted in Figure 2.5, to ensure that larvae would not enter into wells occupied by larvae in other treatments. The larvae were observed at pre-determined points during the bioassays, often on days 1, 2, 3, 4, 5, 7, 9, 10, and 12/14 following the initiation of the assay, depending on the assay duration. Every three days the moistened paper towels, used to provide moisture, were changed to prevent contamination. The trays were also changed every three days, and the larvae were transferred to clean trays using plastic forceps, with different forceps being used for each treatment to prevent cross-contamination. The larvae were also re-fed at the same time and were supplied with fresh carrot cube pieces. During observational periods the larvae were assessed for feeding behaviour, with notes being made on the proportion of carrot eaten, and the colour of the gut, with a darker gut colour and active feeding behaviour indicating health. Following the occurrence of death or the conclusion of the bioassay, larvae which were not macerated were placed into a bag and frozen for at least 24 hours, after which they were autoclaved.

2.5.3 Inoculation of larvae

Overnight cultures used in the inoculation of grass grubs were prepared as detailed in Section 2.3.2, and larvae were typically dosed using coated carrot cube pieces. When using liquid inocula a total of 5 µL of inoculant at the appropriate dosage was pipetted on top of carrot cube pieces contained in ice-cube tray wells and allowed to ‘dry’ for 30 – 60 seconds prior to the addition of larvae to prevent movement from disturbing the inoculant. Alternately, a LB agar plate with a fresh bacterial lawn produced from overnight incubation at 30 °C was used for inoculation, with the carrot cube pieces being rolled in the lawn prior to their addition to the ice-cube tray wells.

2.5.4 Maceration of larvae

Larvae selected for maceration were submerged in a 1.5 mL microcentrifuge tube containing 1 mL of 70 % ethanol for one minute and then shaken vigorously to remove any surfactant waste or dirt. The larvae were removed and dried on a paper towel before being submerged in a clean 1.5 mL microcentrifuge tube containing 1 mL of Milli-Q water to remove any residual ethanol. The larvae were removed and dried again before being submerged in a clean 1.5 mL microcentrifuge tube containing 900 µL of PBS. Larvae were macerated in the PBS using a sterile micropestle, with a different pestle used for each larva. The larval macerate was serially diluted in PBS up to a 10^{-4} dilution with 50 µL of selected dilutions spread across both LB agar and CTA plates, which were incubated at 30 °C overnight and for three to five days, respectively.

2.5.5 Detection of pADAP horizontal transfer within larvae

Horizontal gene transfer (HGT) of pADAP between *Serratia* spp. within larvae was detected via direct dilution plating of 1:10 serially diluted whole-larval macerate samples on LB agar containing both spectinomycin and kanamycin at the required concentrations (Table 2.1). Putative colonies were assessed via patching across agar plates containing either tetracycline, spectinomycin, and kanamycin, and via PCR using the kanamycin and spectinomycin primer sets (Table 2.4). Prospective transconjugants were those which grew on both kanamycin and spectinomycin plates, but not tetracycline, and produced bands of the correct size with the kanamycin and spectinomycin primers sets, but not tetracycline.

2.6 Statistical analysis

2.6.1 Basic statistical analysis and software used

Basic calculations relating to percentages, standard deviation, the calculation of CFUs from growth data, and significant differences between CFU count with a Student's T-test were performed using Microsoft Excel (version 2105). Additional calculations for the area under a curve, used during fluorescence intensity assays, were conducted in GraphPad Prism (version 9.0.1). The calculation of significant difference for disease rates during bioassays and the pot trial observation assay were performed using Minitab (version 19.1.1) using group-wise logistic regression models.

2.6.2 Fitting multiple linear regression models to CFU data

Multiple Linear regression models were fitted to CFU data to determine the rate of change in the CFU counts over assays with durations exceeding five days. The CFU data was initially transformed into $\log_{10}$ values in Excel prior to use. Regressions were performed in Excel, modelling the change in CFU per day,

which corresponded with a one-way analysis of variance (ANOVA) assessment to validate model accuracy.

2.6.3 Pair-wise regression models for *In vivo* and pot trial CFU data

CFU data was imported into Minitab and analysed using the regression option. The log_{10} CFU data was used as the continuous response, the day was chosen as the continuous predictor, and the treatment groups were used as categorical predictors. During pot trial data, when the differing treatments were being compared to the control group for a strain (5.6 and 477 controls), the control group was used as a reference in the categorical predictor.

2.6.4 Calculation of bacteria doubling rates during exponential growth

Prior to the calculation of doubling rates CFU data generated during growth curve experiments was transformed into log_{10} values. The data used was selected from two points during the exponential growth period which formed a straight line. This was often the data recorded over a two-hour period between six- and ten-hours post-inoculation. The fitting of linear regression models to the data provided a slope value, as mentioned above. The slope was transformed from a log_{10} value using the Excel formula '=sum(10^x)', where x is the log_{10} slope value. The resultant value was divided by 120 to provide the doubling time of a strain during exponential growth in minutes, with the use of 120 covering the minutes in the two-hour assessment period.

2.6.5 Lotka/Volterra-style competition models

Mixed inocula competition models were calculated based on previous models by Mallet (2012). The model parameters were calculated in R using the gauseR package (Clark et al., 2021), which derived data from time-lagged per-capita growth rate analyses. This data was used to fit the models, from which the parameters ($r_{1/2}$, $a_{11/22}$, and a_{21}/a_{12}) were collected. The data was derived from log_{10} transformed CFU data obtained during mixed inoculation assessments conducted in both LB broth and M9 medium over a period of seven days. Once the original models had been produced to calculate coefficient values, the data was optimised using the "lv_optim" (gauseR), "optim", and "ode" functions to directly fit the dynamics from the models to the observed data using vectors of the previously fitted log parameter values, which reduces the degree of errors when calculating the original time-lagged observations. The calculated competition coefficients of the strains were compared to quantify competitive behaviour during mixed inoculation.

Chapter 3

Competition between *Serratia* spp. in liquid culture

3.1 Introduction

The characterisation of growth dynamics and the interactions between various pathogenic and non-pathogenic *Serratia* strains is central to the aims of this project. These initial assessments will provide an overview for how the availability of nutrients within the liquid growth media might impact competition between the strains, and how this may impact their fitness. One of the key aspects to be explored are the dynamics between the pathogenic *S. entomophila* strain A1MO2, and its non-pathogenic, isogenic, heat-cured, pADAP-free derivative, 5.6. This will provide insight into the fitness impact of plasmid carriage for A1MO2 and how this might alter factors such as growth rates and generation times between the strains. It is well established that local competition for resources leads to a reduction in cooperation between bacterial species (Fiegna and Velicer, 2005; Kümmerli et al., 2010) in which competition in a mixed infection selects for the bacterium with the greatest competitive ability (Brown et al., 2002). Previous work with non-pathogenic isolates of *B. thuringiensis* showed that non-pathogenic strains had increased growth rates when cultured *in vitro* due to the lack of metabolic pressure of toxin production (Raymond et al., 2007), resulting in a reduction of virulence from the pathogenic strain due to selective pressure and a lack nutrients to sustain toxin production (Brown et al., 2002; Gardner et al., 2004). Investigations will also be conducted using the hyperpathogenic *S. proteamaculans* strain, AGR96X, (Hurst et al., 2018), enabling the assessment of a hypervirulent strain during competition with other *Serratia* spp. in the liquid medium.

These interactions will be explored using various mixed ratio inocula to determine the persistence of A1MO2, a pADAP-bearing strain, when inoculated alongside its isogenic plasmid-free derivative, 5.6, or a natural *S. entomophila* plasmid-free isolate, such as 477, in both nutrient-rich and restricted-nutrient media. This will also allow for comparisons to be made between the dynamics of 5.6 and 477. Furthermore, the inoculation of restricted-nutrient M9 media will highlight the role of the nutrients in competition. The use of a glucose M9 medium will define whether the presence of glucose, a simple monosaccharide sugar, will influence interactions and persistence, as it can be preferentially metabolised ahead of other carbon sources (Koirala et al., 2015). Additionally, a casamino acids M9 medium was chosen to characterise growth when the strains were restricted to amino acids, as the utilisation of amino acids as nutrients in a growth medium has been shown to enhance growth for some bacterial species (Gehring et al., 2014) and increase competitive ability in others (Farrell and Finkel, 2003). The results generated will provide a starting point for the assessment of competition between the pathogenic and non-pathogenic strains in a controlled and simplified system.

3.2 Results

3.2.1 Characterisation of growth dynamics for wild-type *Serratia* spp.

An initial assessment of growth for wild-type *Serratia* strains A1MO2, 5.6, and AGR96X was conducted over a 48-hour period to produce a set of growth curves (method: Chapter 2, section 2.3.3). This indicated whether there were any noticeable differences in growth between the strains when inoculated in a nutrient-rich LB broth medium. The CFU data was also used to calculate the doubling rate of the strains (method: Chapter 2, section 2.6.3).

When inoculated separately into 50 mL LB broth similar dynamics in growth were recorded over the 48-hour assay duration for both wild-type A1MO2 and 5.6. An initial assessment at zero hours post-inoculation (hpi) indicated there was no difference between the average number of viable cells within the cultures, as evident from average CFU counts (n = 9) of 1.19×10^7 and 1.18×10^7 CFU/mL for A1MO2 and 5.6, respectively (Fig. 3.1). However, the initial count for AGR96X was significantly higher than both A1MO2 and 5.6 ($p < 0.05$), with a count of 1.51×10^7 CFU/mL. Over the duration of the lag phase, lasting between zero and two hpi, AGR96X maintained significantly higher CFU counts than both A1MO2 and 5.6. However, upon the onset of exponential growth, lasting between two and eight hpi, both A1MO2 and 5.6 initially grew at a faster rate than AGR96X. The fitting of linear regression models to the exponential growth data specified similar doubling times (T_d) for both A1MO2 (T_d = 38.3 min, R^2 = 0.97) and 5.6 (T_d = 35.3 min, R^2 = 0.96), but a slower time for AGR96X (T_d = 64.3 min, R^2 = 0.87). As a result of this slower replication rate for AGR96X, by the end of the exponential growth period the CFU count for AGR96X was significantly lower than both A1MO2 and 5.6 ($p < 0.05$). Despite this initial difference, over the majority of the stationary phase, between 12 and 36 hpi there was no difference between the CFU counts for all strains. However, by 48 hpi the CFU count of AGR96X decreased so it was significantly lower than both A1MO2 and 5.6 ($p < 0.05$). Upon the conclusion of the assay at 48 hpi the strains had reached CFU counts of 1.66×10^{10} CFU/mL for A1MO2, 1.68×10^{10} CFU/mL for 5.6, and 1.34×10^{10} CFU/mL for AGR96X. These growth curves indicated that when independently cultured in a nutrient-rich LB broth medium there was almost no difference in the recorded growth dynamics for A1MO2 and 5.6, with AGR96X also showing a very similar pattern of growth, albeit a longer initial doubling time.

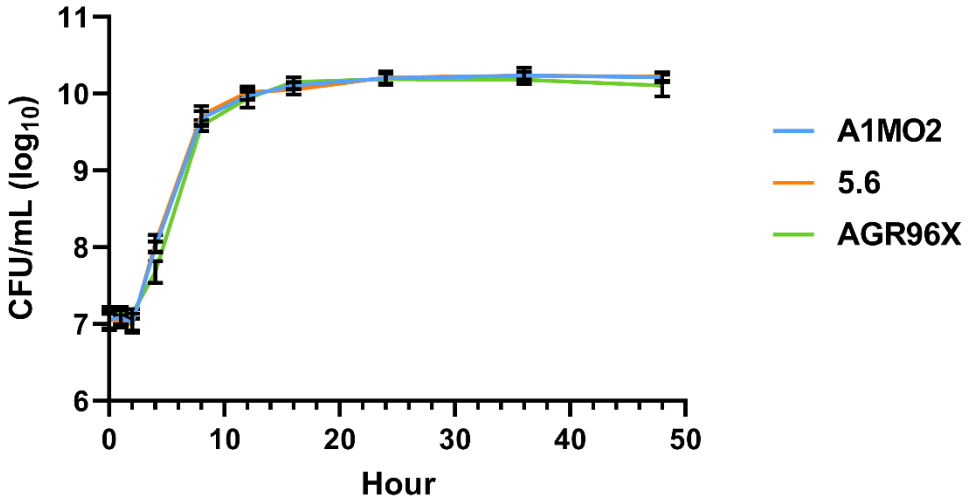


Figure 3.1. Average log_{10} CFU/mL counts (n = 9) for wild-type *Serratia* strains A1MO2, 5.6, and AGR96X inoculated separately and cultured in LB broth over 48 hours. The strains were cultured from respective inocula of 8.31 x 10^8, 6.98 x 10^8, and 8.37 x 10^8 CFU/mL. The results are presented with standard deviation error bars.

3.2.2 Assessing the impact of antibiotic cassette insertion on growth of tagged *Serratia* spp.

As outlined in Chapter 2, section 2.2.16, the insertion site for antibiotic cassettes into the *Serratia* chromosome, RegionA, was chosen as it contained the necessary restriction site and was located between two converging open reading frames. As such, the insertion of the antibiotic cassettes into the chromosome should not interfere with any upstream promotor regions or disrupt the genes located downstream from the insertion site. To validate that the insertion of an antibiotic cassette into RegionA did not impact fitness, the growth dynamics of the tagged *Serratia* strains A1MO2_tk and 5.6_s were compared to the dynamics recorded previously for the wild-type A1MO2 and 5.6 strains (Section 3.2.1).

A comparison between the growth of the strains when inoculated separately in LB broth medium without antibiotics showed very little difference in the growth dynamics of A1MO2_tk and 5.6_s when compared to wild-type A1MO2 and 5.6 (Fig. 3.2). There was only one instance of significant difference recorded between the strains, where the final CFU count for A1MO2_tk at 48 hpi was significantly higher than the count for wild-type A1MO2 ($p < 0.05$). A final average CFU count for A1MO2_tk (n = 3) of 2.04 x 10^{10} CFU/mL was recorded, compared to an average count for wild-type A1MO2 (n = 9) of 1.66 x 10^{10} CFU/mL. Furthermore, similar doubling times were recorded during exponential growth for both A1MO2_tk (T_d = 43.1 min, R^2 = 0.99) and wild-type A1MO2 (T_d = 38.3 min, R^2 = 0.97), and for 5.6_s (T_d = 29.6 min, R^2 = 0.96) and wild-type 5.6 (T_d = 35.3 min, R^2 = 0.96). This indicated that the insertion of the antibiotic cassettes into the A1MO2_tk and 5.6_s construct strains did not have an impact on their growth in liquid cultures.

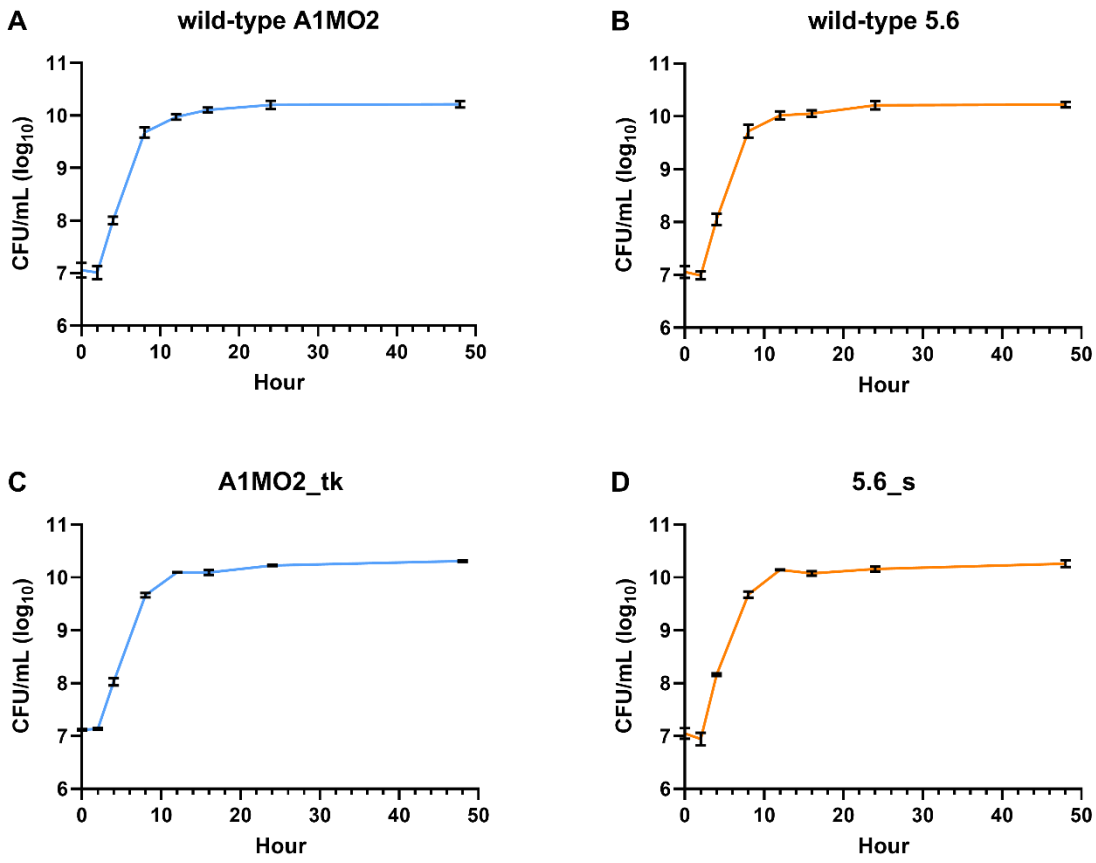


Figure 3.2. Average log_{10} CFU/mL counts for wild-type *Serratia* strains (A) A1MO2 and (B) 5.6 (n = 9) and tagged construct strains (C) A1MO2_tk and (D) 5.6_s (n = 3) when cultured separately in LB broth over 48 hours. The strains were cultured from respective inocula of 8.31 x 10^8, 6.98 x 10^8, 6.40 x 10^8, and 7.10 x 10^8 CFU/mL. The results are presented with standard deviation error bars

3.2.3 The impact of mixed inoculation with tagged *Serratia* spp. on growth dynamics

Following the confirmation that the insertion of the antibiotic cassettes into construct *Serratia* strains did not impact growth in culture, a series of mixed inoculation growth curves were produced. The tagged strains were cultured using the same growth curve method used previously in this chapter (sections 3.2.1 and 3.2.2), but the medium was inoculated with a mixture of A1MO2_tk and 5.6_s produced at a 50:50 ratio (A1MO2_tk:5.6_s) (method: Chapter 2, section 2.3.4).

3.2.3.a. Mixed inoculation of tagged A1MO2_tk and 5.6_s in an LB broth medium

The inoculation of LB broth with the 50:50 A1MO2_tk and 5.6_s mixed ratio inoculant resulted in similar initial CFU counts for both strains at zero hpi, with average CFU counts (n = 3) of 6.50 x 10^6 CFU/mL for A1MO2_tk and 7.73 x 10^6 CFU/mL for 5.6_s (Fig. 3.3. A). No significant changes in CFU were recorded for the strains over the initial lag period, lasting between zero and two hpi. The onset of the

exponential growth phase began between two and four hpi, lasting until eight hpi. A major difference in growth dynamics for the strains was seen during this phase. During the latter portion of exponential growth, between four and eight hpi, a faster doubling time was recorded for 5.6_s (T_d = 49.1 min R^2 = 0.99) than A1MO2_tk (T_d = 53.4 min, R^2 = 0.99). In consequence, 5.6_s had attained a significantly higher CFU count than A1MO2_tk by the end of the exponential growth phase ($p < 0.05$), with this difference being maintained for the remainder of the assay ($p < 0.05$ to < 0.0005). By the end of the assay at 48 hpi, the strains had reached CFU counts of 7.30 x 10^9 CFU/mL for A1MO2_tk and 1.17 x 10^{10} CFU/mL for 5.6_s. These results indicated that when inoculated together, 5.6_s exhibited a competitive advantage over A1MO2_tk during the exponential growth phase.

This conclusion was also reached when comparing the relative proportion of viable cells for each of the strains isolated from the culture over the duration of the assay (Fig. 3.3. B). Whilst an equal proportion was recorded between the strains following inoculation (46:54, A1MO2_tk:5.6_s), the onset of exponential growth phase saw the proportion of 5.6_s cells increase. This was most evident at eight hpi (35:65), when significantly more 5.6_s cells were isolated than A1MO2_tk ($p < 0.05$). However, as this proportion was maintained for the remainder of the assay until 48 hpi (± 4 %), this suggested that the competitive advantage of 5.6_s only occurred during the exponential growth phase.

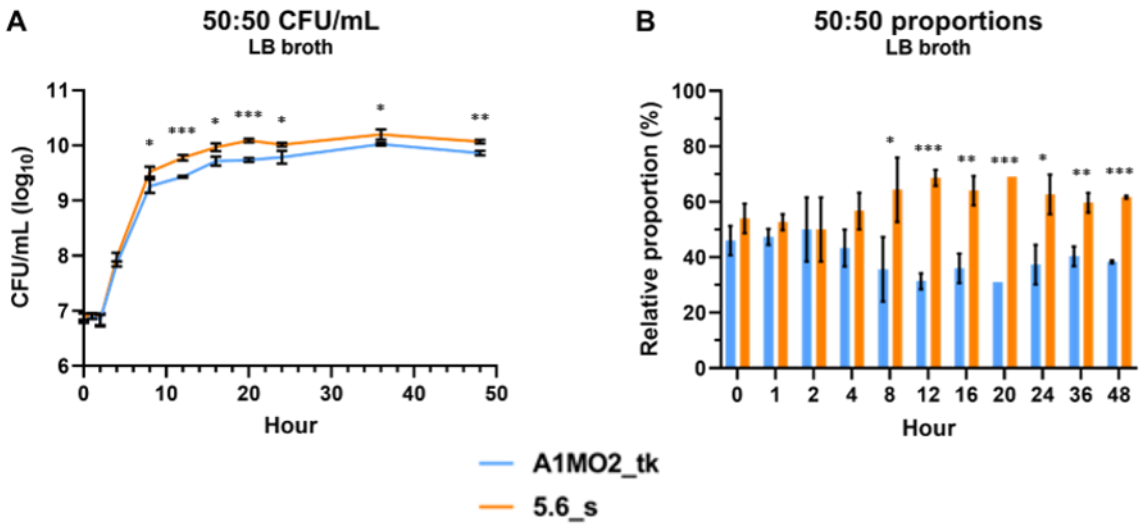


Figure 3.3. (A) Average log_{10} CFU/mL counts (n = 3) for tagged *Serratia* strains A1MO2_tk and 5.6_s when cultured together in LB broth over 48 hours following inoculation at a 50:50 ratio (A1MO2_tk:5.6_s). The strains were cultured from respective inocula of 3.79 x 10^8 and 4.30 x 10^8 CFU/mL. (B) The changes in the average relative proportion (%) (n = 3) of cells isolated from the cultures. Results for both graphs are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.3.b. Mixed inoculation of tagged A1MO2_tk and 5.6_s in a 20 % glucose M9 medium

A1MO2_tk and 5.6_s were inoculated separately into the glucose medium to characterise growth dynamics of the strains in the absence of competition (Fig. 3.4). No significant differences were recorded between the CFU counts of the strains, and similar doubling times for A1MO2_tk (T_d = 34.1 min, R^2 = 0.98) and 5.6_s (T_d = 28.0 min, R^2 = 0.99) were observed during the exponential growth phase. However, a longer initial lag phase was recorded than when the strains were cultured in LB broth, with the lag phase lasting between zero and four hpi, delaying the onset of exponential growth, which occurred between six and 12 hpi. Furthermore, culturing the strains in the glucose medium resulted in final CFU counts one log lower than when the strains were cultured separately in LB broth.

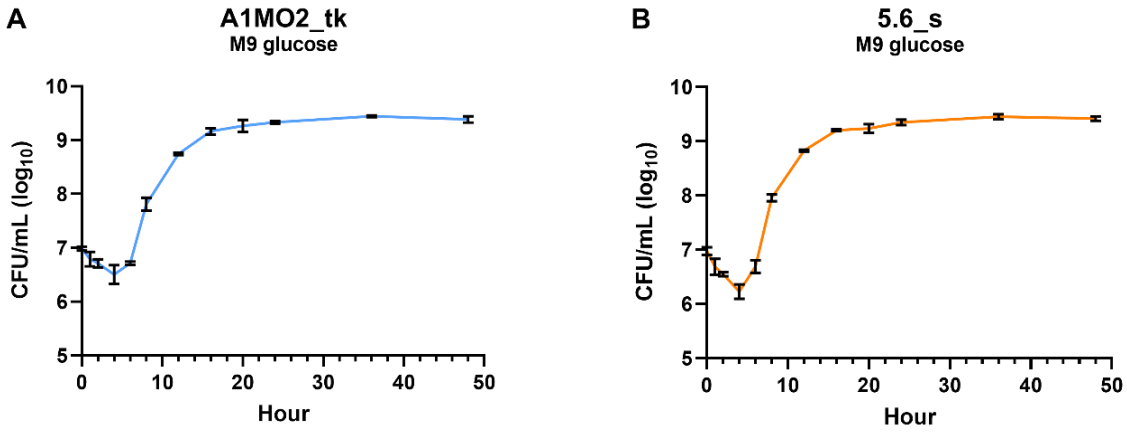


Figure 3.4. Average log_{10} CFU/mL counts (n = 3) for (A) A1MO2_tk and (B) 5.6_s when inoculated separately in a 20 % glucose M9 medium over 48 hours. The strains were cultured from respective inocula of 6.00 x 10^8 and 6.20 x 10^8 CFU/mL. Results are presented with standard deviation error bars.

The inoculation of the glucose medium with the 50:50 mixed inoculant of A1MO2_tk and 5.6_s resulted in similar CFU counts for the strains at zero hpi, with average CFU counts (n = 3) of 4.63 x 10^6 CFU/mL for A1MO2_tk and 5.00 x 10^6 CFU/mL for 5.6_s (Fig. 3.5. A). The inoculation of both strains together caused an increase to the duration of the initial lag phase, which lasted between zero and six hpi. Over this period the CFU count of A1MO2_tk remained stable, whereas the CFU count of 5.6_s decreased. This led to A1MO2_tk having significantly higher CFU counts than 5.6 between two to six hpi ($p < 0.05$ to < 0.005). However, following the onset of exponential growth after six hpi the doubling time for 5.6_s (T_d = 25.0 min, R^2 = 0.98) was much faster than A1MO2_tk (T_d = 65.6 min, R^2 = 0.98) between six to eight hpi. As a result of this growth difference the CFU count for 5.6_s was significantly higher than A1MO2_tk at the end of the exponential growth phase at 12 hpi ($p < 0.05$). Despite this

difference similar CFU counts were recorded for both strains over the stationary phase, leading to final CFU counts at 48 hpi of 1.09 x 10^9 CFU/mL for A1MO2_tk and 1.52 x 10^9 CFU/mL for 5.6_s. This suggested that 5.6_s also had a competitive advantage during the exponential growth phase in the restricted-nutrient medium when compared to A1MO2_tk, however, unlike the growth recorded in lb broth, the growth advantage exhibited by 5.6_s dissipated following the onset of the stationary phase.

These dynamics are also reflected in the relative proportions of cells isolated (Fig. 3.5. B). Following inoculation at an equal ratio (48:52, A1MO2_tk:5.6_s) the proportion of A1MO2_tk cells isolated increased during the initial lag phase over the first six hours of the assay (82:18). However, following the onset of the exponential growth phase at eight hpi the faster doubling time of 5.6_s caused a change in the proportion (40:60), which was maintained for the remainder of the assay at 48 hpi (± 9 %). Similar to culturing in LB broth, whilst this indicated an increase of fitness for 5.6_s during the exponential growth phase, this advantage dissipated following the onset of the stationary phase.

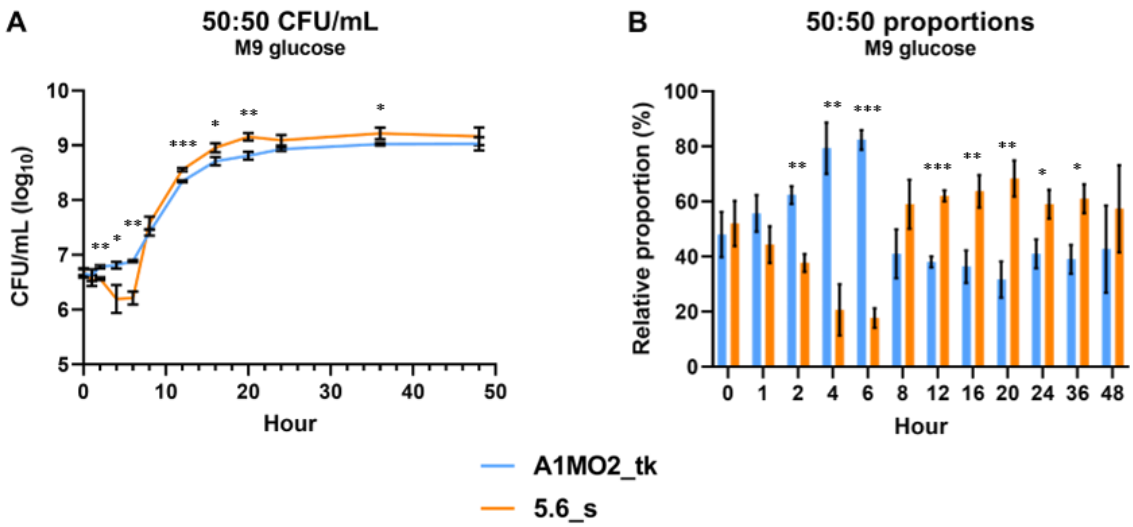


Figure 3.5. (A) Average log_{10} CFU/mL counts (n = 3) for tagged *Serratia* strains A1MO2_tk and 5.6_s when cultured together in a 20 % glucose M9 medium over 48 hours following inoculation at a 50:50 ratio (A1MO2_tk:5.6_s). The strains were cultured from respective inocula of 3.20 x 10^8 and 2.70 x 10^8 CFU/mL. (B) The changes in the average relative proportion (%) (n = 3) of cells isolated from the cultures. Results for both graphs are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.3.c. Mixed inoculation of tagged A1MO2_tk and 5.6_s in a 20 % casamino acids M9 medium

A1MO2_tk and 5.6_s were cultured separately in the casamino acids medium to characterise the growth of the strains in the absence of competition (Fig. 3.6). Following inoculation only one instance of a significant difference was recorded between the strains, occurring at zero hpi, where the average CFU count (n = 3) for A1MO2_tk was significantly higher than 5.6_s ($p < 0.05$). Furthermore, similar

doubling times were recorded for both A1MO2_tk (T_d = 55.1 min, R^2 = 0.99) and 5.6_s (T_d = 60.3 min, R^2 = 0.99) during the exponential growth period. In addition, the durations for both the lag and exponential growth phases were similar to LB broth, though the strains still attained CFU counts one log value lower than when they were cultured in the LB broth.

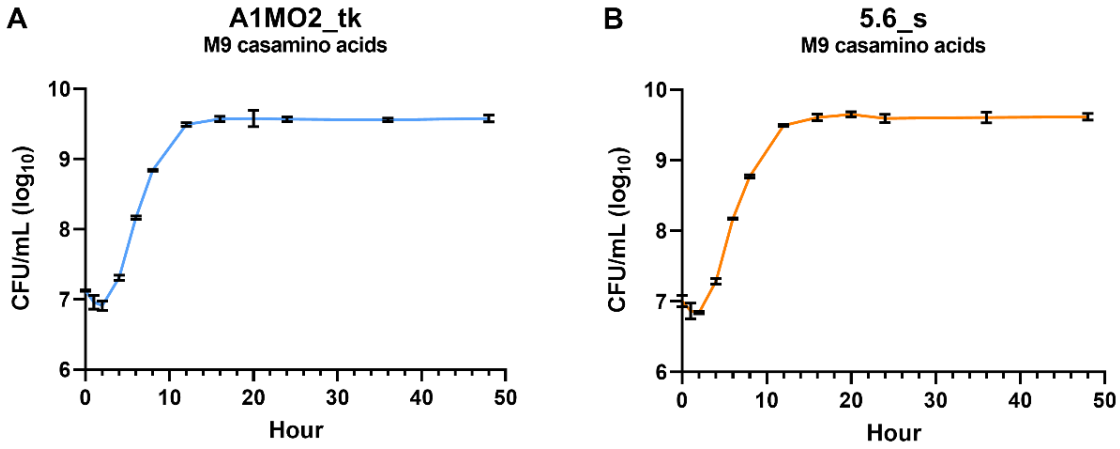


Figure 3.6. Average log_{10} CFU/mL counts (n = 3) for (A) A1MO2_tk and (B) 5.6_s when cultured separately in a 20 % casamino acids M9 medium over 48 hours. The strains were cultured from respective inocula of 6.90 x 10^8 and 5.90 x 10^8 CFU/mL. Results are presented with standard deviation error bars.

The inoculation of the casamino acids medium with an equal proportions (50:50) of A1MO2_tk and 5.6_s resulted in similar CFU counts at zero hpi, with average CFU counts (n = 3) of 5.03 x 10^6 CFU/mL for A1MO2_tk and 6.20 x 10^6 CFU/mL for 5.6_s (Fig. 3.7. A). Similar to when the strains were cultured together in the glucose medium, A1MO2_tk maintained its CFU count during the lag phase, whereas 5.6_s decreased in CFU. This resulted in a significant difference between the strains following the onset of the exponential growth phase at four hpi, where the CFU count for A1MO2_tk was significantly higher than 5.6_s ($p < 0.005$). However, during the latter portion of the exponential growth phase, between six to eight hpi, 5.6_s once again displayed a faster doubling time (T_d = 43.4 min, R^2 = 0.99) than A1MO2_tk (T_d = 73.1 min, R^2 = 0.99). As a result of this difference, 5.6_s attained a significantly higher CFU count than A1MO2_tk by the end of the exponential growth phase at 12 hpi ($p < 0.05$). Whilst this difference was maintained over the initial portion of the stationary phase, by 24 hpi there was no longer a difference between the CFU counts of the strains, with this similarity persisting for the remainder of the assay at 48 hpi. This resulted in final CFU counts of 1.51 x 10^9 CFU/mL for A1MO2_tk and 1.61 x 10^9 CFU/mL for 5.6_s. Similar to previous assays, the faster doubling time for 5.6_s during the exponential growth phase provided evidence of a competitive advantage for the strain.

These changes in dynamics are also reflected in the changes in relative proportion of cells isolated from the cultures (Fig. 3.7. B). Following inoculation at an equal ratio (45:55, A1MO2_tk:5.6_s), A1MO2_tk increases in proportion over the duration of the lag phase (66:34) However, due to the faster doubling time of 5.6_s during the early exponential growth period at six hpi more cells were being isolated from the culture (40:60). These proportions were maintained into the beginning of the stationary phase at 20 hpi (± 6 %), however, a shift at 24 hpi resulted in an equal ratio of cells being isolated (49:51), which was maintained until 48 hpi (± 2 %). This indicated that whilst there was a competitive advantage for 5.6_s during the exponential growth phase, a shift in dynamics at 24 hpi resulted in a similar fitness for both strains.

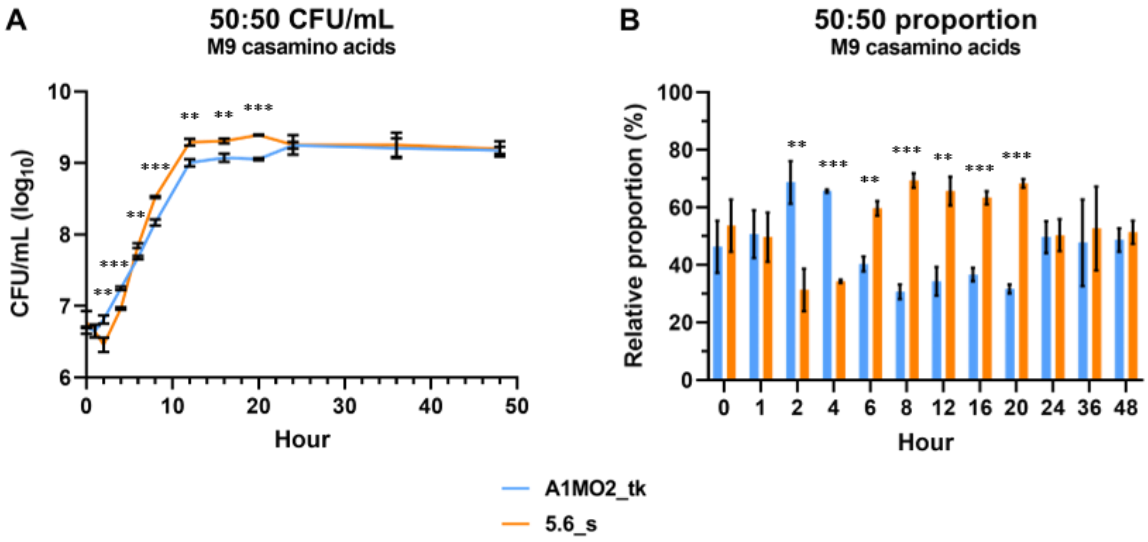


Figure 3.7. (A) Average log_{10} CFU/mL counts (n = 3) for tagged *Serratia* strains A1MO2_tk and 5.6_s when cultured together in a 20 % casamino acids M9 medium over 48 hours following inoculation at a 50:50 ratio (A1MO2_tk:5.6_s). The strains were cultured from respective inocula of 3.15 x 10^8 CFU/mL. (B) The changes in the average relative proportion (%) (n = 3) of cells isolated from the cultures. Results for both graphs are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.4 Assessing the persistence of tagged *Serratia* spp. when inoculated at mixed ratios

The growth assessments conducted over a 48-hour period showed that when A1MO2 and 5.6 were inoculated and co-cultured at equal proportions 5.6_s displayed a competitive advantage over A1MO2_tk during the exponential growth phase with a faster replication rate during mixed inoculation, leading to significantly higher CFU counts (Section 3.2.3). Further investigation was conducted to determine how this might impact the outcome of competition between the strains over

a seven-day period (method: Chapter 2, section 2.3.5), and whether further differences in the dynamics of the strains would be observed through differences in the persistence of the strains in medium. This helped to provide an insight into how the gradual reduction of nutrients would shape the outcome of competition. The selected media (LB broth and M9 media) were inoculated separately with both A1MO2_tk (100:0) and 5.6_s (0:100), and with a range of mixed ratio inocula (90:10, 70:30, 50:50, 30:70, 10:90, A1MO2_tk:5.6_s). Simple linear regression models were used to determine the differences between the persistence of the strains in the medium (method: Chapter 2, section 2.6.2). In addition to competition between A1MO2 and 5.6, additional experiments were conducted between A1MO2_tk and 477_s and between A1MO2_tk and AGR96X_s. This allowed for the outcome of competition over a longer period to be determined between A1MO2 and 477, a naturally occurring non-pathogenic strain non-isogenic to A1MO2, and between two pathogenic *Serratia* species. The persistence of the strains would be characterised by a slower rate of CFU decrease in the medium, where a slower rate of decrease may denote an increased level of bacterial fitness over a longer period of time. A previous assessment with *E. coli* strain hipQ (1000-fold higher rate of switching to persister cells) demonstrated that slower growth due to increased persistence mitigated treatment against antibiotics and enabled a trade-off between slower growth and increased survivability through persister cells (Kussell et al., 2005).

3.2.4.a. Measuring persistence of tagged A1MO2_tk and 5.6_s at mixed ratios in LB broth

Prior to competition both A1MO2_tk (100:0) and 5.6_s (0:100) were assessed separately following inoculation to characterise the dynamics of the strains over an extended period of time in the absence of competition. The cultures were first assessed one day post-inoculation (dpi), where the average CFU count for A1MO2_tk was significantly lower than 5.6_s, with average CFU counts of 1.19×10^{10} CFU/mL for A1MO2_tk and 1.53×10^{10} CFU/mL for 5.6_s (Fig. 3.8. A - B). Over the duration of the assay both strains continually decreased in CFU, with similar final CFU counts at seven dpi of 1.91×10^{8} CFU/mL for A1MO2_tk and 1.98×10^{8} CFU/mL for 5.6_s. Fitting a multiple regression model to the $\log_{10}$ CFU data revealed that there was no significant difference between the dynamics of the strains, indicating that both strains were exhibiting a similar decrease in CFU. This model was validated with a subsequent one-way ANOVA, with an F statistic value (F (2, 4) = 24.2, p = 0.006) signifying significance.

Whilst the separate culturing of A1MO2_tk and 5.6_s resulted in a similar pattern of CFU decrease between the strains, the results of mixed inoculation hint at the onset of competition between the strains. These differences in persistence as a result of competition were most evident in the mixed ratio treatments comprised primarily of A1MO2_tk: the 90:10 and 70:30 treatments (A1MO2_tk:5.6_s) (Fig. 3.8. C - D). Whilst both strains decreased in CFU, as was to be expected during this experiment,

the CFU counts for 5.6_s decreased at a rate significantly slower than A1MO2_tk ($p < 0.0005$), indicating an increased persistence of the cells within the cultures. This was most apparent in the 90:10 culture, where, at one dpi, A1MO2_tk had attained a significantly higher average CFU count (n = 3) than 5.6_s ($p < 0.0005$). However, due to the slower rate of 5.6_s decrease, by three dpi there was no longer a significant difference between the strains, and by five dpi 5.6_s had attained CFU counts significantly higher than A1MO2_tk ($p < 0.0005$), which was maintained until the conclusion of the assay at seven dpi.

A higher persistence for 5.6_s was also recorded when both strains were inoculated at an equal ratio (50:50, A1MO2_tk:5.6_s) (Fig. 3.8. E). However, in this culture 5.6_s had already attained a CFU count significantly higher than A1MO2_tk by one dpi ($p < 0.005$). This suggests that competition had already occurred within the first 24 hpi, which correlated with the outcome of competition observed during the culturing of A1MO2 and 5.6 over 48 in LB broth (Section 3.2.3.a). Furthermore, a significant difference was calculated for the rate of decline between the strains ($p < 0.0005$), which also suggests an increased persistence for 5.6_s in this treatment.

A second dynamic was noted in the cultures treated with inocula comprised primarily of 5.6_s (30:70, 10:90, A1MO2_tk:5.6_s) (Fig. 3.8. F - G), where a similar rate of CFU decrease was noted for both strains. However, whilst there was no longer a statistical difference between the persistence of the strains, 5.6_s maintained significantly higher CFU counts than A1MO2_tk over the duration of the assay ($p < 0.05$ to < 0.0005) relative to A1MO2_tk. The lack of significant difference between the rate of CFU decrease for A1MO2_tk and 5.6_s in these treatments during the assay suggested that the increased persistence of 5.6_s diminished as its proportion within the inoculant increased.

Fitting multiple linear regression models to the mixed inocula CFU data yielded a similar outcome to the models derived from the separate inoculation data, in which there was no significant difference between the coefficients for the strains. Each of the models themselves were statistically significant ($p < 0.05$). This signifies that there were no significant differences between the rate of CFU change over time between the strains. This was supported by a measurement of the area under the curve (AUC) of the graphs in Figure 3.8, which revealed no significant difference between the AUC of the strains when cultured separately, nor were any significant differences calculated in the AUC measurements for the 90:10, 70:30, and 50:50 treatments (A1MO2_tk:5.6_s). However, in both the 30:70 and 10:90 treatments (A1MO2_tk:5.6_s) the total AUC for 5.6_s was significantly higher than A1MO2_tk ($p < 0.05$). This aligns with a higher persistence of 5.6_s in the mixed inoculation, and with the inoculants for these treatments comprising primarily of 5.6_s.

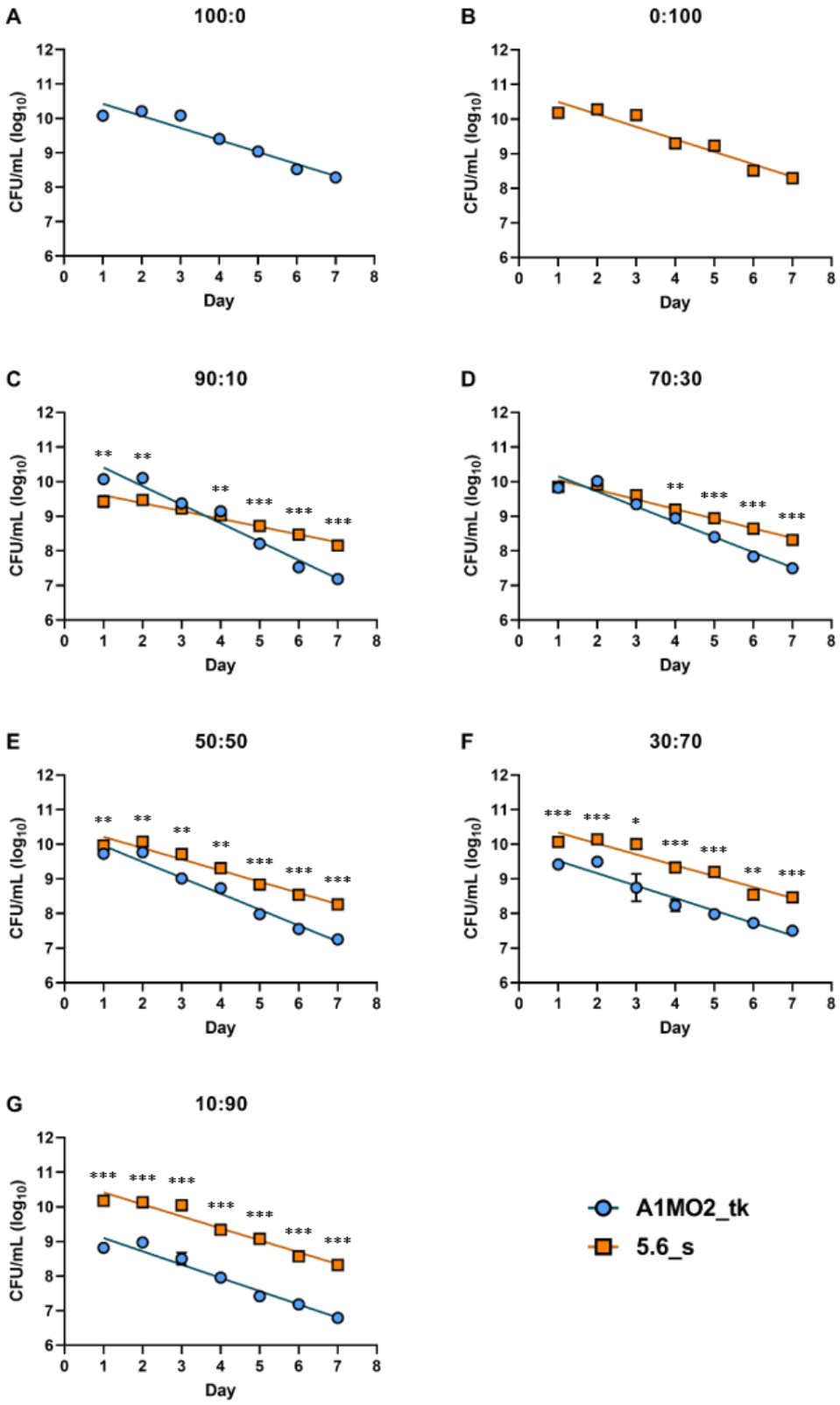


Figure 3.8. Average $\log_{10}$ CFU/mL counts (n = 3) for A1MO2_tk and 5.6_s when cultured in LB broth over seven days following inoculation at separate (A) 100:0 (B) 0:100, and mixed ratios of (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:5.6_s). Inocula were prepared from cultures of 1.07 x 10^9 CFU/mL for A1MO2_tk and 1.21 x 10^9 CFU/mL for 5.6_s. Results are presented with fitted linear regression models for both strains and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU and inocula data is available in Appendix B.1.

Table 3.1. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 5.6_s cultured in LB broth following inoculation at various ratios (A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.89	-1.489	3.336	0.678	24.21	0.006
0:100	5.6_s		-1.117	3.234	0.747		
90:10	A1MO2_tk	0.95	-0.967	1.390	0.525	52.95	0.001
	5.6_s		-1.955	3.229	0.578		
70:30	A1MO2_tk	0.95	-0.517	2.086	0.816	54.04	0.001
	5.6_s		-2.632	3.283	0.468		
50:50	A1MO2_tk	0.95	-2.125	1.682	0.275	63.63	0.001
	5.6_s		0.024	2.376	0.992		
30:70	A1MO2_tk	0.94	-1.861	0.975	0.129	45.89	0.002
	5.6_s		-0.902	1.106	0.460		
10:90	A1MO2_tk	0.94	-1.766	1.718	0.362	46.69	0.002
	5.6_S		-0.832	1.884	0.682		

The impact of the increased persistence of 5.6_s was most clearly seen in the relative proportion of 5.6_s cells isolated from the culture over the duration of the assay. A clear increase in proportion for 5.6_s was seen in the 90:10, 70:30, and 50:50 cultures (Fig. 3.9. A - C), where in the 90:10 culture a relative proportion of 81:19 was recorded one dpi (A1MO2_tk:5.6_s). Due to the increased persistence of 5.6_s the proportion of 5.6_s cells isolated continually increased, with a near-equal proportion of cells being isolated by four dpi (57:43), and then significantly more 5.6_s cells being isolated than A1MO2_tk at seven dpi (10:90) ($p < 0.0005$). As mentioned previously, in both the 30:70 and 10:90 cultures very little difference in persistence between the strains was noted (Fig. 3.9. D - E), however, in these cultures the relative proportion of 5.6_s was still seen to increase by a small amount, suggesting it was not as competitive over a longer period of time when inoculated at these proportions, when compared to being inoculated as the minority strain in the inoculant, such as in the 90:10 and 70:30 treatments. This may also suggest the exhaustion of nutrients at an earlier stage in the assay due to the increased abundance and replication of 5.6_s relative to A1MO2_tk.

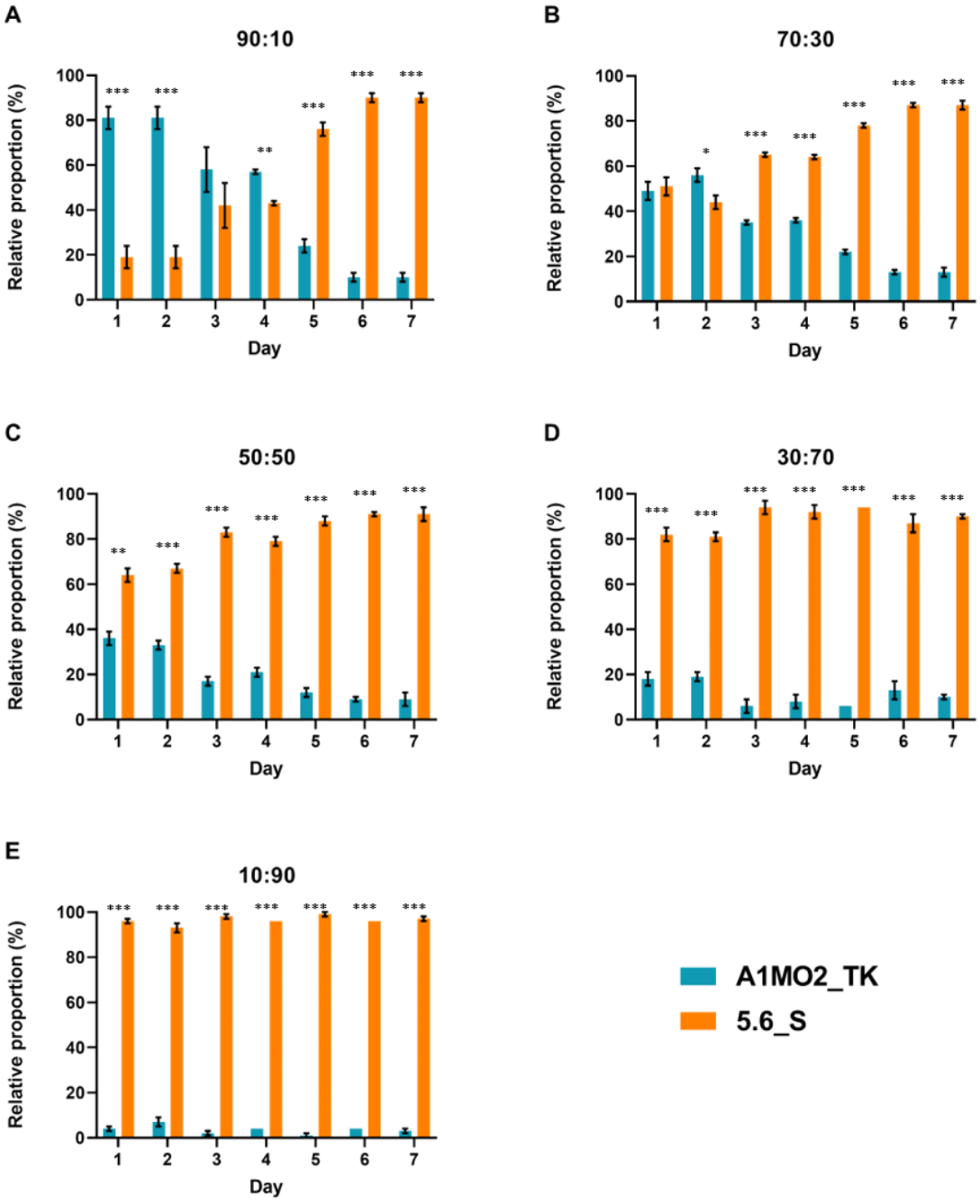


Figure 3.9. Changes in the average relative proportion (%) (n = 3) of viable A1MO2_tk and 5.6_s cells isolated from LB broth cultures over a seven-day period. The medium was inoculated with various mixed ratio A1MO2_tk and 5.6_s inocula: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.4.b. Measuring persistence of tagged A1MO2_tk and 477_s at mixed ratios in LB broth

Prior to an assessment of mixed ratio inocula both A1MO2_tk (100:0) and 477_s (0:100) were inoculated separately in LB broth to characterise the persistence of the strains in the absence of competition. Based on the results, 477_s showed a slightly slower rate of CFU decrease than A1MO2_tk over the seven-day assay duration, resulting in 477_s attaining a significantly higher CFU count than A1MO2_tk at six dpi ($p < 0.0005$), which was maintained until seven dpi (Fig. 3.10. A - B). Whilst fitting the $\log_{10}$ CFU data to a multiple regression model did not indicate a statistical difference between the coefficients for the strains (Table 3.2), the coefficient for A1MO2_tk was just over the threshold required for significance ($p = 0.06$).

A1MO2_tk displayed an increased persistence within the culture compared to 477_s when A1MO2_tk predominated as the majority strain in the initial inocula (90:10, 70:30, A1MO2_tk:477_s) (Fig. 3.10. C - D). In both of these treatment groups A1MO2_tk and 477_s decreased in CFU at a similar rate over the duration of the assay, as described by the linear regression equations (Table 3.2). This was most evident in the 90:10 culture, where, despite A1MO2_tk attaining a CFU counts significantly higher than 477_s at one dpi ($p < 0.0005$), by seven dpi there was no longer a difference between the strains. Furthermore, there was no significant difference in the rate of CFU decrease between the strains over the assay duration This provided a contrast to the culturing of A1MO2 and 5.6 over seven days, where 5.6 decreased in CFU at a significantly slower rate than A1MO2.

When A1MO2_tk and 477_s were inoculated at equal proportions (50:50, A1MO2_tk:477_s) (Fig 3.10. E), a different outcome was reported. 477_s reached a significantly higher CFU count than A1MO2_tk by one dpi ($p < 0.05$), but then decreased in CFU at a faster rate than A1MO2_tk towards the latter portion of the assay (five to seven dpi), when A1MO2_tk CFU counts were significantly higher than 477_s ($p < 0.05$). These changes led to a significantly slower rate of CFU decrease in the culture for A1MO2_tk than 477_s ($p < 0.05$).

Decreasing the proportion of A1MO2_tk in the inoculant (30:70, 10:90, A1MO2_tk:477_s) (Fig. 3.10. F - G) corresponded with an increase in the persistence of A1MO2_tk within the culture, relative to 477_s. In both of these treatment groups 477_s attained significantly higher CFU counts that A1MO2_tk at one dpi ($p < 0.0005$), however, due to a significantly slower rate of CFU decrease for A1MO2_tk compared to 477_s in both of these treatments ($p < 0.0005$), A1MO2_tk had either attained CFU counts significantly higher than 477_s (30:70) by seven dpi, or similar CFU counts were recorded for both strains (10:90) by seven dpi. This suggested that as the proportion of A1MO2_tk within the inoculant decreased, its growth, relative to 477_s, increased, which was not observed during the previous assessment of A1MO2 and 5.6.

Fitting multiple regression models to the CFU data obtained from mixed inoculation revealed no significant difference between the coefficients for the strains in the 90:10, 70:30, 50:50, of 30:70 treatments (A1MO2_tk:477_s) (Table 3.2). A difference was noted between the strains in the 10:90 treatment, in which the *p* value for the 477_s coefficient was below the level of significance ($p < 0.05$), indicating a significant amount of CFU change per day, whereas the change for A1MO2_tk was not significant. No significant differences were recorded for the AUC measurements between A1MO2_tk and 477_s in each of the 90:10, 70:30, 50:50, of 30:70 treatments (A1MO2_tk:477_s). Whilst no significant difference was recorded between the 10:90 treatment, the difference between AUC measurements for the strains falls just outside of the significance level ($p = 0.06$).

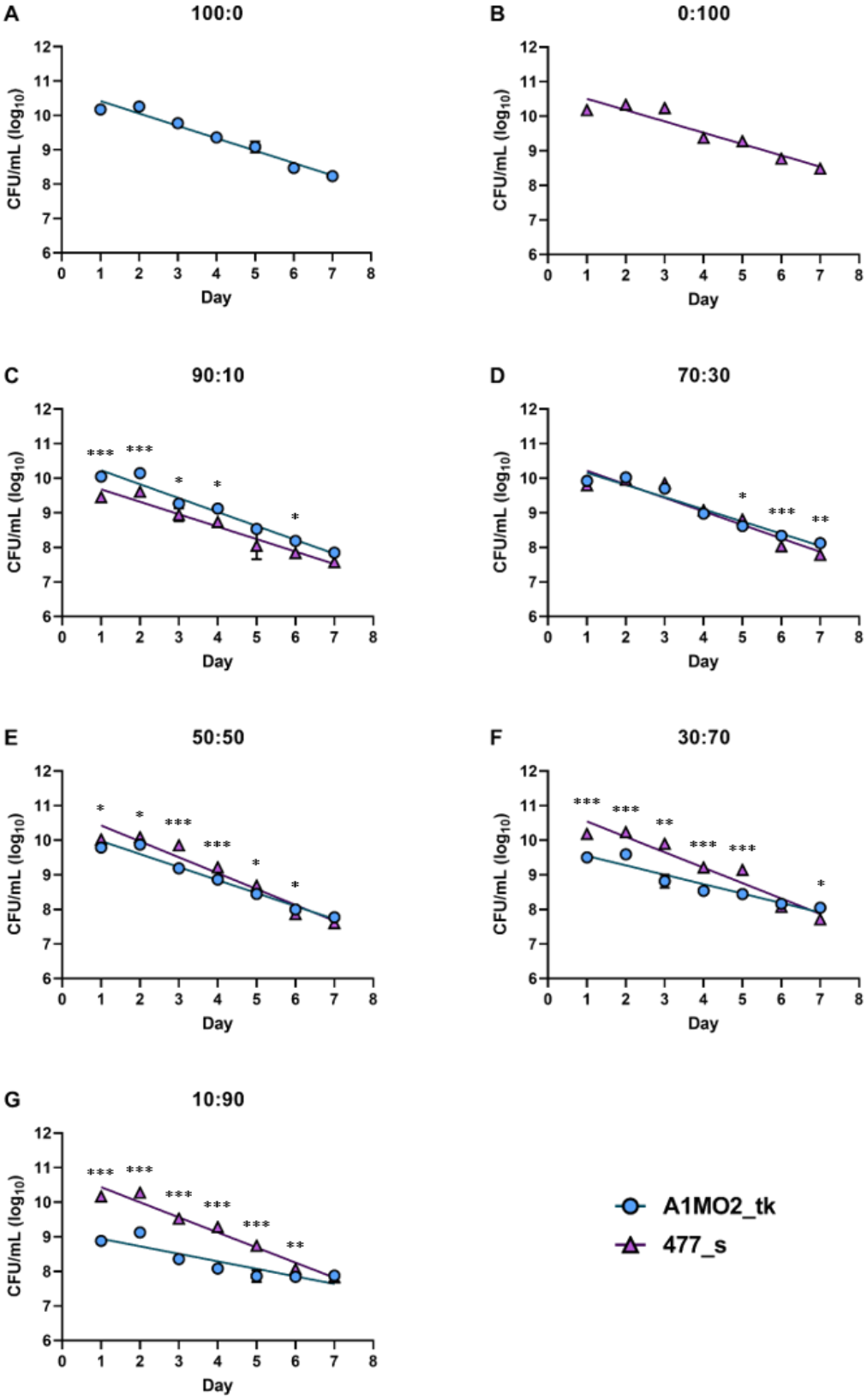


Figure 3.10. Average log_{10} CFU/mL counts (n = 3) for A1MO2_tk and 477_s when cultured in LB broth over seven days following inoculation at separate (A) 100:0 (B) 0:100, and mixed ratios of (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:477_s). Inocula were prepared from cultures of 1.19 x 10^9 CFU/mL for A1MO2_tk and 1.25 x 10^9 CFU/mL for 477_s. Results are presented with fitted linear regression models for both strains and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU and inocula data is available in Appendix B.2.

Table 3.2. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 477_s cultured in LB broth following inoculation at various ratios (A1MO2_tk:477_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.94	-3.208	1.258	0.063	52.14	0.001
0:100	477_s		0.588	1.342	0.684		
90:10	A1MO2_tk	0.94	-2.150	2.932	0.503	49.72	0.001
	477_s		-0.280	3.287	0.936		
70:30	A1MO2_tk	0.92	-2.499	1.456	0.161	33.73	0.003
	477_s		-0.162	1.286	0.906		
50:50	A1MO2_tk	0.95	-2.258	1.057	0.100	64.66	0.001
	477_s		-0.261	0.850	0.775		
30:70	A1MO2_tk	0.94	-1.668	0.905	0.139	47.95	0.002
	477_s		-1.136	0.558	0.111		
10:90	A1MO2_tk	0.94	-0.091	0.975	0.930	50.09	0.001
	477_s		-2.159	0.531	0.015		

By comparing the relative proportion of A1MO2_tk and 477_s cells isolated from the mixed inocula cultures it is also possible to see the fitness of A1MO2_tk increasing as its proportion in the inoculant decreased. A slight increase in the number of 477_s cells isolated over the duration of the assay was recorded in the 90:10 culture (Fig. 3.11. A), resulting in a small change for 477_s, but potentially indicating an increased fitness over a longer period, shifting from 17:83 by one dpi (A1MO2_tk:477_s) to 65:35 at seven dpi. In contrast, each of the 70:30, 50:50, 30:70, and 10:90 cultures (Fig. 3.11. B - E) showed a continual increase in proportion for A1MO2_tk following inoculation. This increase was most noticeable in both the 30:70 and 10:90 cultures, where in the 10:90 culture the proportion shifted from 5:95 at one dpi (A1MO2_tk:477_s) to 53:47 by seven dpi.

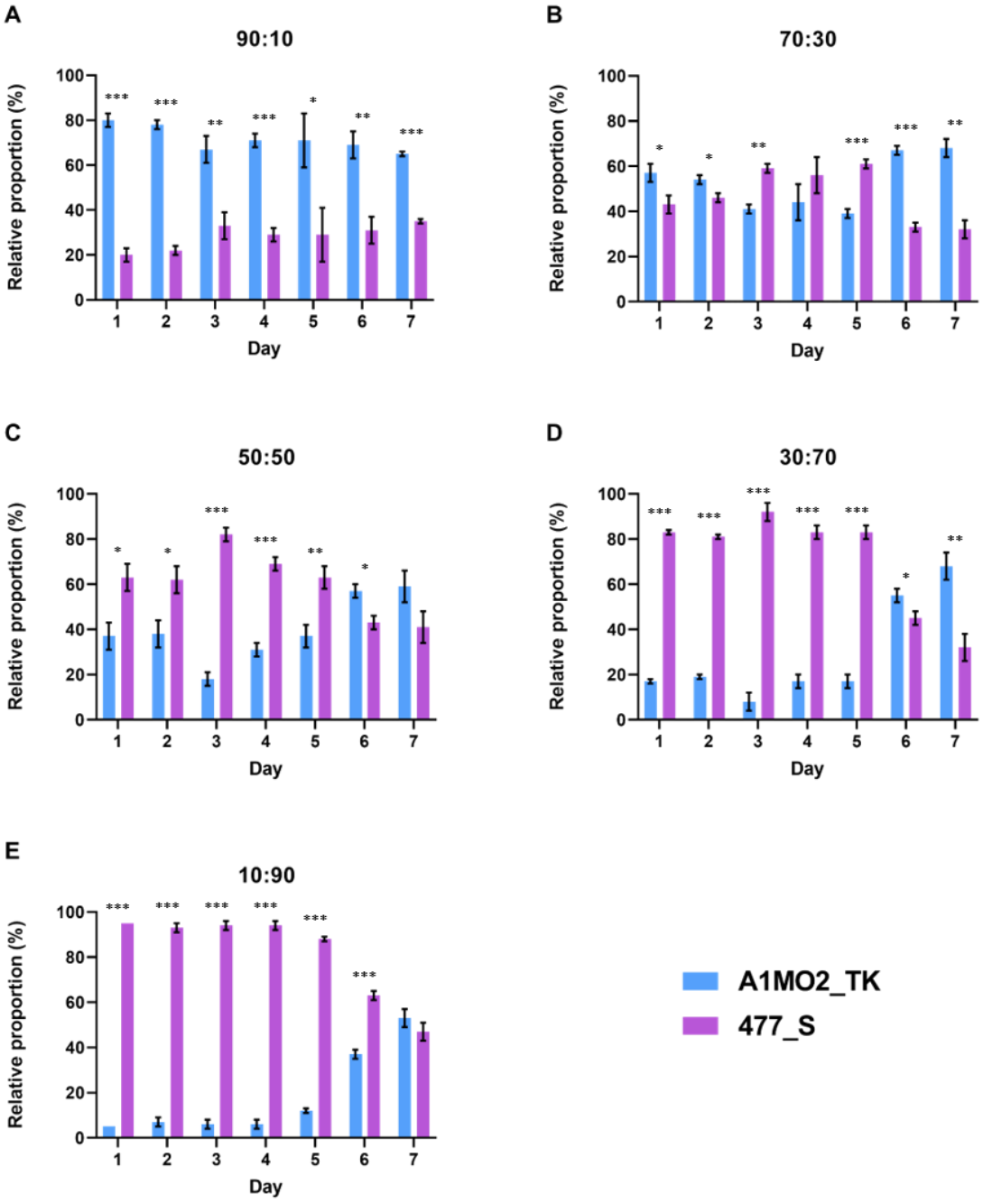


Figure 3.11. Changes in the average relative proportion (%) (n = 3) of viable A1MO2_tk and 477_s cells isolated from LB broth cultures over a seven-day period. The medium was inoculated with various mixed ratio A1MO2_tk and 477_s inocula: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:477_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.4.c. Measuring persistence of tagged A1MO2_tk and AGR96X_s at mixed ratios in LB broth

Competition between the chronic pathogen A1MO2 and the hypervirulent *S. proteamaculans* strain, AGR96X was also examined in a nutrient-rich LB broth medium. As with other mixed inoculation competition experiments, both A1MO2_tk (100:0) and AGR96X_s (0:100) were inoculated separately in the LB broth to determine the persistence of the strains in the absence of competition. Over the assay duration the CFU counts for A1MO2_tk decreased at a slower rate than AGR96X_s, leading to a significant difference between the CFU counts by three dpi ($p < 0.05$), which was maintained until the end of the assay at seven dpi ($p < 0.005$) (Fig. 3.12. A - B). Fitting multiple regression models to the data resulted in no significant differences being observed for the coefficients of either strain (Table 3.3), signifying a non-significant CFU change per day.

The CFU data obtained during the mixed inoculation of A1MO2_tk and AGR96X_s showed that by the end of the assay at seven dpi there was either no significant difference between the CFU counts for the strains, or the CFU count for A1MO2_tk was significantly higher than AGR96X_s ($p < 0.05$). This suggested that A1MO2_tk had a higher level of persistence in the medium than AGR96X_s. The fitting of linear regression models to $\log_{10}$ transformed CFU data revealed this difference in persistence was based on the proportion of the strain in the initial inoculant. A higher level of persistence and a significantly slower rate of CFU decrease was recorded for AGR96X_s compared to A1MO2_tk when using the inocula comprised primarily of A1MO2_tk (90:10, 70:30, A1MO2_tk:AGR96X_s) (Fig. 3.12. C - D) ($p < 0.005$). Both strains showed a similar level of persistence and rate of CFU decline when inoculated at equal ratios (50:50, A1MO2_tk:AGR96X_s) (Fig. 3.12. E). Finally, A1MO2_tk displayed a higher level of persistence and significantly slower rate of CFU decrease than AGR96X_s within the cultures when using inocula comprised primarily of AGR96X_s (30:70, 10:90, A1MO2_tk:AGR96X_s) (Fig. 3.12. F - G) ($p < 0.05$).

An interesting dynamic was observed in the output of the multiple regression models (Table 3.3), where the significance of the coefficients varied depending on which strain comprised a majority of the inoculant. When A1MO2_tk comprised the majority, in the 90:10 and 70:30 treatments (A1MO2_tk:AGR96X_s) a significant difference in CFU change was noted for the A1MO2 coefficient ($p < 0.05$), favouring AGR96X_s, whereas in the AGR96X_s majority treatments, 30:70 and 10:90, a significant difference was noted for AGR96X_s ($p < 0.05$), favouring A1MO2_tk. No significant differences were observed in the 50:50 treatment. This indicated that the more of the inoculant that the strain comprised, the more likely significant changes in CFU were to be expected. Further similarities between the strains were evident in the AUC measurements, wherein no significant differences were calculated between the AUC of either strain in each of the mixed ratio and separate culture treatments.

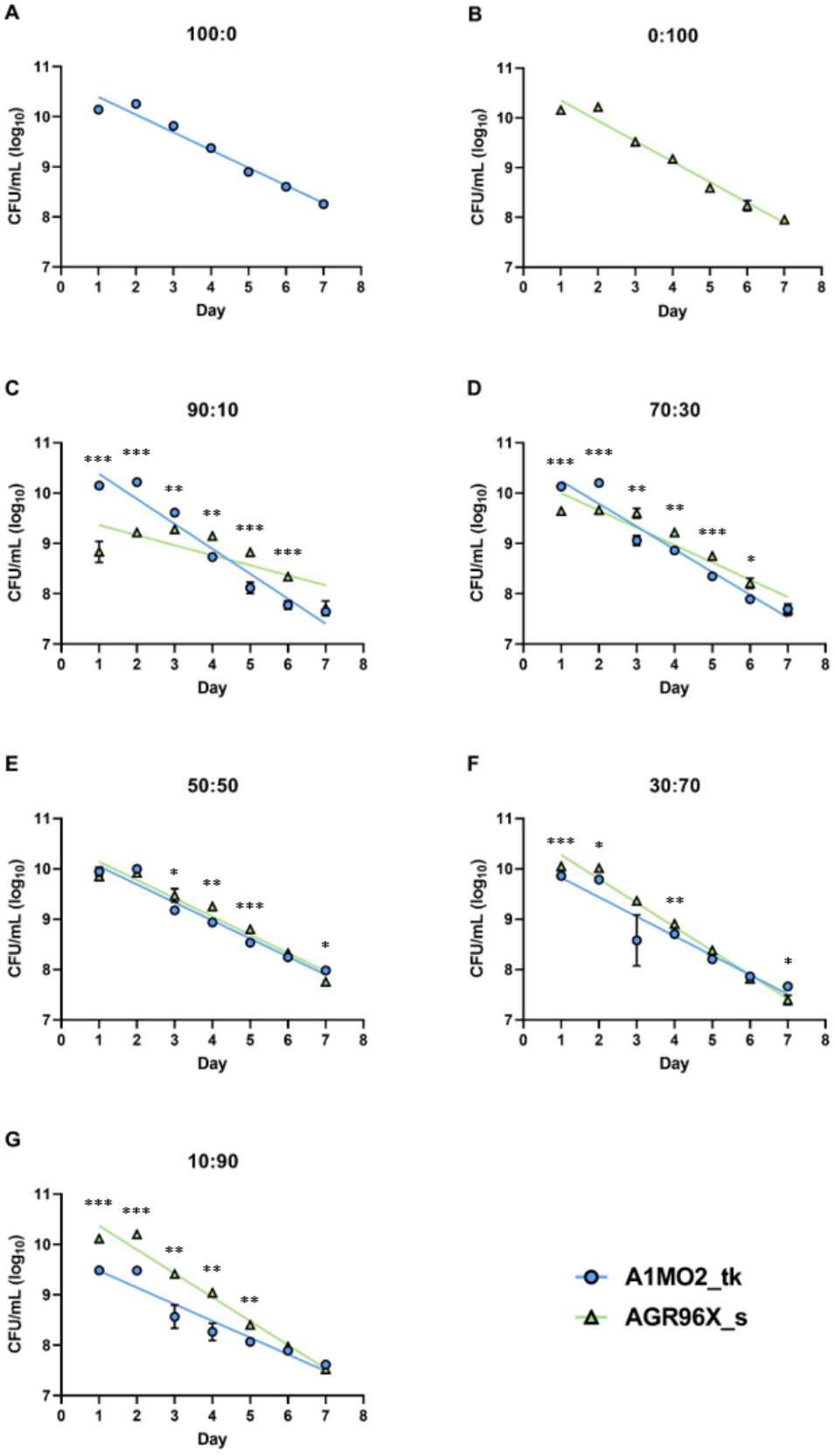


Figure 3.12. Average log_{10} CFU/mL counts (n = 3) for A1MO2_tk and AGR96X_s when cultured in LB broth over seven days following inoculation at separate (A) 100:0 (B) 0:100, and mixed ratios of (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:AGR96X_s). Inocula were prepared from cultures of 1.19 x 10^9 CFU/mL for A1MO2_tk and 1.25 x 10^9 CFU/mL for AGR96X_s. Results are presented with fitted linear regression models for both strains and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU and inocula data is available in Appendix B.3.

Table 3.3. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and AGR96X_s cultured in LB broth following inoculation at various ratios (A1MO2_tk:AGR96X_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.96	-0.003	2.471	0.999	68.02	0.001
0:100	AGR96X_s		-2.368	2.141	0.331		
90:10	A1MO2_tk	0.93	-1.754	0.324	0.006	39.49	0.002
	AGR96X_s		-0.418	0.643	0.545		
70:30	A1MO2_tk	0.96	-1.369	0.405	0.028	78.21	0.001
	AGR96X_s		-1.043	0.520	0.116		
50:50	A1MO2_tk	0.96	-1.614	0.807	0.116	75.14	0.001
	AGR96X_s		-1.088	0.792	0.242		
30:70	A1MO2_tk	0.98	-0.298	0.680	0.684	122.44	0.001
	AGR96X_s		-1.824	0.571	0.033		
10:90	A1MO2_tk	0.96	-0.254	0.849	0.780	74.31	0.001
	AGR96X_s		-1.882	0.608	0.036		

The changes in relative proportion for the cells isolated from the mixed culture show an initial increase in the proportion of AGR96X_s cells over the first several days of the assay (Fig. 3.13. A - E). This was most evident in the 90:10 culture (A1MO2_tk:AGR96X_s), where the proportion of AGR96X_s continually increased over the first five days of the assay, with significantly more AGR96X_s cells being isolated than A1MO2_tk ($p < 0.005$). However, by seven dpi the proportion of A1MO2_tk began to increase, and there was no longer a significant difference between the number of viable cells isolated for each strain. This suggested an increase in the competitive ability for A1MO2_tk as the assay progressed. Additionally, the outcome of competition between A1MO2 and AGR96X resembled the outcome of competition between A1MO2 and 477, depicted previously. The results obtained presented a clear in the dynamics of competition for these strains when compared to A1MO2 and 5.6, suggesting differences in 5.6 which, due to its isogenic nature to A1MO2, may facilitate its increased competitive behaviour in the cultures.

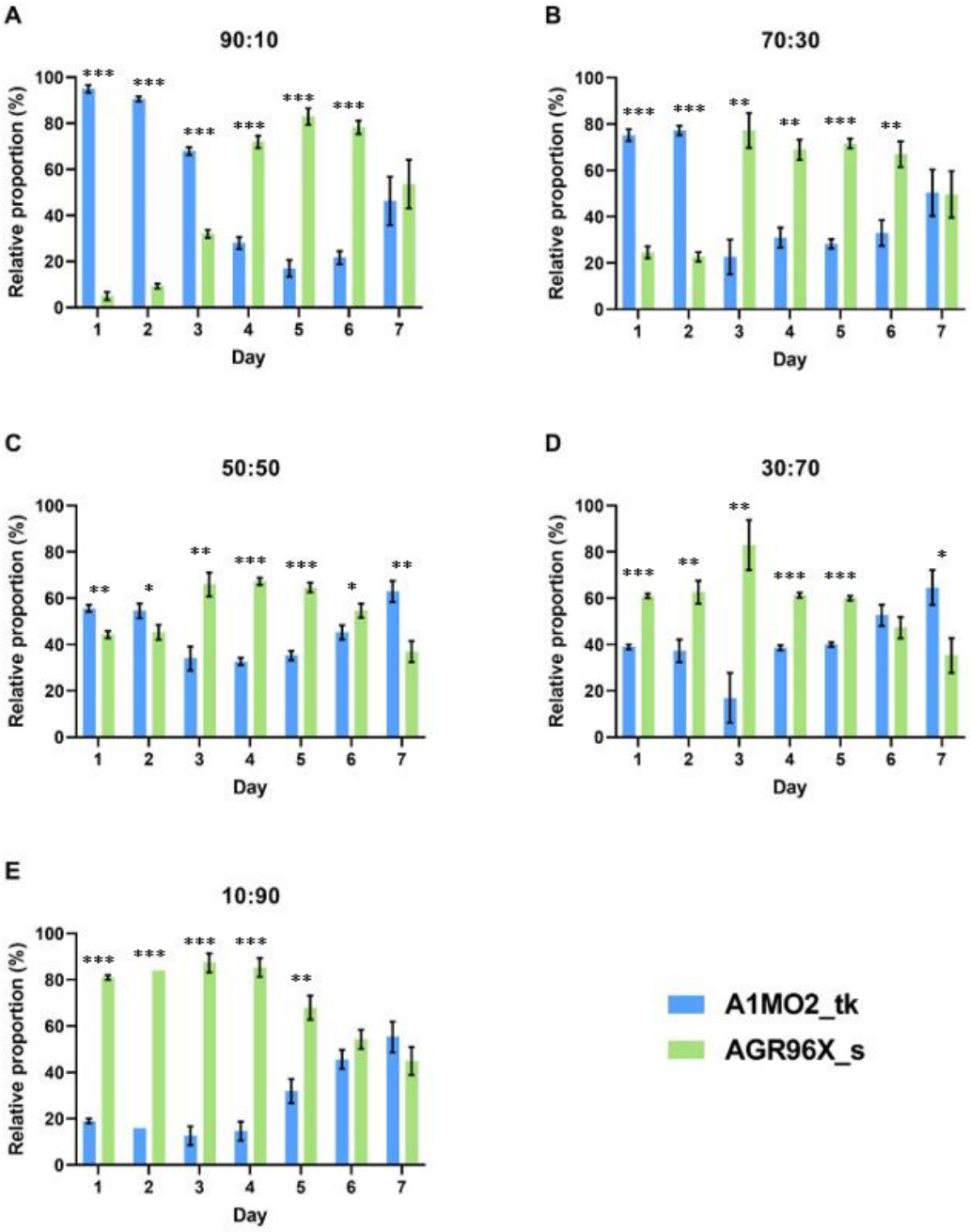


Figure 3.13. Changes in the average relative proportion (%) (n = 3) of viable A1MO2_tk and ARGR96X_s cells isolated from LB broth cultures over a seven-day period. The medium was inoculated with various mixed ratio A1MO2_tk and AGR96X_s inocula: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:AGR96X_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.5 Measuring persistence of tagged A1MO2_tk and 5.6_s in a daily re-seeded LB broth culture

The results obtained during the culturing of A1MO2_tk and 5.6_s over a seven-day period indicated that 5.6_s had a higher persistence within the aging culture than A1MO2_tk. However, over the duration of the assay the amount of available nutrients within the culture decreased, increasing the likelihood of competition. As such, the mixed ratio inocula of A1MO2_tk and 5.6_s were assessed again, where a portion of the aged culture was used to inoculate fresh medium each day, ensuring a continual supply of nutrients for the cells (method: Chapter 2, section 2.3.6).

The separate inoculation and re-seeding of A1MO2_tk (100:0) and 5.6_s (0:100) resulted in very little change to the number of cells in the culture over the duration of the assay, indicating a high level of persistence when continually seeded into fresh medium. Furthermore, no significant changes in CFU were recorded for either A1MO2_tk or 5.6_s over the assay duration, with both strains attaining average CFU counts (n = 3) at one dpi of 1.72 x 10^{10} CFU/mL for A1MO2_tk and 2.08 x 10^{10} CFU/mL for 5.6_s (Fig. 3.14. A - B). Whilst the multiple regression models do not show a significant change in CFU per day for either A1MO2_tk or 5.6_s, the low R^2 output (0.08) and high *p* value in relation to the *F* statistic ($F(2,4) = 0.17$, $p = 0.846$) (Table 3.4) may signify either that the CFU changes depicted by the strains are non-linear, and thus may require a different type of modelling.

The inoculation and re-seeding of the mixed ratio inocula resulted in a different outcome, where 5.6_s showed an increased persistence over the assay duration, relative to A1MO2_tk, and may also reflect the ability of 5.6 to outcompete A1MO2 during growth in an environment with continually replenished nutrients. In addition to an increased persistence within the re-seeded cultures, in several instances 5.6_s continued to increase in CFU over the assay duration. This is most evident in the re-seeded cultures initially inoculated with inocula consisting primarily of A1MO2_tk (90:10, 70:30, A1MO2_tk:5.6_s) (Fig. 3.14. C - D), or with an equal proportion of both strains (50:50, A1MO2_tk:5.6_s) (Fig. 3.14. E). In each of these cultures the CFU count of 5.6_s increased significantly between one and seven dpi ($p < 0.005$ to < 0.0005) whereas A1MO2_tk decreased in CFU significantly between one and seven dpi ($p < 0.005$ to < 0.0005). These changes were most evident in the 90:10 re-seeded cultures, where A1MO2_tk decreased from 2.00 x 10^{10} to 5.10 x 10^{9} CFU/mL over the assay duration, whereas 5.6_s increased from 4.87 x 10^{9} to 1.39 x 10^{10} CFU/mL. These changes were also reflected in the fitted linear regression models, showing a daily CFU decrease for A1MO2_tk, and an increase in CFU for 5.6_s over the duration of the assay, with a significant difference between these rates of change in each of the 90:10 70:30, and 50:50 treatments ($p < 0.0005$).

The re-seeding of the cultures initially inoculated primarily with 5.6_s (30:70, 10:90, A1MO2_tk:5.6_s) (Fig. 3.14. F - G) resulted in a continual maintenance of CFU for 5.6_s over the assay duration, and a decrease in CFU for A1MO2_tk. This difference in dynamics led to a significant difference between the rates of CFU change over the duration of the assay between the strains ($p < 0.0005$). Additionally, the CFU of A1MO2_tk in the 10:90 culture decreased over the first three dpi of the assay but stabilised in CFU after this period which lasted over the remainder of the assay with no significant changes in CFU being recorded between three and seven dpi. Additionally, no significant difference was recorded in the rates of CFU change in this treatment. This indicated that when the strains were continually re-seeded 5.6_s was more able to maintain a higher number of cells than A1MO2_tk, with these cells showing an increased persistence. In addition, the re-seeding of the cultures facilitated the maintenance of higher CFU counts for both A1MO2_tk and 5.6_s when compared to their culturing in a single flask (Section 3.2.4.a).

The multiple regression models fitted to the mixed inoculation CFU data had a much higher level of accuracy than those derived from the separate inoculation data (Table 3.4). In each of the 90:10, 70:30, 50:50, and 30:70 treatments (A1MO2_tk:5.6_s) the coefficient for A1MO2_tk indicated a significant change in CFU per day ($p < 0.05$), whereas 5.6_s only exhibited a significant change per day in the 10:90 treatment ($p < 0.05$). This indicates that 5.6_s was more able to maintain its CFU counts in the re-seeded medium than A1MO2_tk. The AUC measurements also indicate a large degree of difference between the persistence of the strains, wherein 5.6_s had an AUC value significantly higher than A1MO2_tk in the 70:30, 50:50, 30:70, and 10:90 treatments (A1MO2_tk:5.6_s) ($p < 0.05$), and also when cultured separately ($p < 0.05$).

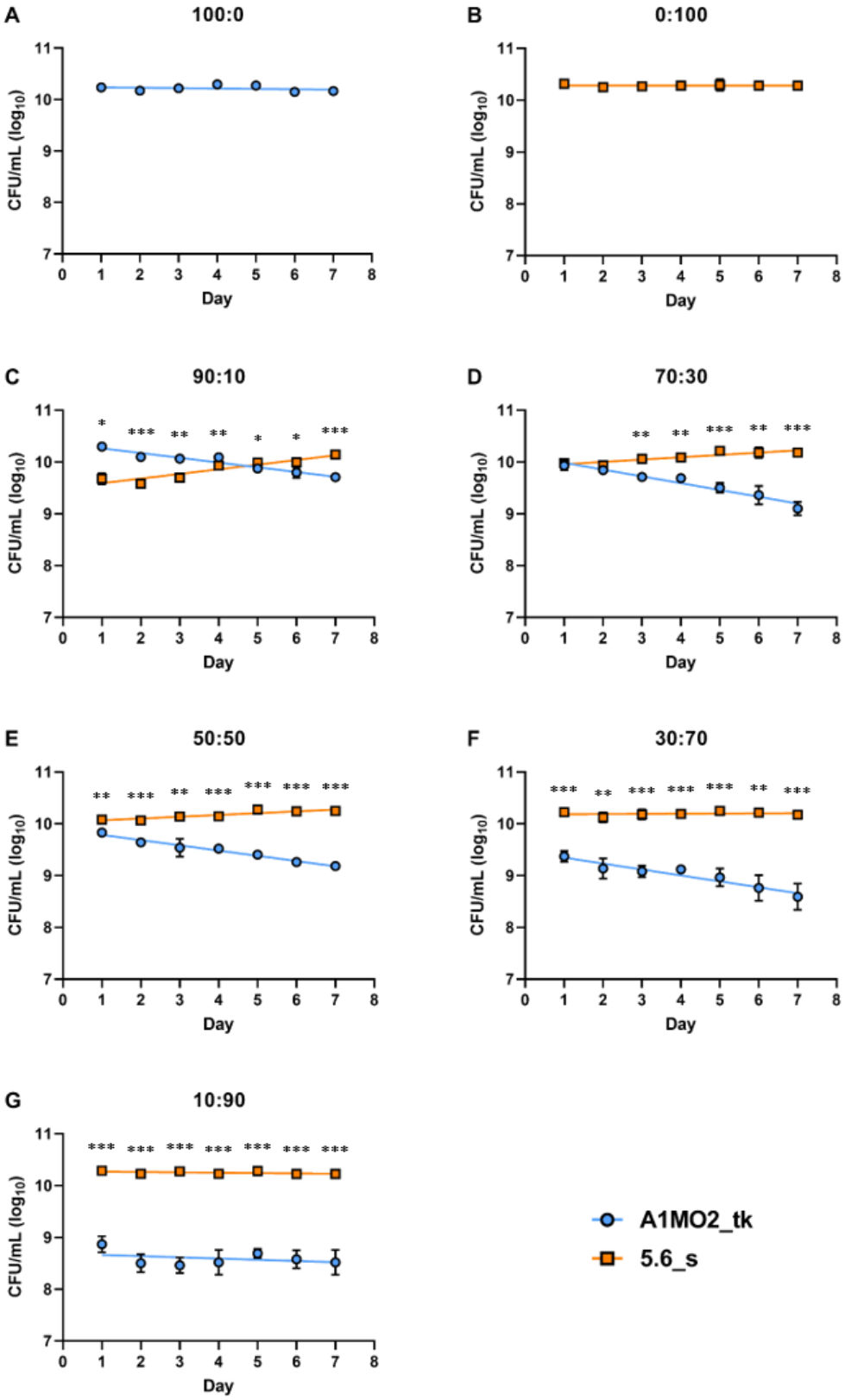


Figure 3.14. Average log_{10} CFU/mL counts (n = 3) for A1MO2_tk and 5.6_s when re-seeded in LB broth daily over seven days following inoculation at separate (A) 100:0 (B) 0:100, and mixed ratios of (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:5.6_s). Inocula were prepared from cultures of 1.19 x 10^9 CFU/mL for A1MO2_tk and 1.33 x 10^9 CFU/mL for 5.6_s. Results are presented with fitted linear regression models for both strains and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU and inocula data is available in Appendix B.4.

Table 3.4. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 5.6_s cultured in LB broth following inoculation at various ratios (A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.08	-12.229	20.742	0.587	0.17	0.846
0:100	5.6_s		12.568	54.350	0.828		
90:10	A1MO2_tk	0.98	-6.294	1.074	0.004	152.75	0.001
	5.6_s		4.670	1.077	0.012		
70:30	A1MO2_tk	0.96	-5.590	1.152	0.008	73.38	0.001
	5.6_s		5.641	3.060	0.139		
50:50	A1MO2_tk	0.98	-8.071	1.235	0.003	121.09	0.001
	5.6_s		4.649	3.296	0.231		
30:70	A1MO2_tk	0.95	-8.545	0.835	0.001	53.53	0.001
	5.6_s		10.957	5.293	0.107		
10:90	A1MO2_tk	0.25	-5.576	3.788	0.215	2.01	0.249
	5.6_s		-10.817	30.873	0.744		

The increased fitness of 5.6_s in the re-seeded cultures is evident from the changes in the proportion of cells isolated for each strain over the duration of the assay. In each of the 90:10, 70:30, 50:50, and 30:70 cultures the proportion of 5.6_s can be seen continuously increasing (Fig. 3.15. A - D). This was most apparent in the 90:10 culture, which attained a proportion of 80:20 (A1MO2_tk:5.6_s) one dpi. A majority of 5.6_s cells were first isolated five dpi (43:57), progressing to a solid majority for 5.6_s by seven dpi (27:73). Whilst 5.6_s also increased in proportion in the 10:90 culture (Fig. 3.15. E), with a proportion of 4:96 (A1MO2_tk:5.6_s) one dpi, the change was less noticeable by seven dpi (2:98) in comparison to the other cultures. Furthermore, the results obtained for the proportional changes in cells isolated from the re-seeded cultures resembled the proportional changes seen earlier when A1MO2_tk and 5.6_s were incubated in the same culture over a seven-day period, depicted previously in Figure 3.9 (Section 3.2.4.a).

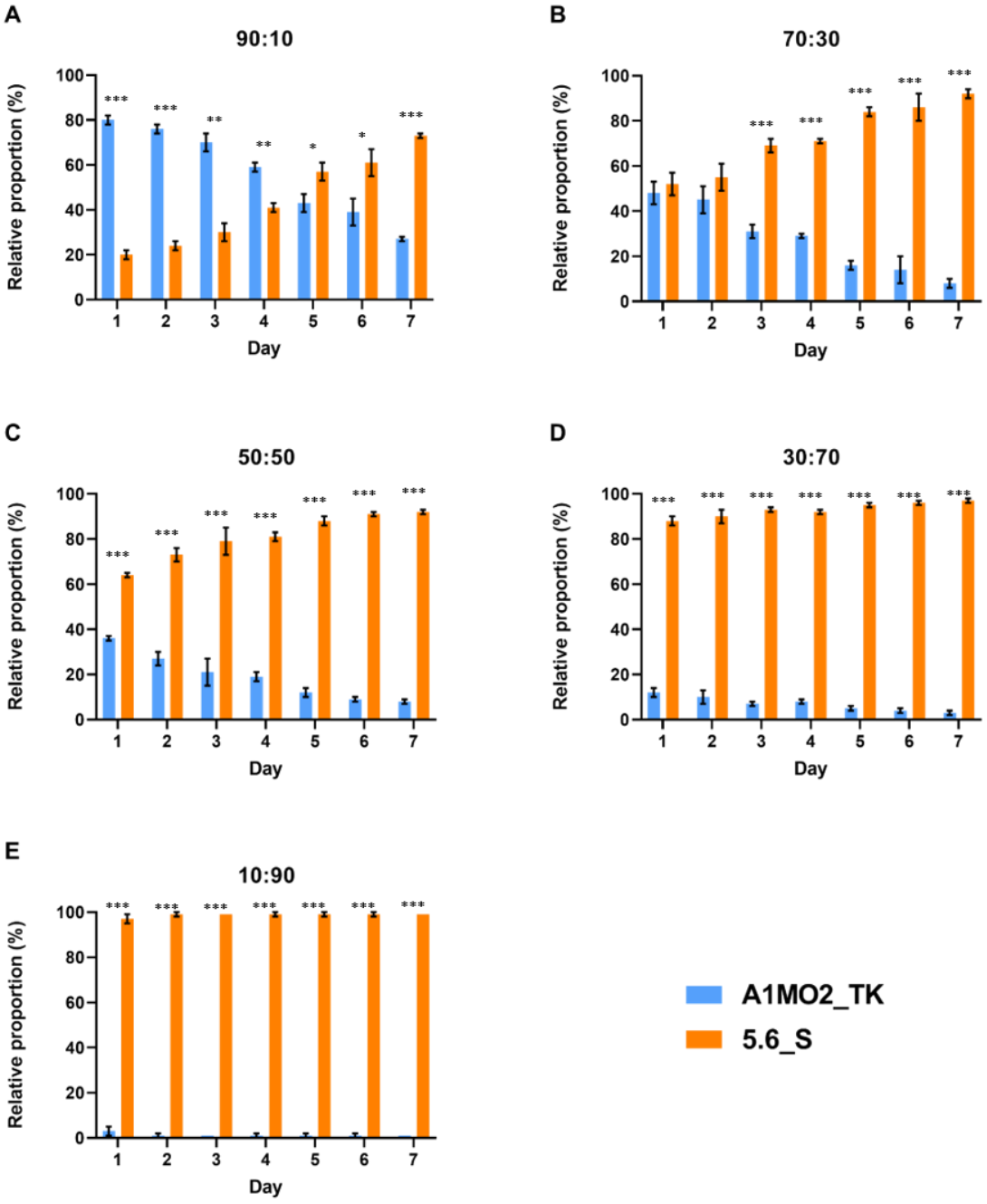


Figure 3.15. Changes in the average relative proportion (%) (n = 3) of viable A1MO2_tk and 5.6_s cells isolated from continually re-seeded LB broth cultures over a seven-day period. The medium was inoculated with various mixed ratio A1MO2_tk and 5.6_s inocula: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.6 Measuring persistence of tagged A1MO2_tk and 5.6_s at mixed ratios in an M9 medium

In addition to LB broth, the persistence of A1MO2_tk and 5.6_s was also assessed following inoculation in a restricted-nutrient M9 medium, to characterise how nutrient availability might alter the persistence of the strains. The separate inoculation of A1MO2_tk (100:0) and 5.6_s (0:100) into a 20 % glucose M9 medium yielded a distinct set of results where the average CFU counts (n = 3) for the strains remained relatively stable over the duration of the assay, in contrast to the continuous decrease in CFU reported in LB broth. Following inoculation, CFU counts of 3.93×10^9 and 4.07×10^9 CFU/mL were recorded one dpi for A1MO2_tk and 5.6_s, respectively (Fig. 3.16. A - B). By seven dpi these counts had decreased to 1.79×10^9 CFU/mL for A1MO2_tk and 2.29×10^9 CFU/mL for 5.6_s. Whilst this indicated a high degree of persistence within the culture over the time assessed, the fitting of multiple regression models showed a similar persistence for 5.6_s and A1MO2_tk when cultured separately (Table 3.5), with no significant differences in the rate of CFU change per day between the strains.

A similar CFU stability was recorded when both A1MO2_tk and 5.6_s were cultured together at mixed ratios in the glucose medium. Additionally, no significant difference was recorded between the rates of CFU change in any of the mixed ratio treatment groups (Fig. 3.16. C - G). However, the fitting of multiple regression models to the mixed inocula data (Table 3.5) indicated that in most cases the CFU changes per day were non-significant. Only 5.6_s in the 50:50 and 10:90 treatments (A1MO2_tk:5.6_s) exhibited a statistically significant CFU change per day ($p < 0.05$). Furthermore, in both the 90:10 and 30:70 multiple regression models the one-way ANOVA *p* value exceeds the *F* statistic, indicating that more data is required in these treatments to create more accurate models. In addition, an assessment of the AUC revealed that in the 90:10, 50:50, 30:70, and 10:90 mixed ratio treatments (A1MO2_tk:5.6_s), the AUC for 5.6_s was significantly higher than A1MO2_tk ($p < 0.05$). The AUC for 5.6_s was also significantly higher than A1MO2_tk when cultured separately ($p < 0.05$), suggesting an overall better growth of 5.6_s in the medium due to its early competitive advantages.

The assay was repeated using a 20 % casamino acids M9 medium inoculated with the varying ratios of A1MO2_tk and 5.6_s using the same method. The results obtained mirrored those produced when the strains were cultured in the 20 % glucose medium with both A1MO2_tk and 5.6_s showing a high level of persistence in the cultures over the assay duration, with no significant difference between the rates of change for the strains. This suggested an increased persistence in the restricted-nutrient media, and a different outcome when compared to the nutrient-rich LB broth, where both strains were shown to continually decrease in CFU counts. These results are available in Appendix B.6.

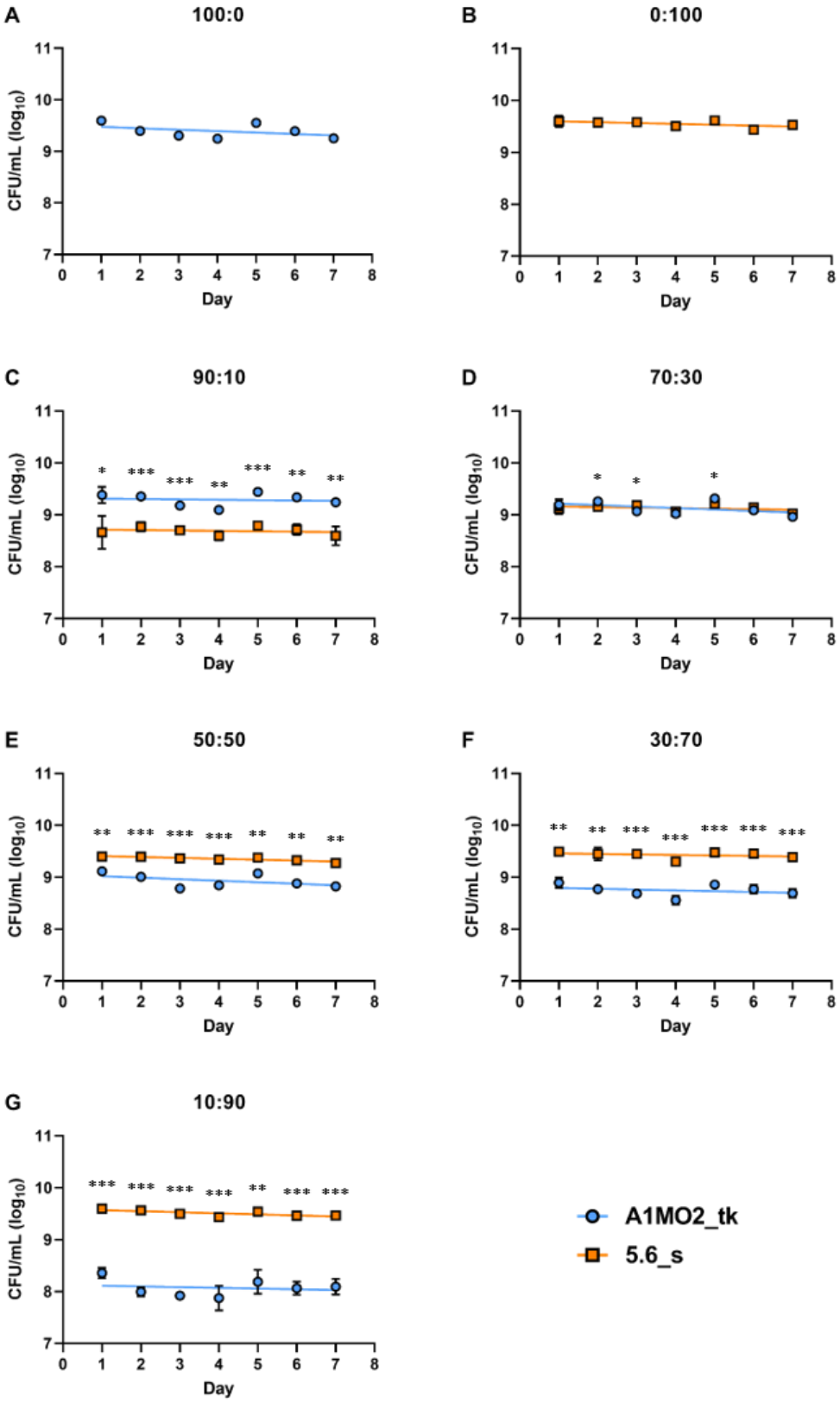


Figure 3.16. Average log_{10} CFU/mL counts (n = 3) for A1MO2_tk and 5.6_s when cultured in a 20 % glucose M9 medium over seven days following inoculation at separate (A) 100:0 (B) 0:100, and mixed ratios of (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:5.6_s). Inocula were prepared from cultures of 1.21 x 10^9 CFU/mL for A1MO2_tk and 1.30 x 10^9 CFU/mL for 5.6_s. Results are presented with fitted linear regression models for both strains and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU and inocula data is available in Appendix B.5.

Table 3.5. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 5.6_s cultured in 20% M9 glucose medium following inoculation at various ratios (A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.03	-2.682	7.423	0.736	1.11	0.414
0:100	5.6_s		-16.240	15.881	0.364		
90:10	A1MO2_tk	0.10	-4.203	8.705	0.654	0.22	0.811
	5.6_s		6.408	10.873	0.587		
70:30	A1MO2_tk	0.23	-8.208	10.972	0.496	0.61	0.588
	5.6_s		0.469	21.380	0.984		
50:50	A1MO2_tk	0.71	4.489	5.131	0.431	8.41	0.037
	5.6_s		-53.238	15.361	0.026		
30:70	A1MO2_tk	0.12	2.284	21.458	0.920	0.29	0.766
	5.6_s		-14.849	36.460	0.705		
10:90	A1MO2_tk	0.58	7.422	4.523	0.176	5.16	0.078
	5.6_s		-39.003	12.302	0.034		

An assessment of the relative proportion of cells isolated from the cultures also showed a potential increased persistence for 5.6_s, relative to A1MO2_tk. In each of the mixed ratio cultures there was an increase in the proportion of 5.6_s when measured at one and seven dpi (Fig. 3.17. A - E). This difference was most noticeable in the 70:30 and 50:50 cultures, where the proportion of 5.6_s cells isolated had increased by 8 % by the end of the assay at seven dpi. However, in the 90:10, 30:70, and 10:90 cultures (A1MO2_tk:5.6_s) this was marked by a 3 % increase in the proportion of 5.6_s cells isolated, indicating that when inoculated at these ratios both strains were coexisting without competing against each other. These findings contrasted those of the previous mixed inoculation assays with A1MO2 and 5.6 conducted in the LB broth, suggesting a difference in growth dynamics stemming from the type of medium used, or the resources available in the medium used in cellular growth. This demonstrates how a lower nutrient availability, particularly with the lack of nutrients in the M9 medium in comparison to the LB can alter the appearance of competition within a mixed inoculant, leading to less overall competition between the strains.

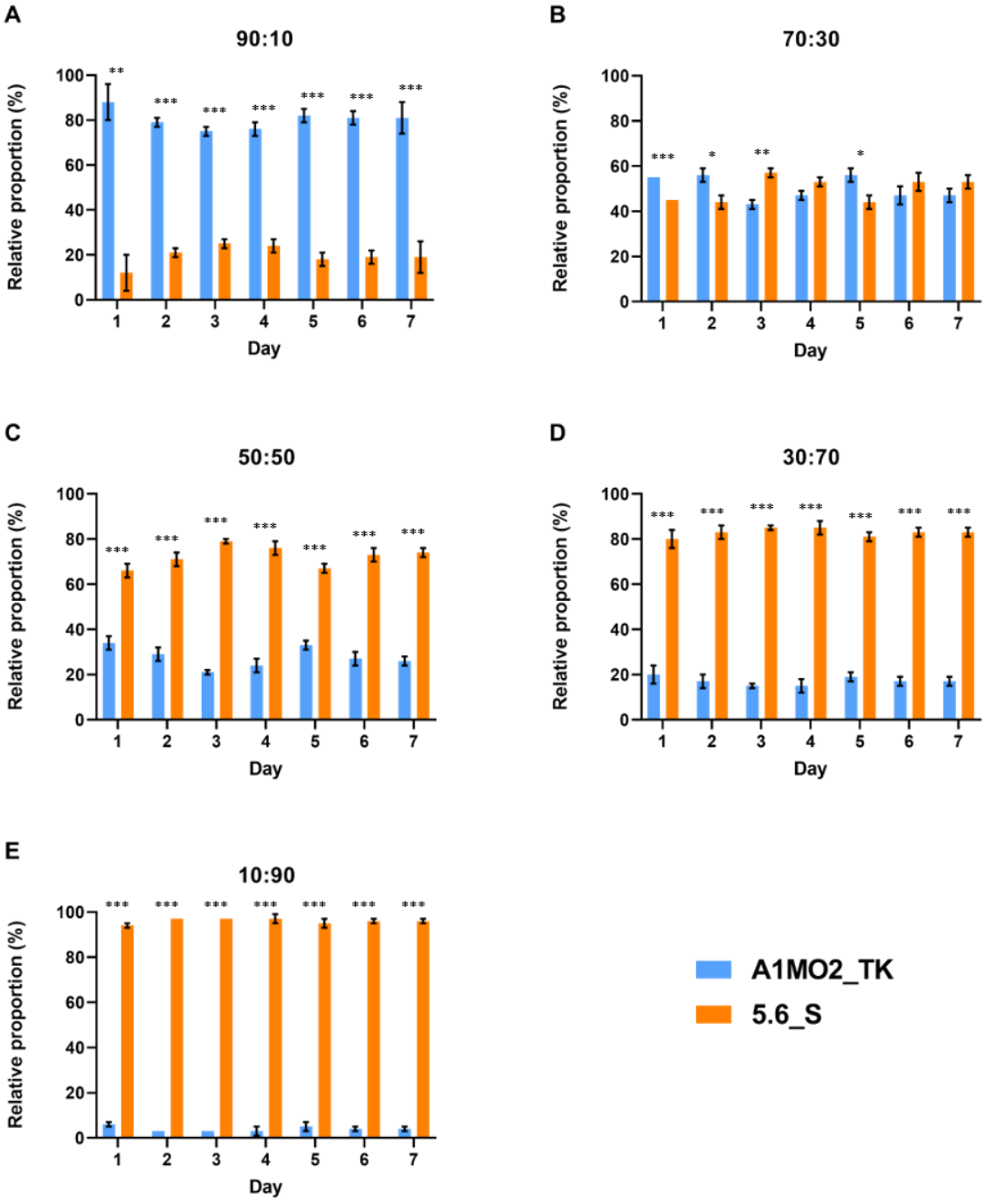


Figure 3.17. Changes in the average relative proportion (%) (n = 3) of viable A1MO2_tk and 5.6_s cells isolated from 20 % glucose M9 medium cultures over a seven-day period. The medium was inoculated with various mixed ratio A1MO2_tk and 5.6_s inocula: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:477_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

3.2.7 Quantifying the impact of competition with Lotka/Volterra-style models

Using the $\log_{10}$ CFU data it was possible to quantify the impact of competition between two strains over a period of time in a mixed inoculant using a simple Lotka/Volterra-style competition model, calculated using the gauseR package (Methods, Section 2.6.4). The output of this model, chiefly the a_{12} and a_{21} parameter values, can be used to more easily determine which of the two strains in the mixed inoculant was more competitive, and predict potential future dynamics. The model takes the following forms, where equation three describes the impact of strain 2 on strain 1, and equation four describes the impact of strain 1 on strain 2:

$$\frac{1}{n_1}\frac{dn_1}{dt} = r_1 - a_1 n_1 - a_{12} n_2 \quad (3)$$

$$\frac{1}{n_2}\frac{dn_2}{dt} = r_2 - a_2 n_2 - a_{21} n_1 \quad (4)$$

In which $\boldsymbol{r_1}$ and $\boldsymbol{r_2}$ denote the respective growth rates of species 1 and 2, $\boldsymbol{a_1}$ and $\boldsymbol{a_2}$ denote the strength of self-limitation, $\boldsymbol{a_{12}}$ denotes the impact of strain 2 on strain 1, $\boldsymbol{a_{21}}$ denotes the impact of strain 1 on strain 2, $\boldsymbol{n_1}$ and $\boldsymbol{n_2}$ denote the population of each species, and $\boldsymbol{t}$ denotes time (days). In these equations, A1MO2_tk is considered as strain 1, and the accompanying strain (5.6_s, 477_s, and AGR96X_s) is considered as strain 2. The parameters are included in Appendix B7.

The parameters calculated from the $\log_{10}$ CFU data for A1MO2_tk and 5.6_s when cultured together in LB broth over a period of seven days show mainly positive competition coefficient values for both strains, with the value of the coefficient varying depending on the ratio of the strain in the inoculant. When both strains exhibit positive coefficients, this signifies a potential mutualism dynamic, where one species exerts a beneficial effect on the growth rate of the other. The highest recorded competition coefficient for A1MO2_tk during mixed inoculation with 5.6_s was in the 90:10 treatment (A1MO2_tk:5.6_s), with an a_{12} value of 0.59, and an a_{21} value of 0.00 for 5.6_s. These results indicate that the presence of 5.6_s has a strong impact on the growth of A1MO2_tk, whereas, in this treatment, the presence of A1MO2_tk has little effect on 5.6_s.

A different outcome was recorded when A1MO2_tk and 477_s were inoculated together, in which the competition coefficient values for A1MO2_tk are negative (except in the 10:90 treatment), whilst the coefficient values for 477_s are all positive. This signifies that in mixed inoculation alongside 477_s was detrimental to the growth of A1MO2_tk. Furthermore, the negative a_{22} values for 477_s suggesst a higher degree of self-limitation, hindering the growth of 477_s in the mixed inoculant further. Very minor positive coefficients were calculated when A1MO2_tk and AGR96X_s were inoculated and

cultured together in mixed ratio treatments. This correlates with what was seen during the assay, in which both strains were able to coexist within the flasks, with neither strain outcompeting the other.

During the mixed inoculation of A1MO2_tk and 5.6_s in the glucose M9 medium, negative competition coefficient values were calculated for A1MO2_tk in each of the mixed ratio treatments, whereas positive values were calculated for 5.6_s. This indicates that mixed inoculation in this medium was detrimental to A1MO2_tk, and beneficial to 5.6_s. However, as can be seen in CFU data, both strains were still able to coexist in the medium, as such any impact of competition may not be apparent until a much longer period of time had passed.

3.2.8 Assessing the viability of *Serratia* spp. over seven days in culture with Live/Dead staining

The culturing of A1MO2_tk and 5.6_s over seven days in the previous assays indicated an increased persistence for 5.6_s within cultures when inoculated alongside A1MO2_tk, but a similar persistence between the strains when cultured together. Further investigation was conducted using a Live/Dead stain with seven-day cultures to determine how many of the cells within these cultures were viable, and how many were dead (method Chapter 2. section 2.3.7). Due to the recorded differences in persistence of the bacteria between the media, both LB broth and a 20 % casamino acids medium were assessed.

3.2.7.a. The viability of *Serratia* spp. when cultured in LB broth

The separate inoculation of wild-type A1MO2, 5.6, and AGR96X in the LB broth resulted in the generation of similar average CFU counts (n = 3) for the strains one dpi, with respective counts of 1.74 x 10^{10}, 1.76 x 10^{10}, and 1.60 x 10^{10} CFU/mL (Fig. 3.18). Similar to the previous assay the CFU counts for each of the strains continually decreased over the duration of the assay, resulting in CFU counts of 9.13 x 10^{7} CFU/mL for A1MO2, 1.75 x 10^{8} CFU/mL for 5.6, and 1.93 x 10^{7} CFU/mL for AGR96X by seven dpi. Furthermore, both A1MO2 and 5.6 attained significantly higher CFU counts than AGR96X four dpi ($p <$ 0.05 to < 0.005), which were maintained over the assay duration. The fitting of linear regression models to the data showed that 5.6 had the highest persistence within the culture (R^2 = 0.96), followed by A1MO2 (R^2 = 0.94), with AGR96X displaying the lowest persistence (R^2 = 0.95) (Table 3.6).

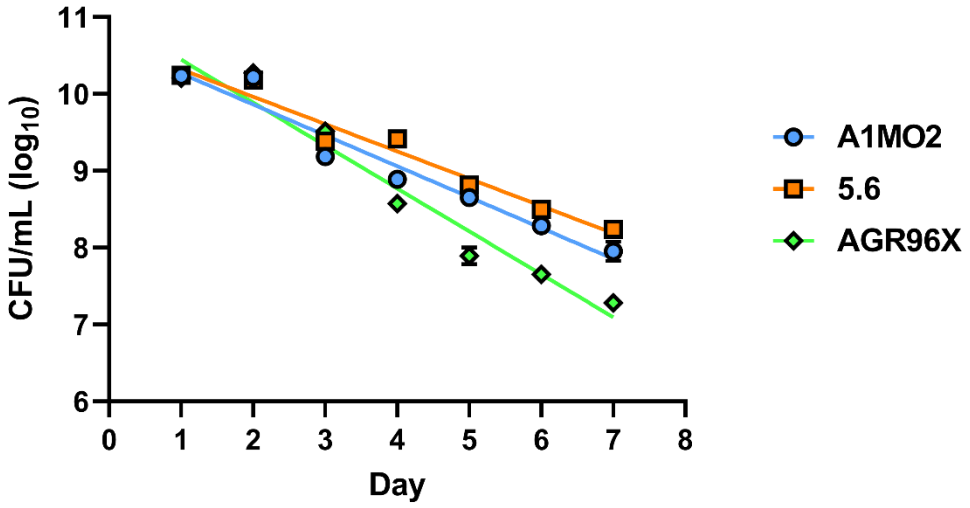


Figure 3.18. Average log_{10} CFU/mL counts (n = 3) for wild-type *Serratia* strains A1MO2, 5.6, and AGR96X cultured separately in LB broth over seven days. The strains were cultured from respective inocula of 7.40 x 10^8, 6.27 x 10^8, and 6.27 x 10^8 CFU/mL. The results are presented with standard deviation error bars.

Table 3.6. The linear regression models derived from log_{10} CFU/mL data for wild-type A1MO2, 5.6, and AGR96X cultured in LB broth over seven days, used to calculate the change in log_{10} CFU (*y*) per day (*x*), the standard error of the slope, the model goodness of fit (R^2), and the significance of the slope (*p*). Models were calculated to 19 degrees of freedom.

Strain	Regression equation	Slope error	R^2	*p*
A1MO2	$y = -0.402*x + 10.67$	0.022	0.94	< 0.001
5.6	$y = -0.355*x + 10.67$	0.017	0.96	< 0.001
AGR96X	$y = -0.559*x + 11.00$	0.029	0.95	< 0.001

The staining of a 3 µL culture sample showed that many of the cells for A1MO2, 5.6, and AGR96X were still viable one dpi (Fig. 3.19). In an approximate area of 113,950 μm^2, an average total of 7,197, 17,262, and 9,441 cells were counted for the respective strains across three images captured randomly on the microscope slide. Of these cells, 99.8 % were still viable, producing green fluorescence. By three dpi the proportion of viable cells decreased slightly for A1MO2 and 5.6, with 98.3 % of 2,747 cells viable for A1MO2, and 98.7 % of 6,379 cells viable for 5.6. The proportion of viable cells for AGR96X had remained similar to the proportion at one dpi, with 99.6 % of 10,168 cells viable. By five dpi all three strains exhibited a large decrease in the number of viable cells, with a proportion of 95.3 % (± 0.3 %) of viable cells for A1MO2 from 4,448 total cells, 6,932 total cells for 5.6, and 8.914 total cells for AGR96X. Further decreases in viable cells were recorded at seven dpi. For A1MO2, of 6,559 total cells 92.5 % were viable, from 6,216 total cells for 5.6, 83.1 % were still viable, and for AGR96X a total of 79.9 % from 3,276 cells were still viable.

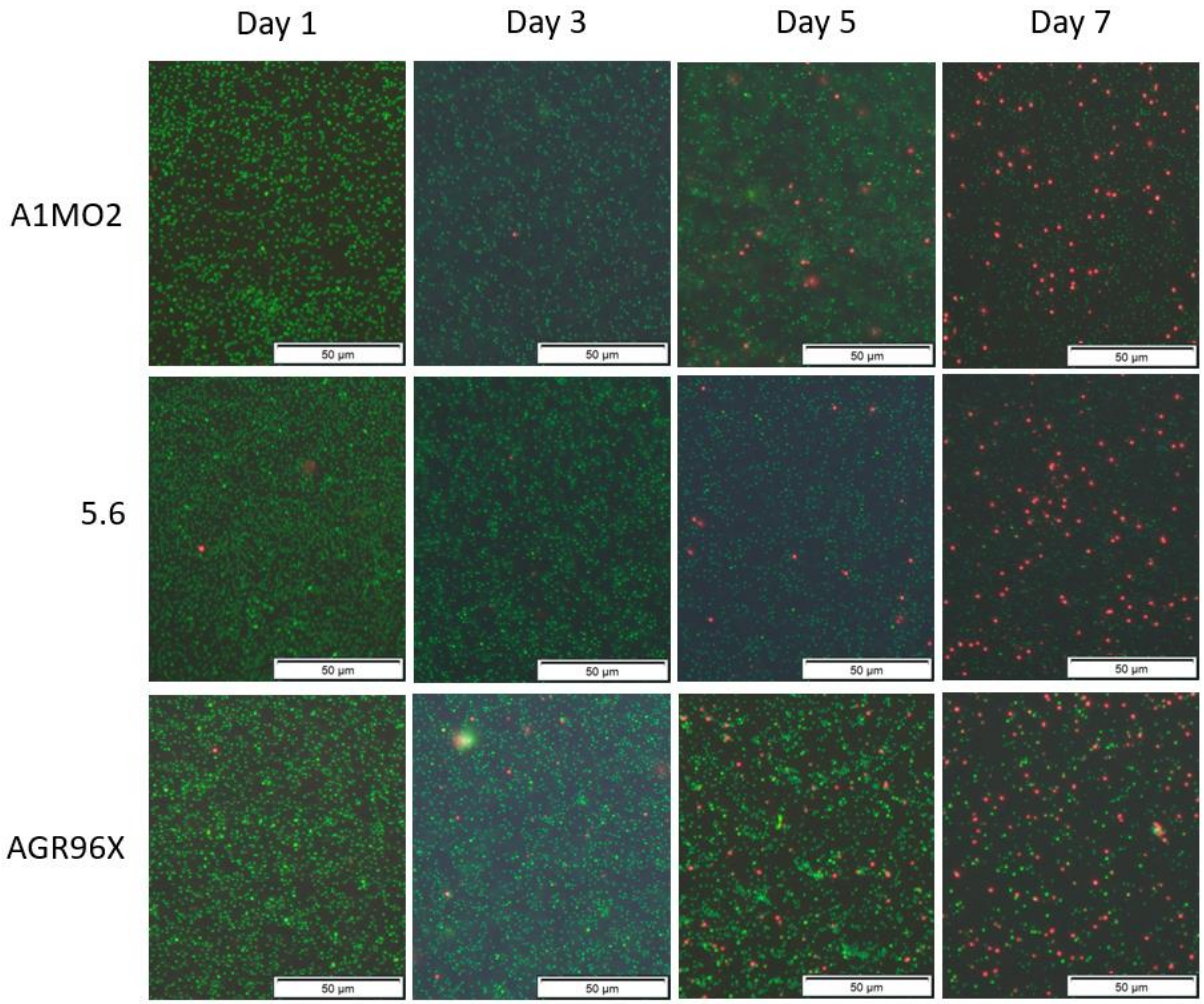


Figure 3.19. Microscopy images of viable (green) and dead (red) cells for wild-type *Serratia* spp. A1MO2, 5.6, and AGR96X cultured in LB broth over seven days. Images were captured at 40x magnification and are representative of three images captured at random areas on the slide, with each image covering an area of approximately 60,950 µm^2. Image scales represent 50 µm.

3.2.7.b. The viability of *Serratia* spp. when cultured in a 20 % casamino acids M9 medium

The separate inoculation of wild-type A1MO2, 5.6, and AGR96X in the casamino acids medium results in the generation of similar average CFU counts (n = 3) one dpi, with respective counts of 2.20 x 10^9, 2.73 x 10^9, and 2.53x 10^9 CFU/mL (Fig. 3.20). Similar to the previous culturing of the strains in the M9 medium the strains each maintained a high persistence within the cultures. This was most evident for 5.6, as there were no significant changes in CFU over the assay duration. However, between one and seven dpi the CFU count of A1MO2 increased significantly to 2.59 x 10^9 CFU/mL ($p < 0.05$), and AGR96X decreased significantly to 1.18 x 10^9 CFU/mL ($p < 0.05$). These trends were reflected in the regression models, showing a similar level of persistence for both A1MO2 ($R^2 = 0.15$) and 5.6 ($R^2 = 0.01$), and a reduced persistence for AGR96X ($R^2 = 0.75$) in the culture (Table 3.7), relative to A1MO2 and 5.6.

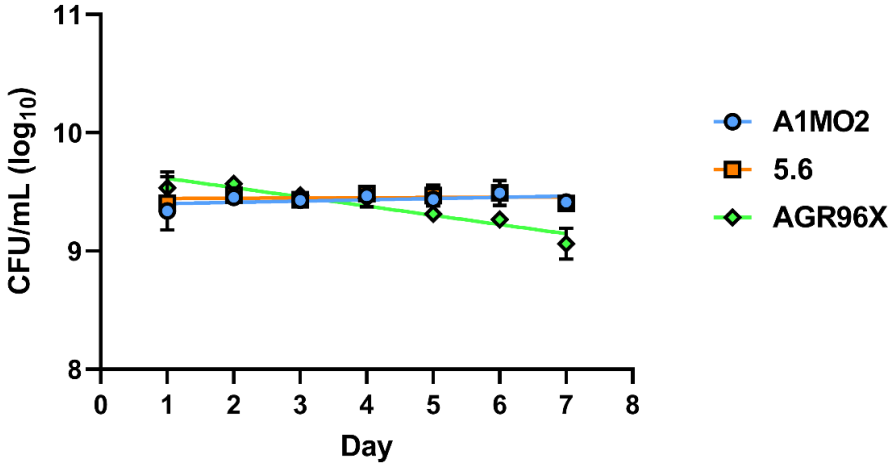


Figure 3.20. Average $\log_{10}$ CFU/mL counts (n = 3) for wild-type *Serratia* strains A1MO2, 5.6, and AGR96X cultured separately in an M9 20 % casamino acids medium over seven days. The strains were cultured from respective inocula of 7.40 x 10^8, 6.27 x 10^8, and 6.27 x 10^8 CFU/mL. The results are presented with standard deviation error bars.

Table 3.7. The linear regression models derived from $\log_{10}$ CFU/mL data for wild-type A1MO2, 5.6, and AGR96X cultured in an M9 20 % casamino acids medium over seven days, used to calculate the change in $\log_{10}$ CFU (*y*) per day (*x*), the standard error of the slope, the model goodness of fit (R^2), and the significance of the slope (*p*). Models were calculated to 19 degrees of freedom.

Strain	Regression equation	Slope error	R^2	*p*
A1MO2	$y = -0.011*x + 9.39$	0.006	0.15	0.084
5.6	$y = 0.002*x + 9.44$	0.011	0.01	0.843
AGR96X	$y = -0.078*x + 9.69$	0.010	0.75	< 0.001

There was a clear difference in the number of total cells when the strains were cultured in the casamino acids medium when compared to the LB broth, with fewer cells on average growing in the reduced-nutrient casamino acids medium (Fig. 3.21). Over the seven days of the assay very little change was recorded in the proportion of viable cells for A1MO2. From a total of 3,762 cells for A1MO2 at one dpi 99.0 % were still viable, and by seven dpi from a total of 3,960 cells 99.2 % were still viable. A similar outcome was recorded for 5.6, with 99.8 % of 4,007 total cells being viable at one dpi, and then 99.3 % of 3,445 total cells being viable by seven dpi. In contrast, the number of viable AGR96X cells in the culture showed some fluctuation, with 99.7 % of 2,329 cells being viable one dpi, decreasing to 96.0 % of 2,313 cells being viable at three dpi, and further to 87.6 % of 1,420 cells being viable by five dpi. The number of viable cells had increased by seven dpi, resulting in 94.6 % of 3,044 total AGR96X cells being viable. These results indicated that whilst fewer viable cells were present within the casamino acids medium, they had an increased persistence within the culture, when compared to a higher abundance of cells, as seen in the LB broth.

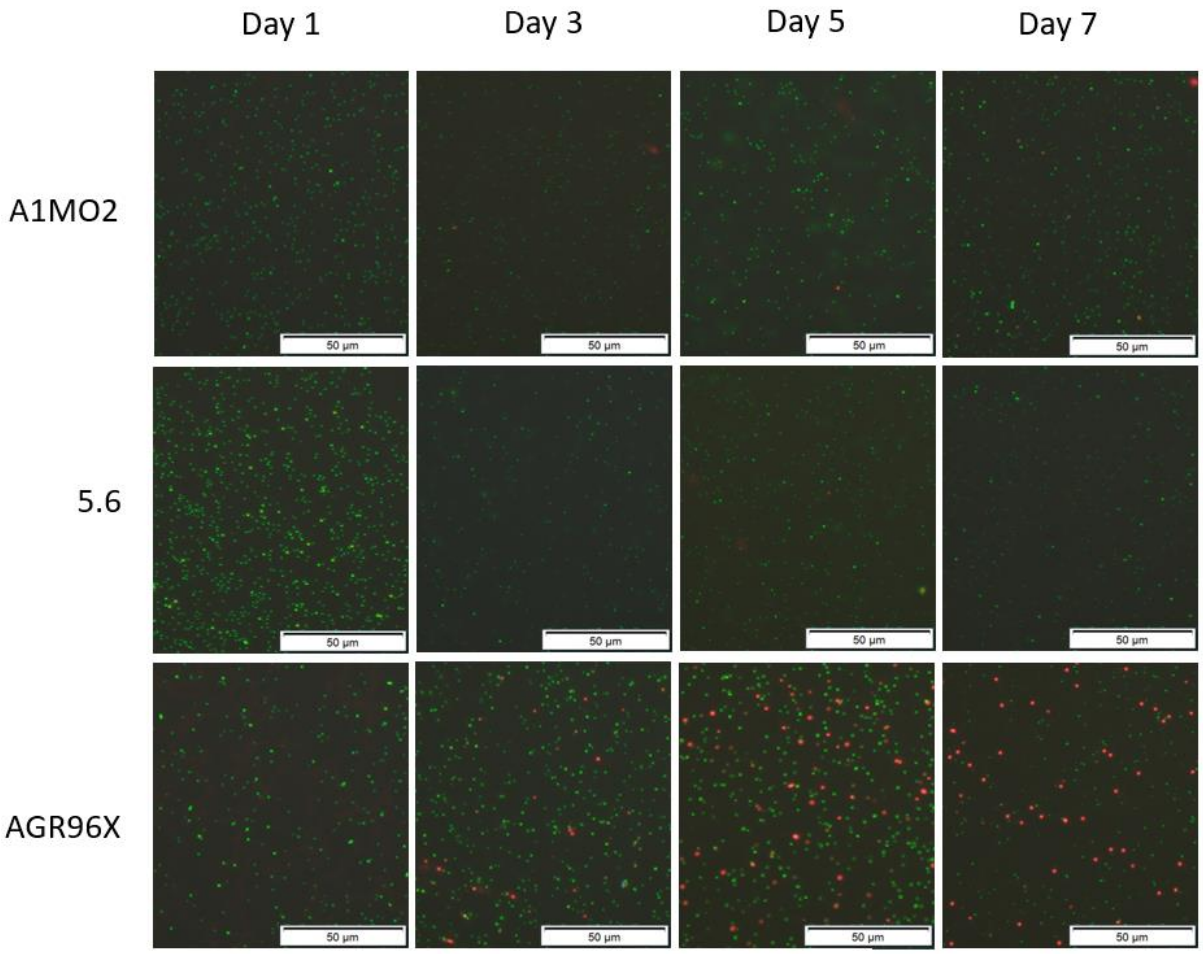


Figure 3.21. Microscopy images of viable (green) and dead (red) cells for wild-type *Serratia* spp. A1MO2, 5.6, and AGR96X cultured in an M9 20 % casamino acids medium over seven days. Images were captured at 40x magnification and are representative of three images captured at random areas on the slide, with each image covering an area of approximately 60,950 µm^2. Image scales represent 50 µm.

3.2.9 Characterising growth of fluorescence tagged *Serratia* spp. with fluorescence intensity

Further assessments were conducted into the growth dynamics of A1MO2 and 5.6 to build upon previous results showing a competitive advantage for 5.6 of a shorter doubling time during the exponential growth phase. The assays were conducted using fluorescence-tagged A1MO2 and 5.6 constructs (A1MO2_gfp and 5.6_rfp) (method: Chapter 2, section 2.3.8), allowing for differentiation of the strains based on green fluorescence (gfp) and red fluorescence (rfp), respectively, during growth over a 48-hour period (method: Chapter 2, section 2.3.10). The selected media (LB broth, 20 % glucose M9, 20 % casamino acids M9) were inoculated separately with both A1MO2_gfp (100:0) and 5.6_rfp (0:100), and with each of the strains in mixed ratios (75:25, 50:50, 25:75, A1MO2_gfp:5.6_rfp). The M9 media were inoculated just with the separate (100:0 and 0:100) and equal proportion (50:50, A1MO2_gfp:5.6_rfp) inocula. However, exposure of the strains to light at a wavelength of ~ 550 nm

resulted in a high amount of autofluorescence between construct and wild-type strains. Therefore, alternate constructs were produced (A1MO2_rfp and 5.6_gfp), and all assays were conducted using red-light fluorescence.

3.2.8.a. Fluorescence intensity of A1MO2 and 5.6 fluorescence constructs in LB broth

When inoculated in the LB broth there was a clear increase in fluorescence intensity as the proportion of A1MO2_rfp within the inoculant increased. The highest fluorescence intensity measurements were recorded when A1MO2_rfp was cultured separately (100:0), with an intensity reading of 1,747 a.u by 48 hpi (Fig. 3.22. A). However, reducing the proportion of A1MO2_rfp in the initial inoculant to 75:25 (A1MO2_rfp:5.6_gfp) also yielded similar intensity values, with an intensity reading of 1,571 a.u by 48 hpi. Inoculation with an equal proportion of both strains (50:50, A1MO2_rfp:5.6_gfp) produced an intensity reading of 1,180 a.u at 48 hpi. Further reducing the proportion of A1MO2_rfp in the initial inoculant (25:75, A1MO2_rfp:5.6_gfp) resulted in the lowest of the competition intensity measurements, with a reading of 732 a.u at 48 hpi. When cultured separately none of the control strains of 5.6_gfp, non-fluorescent wild-type A1MO2, and non-fluorescent wild-type 5.6 showed any major changes in fluorescence between inoculation and 48 hpi, with intensity readings between 375 and 378 a.u by 48 hpi (Fig. 3.22. B). Furthermore, the strains entered into exponential growth by ~ 2.5 hpi (150 minutes), which was maintained until ~ five hpi (300 minutes). Following exponential growth all treatments remained in the stationary phase until the end of the assay at 48 hpi.

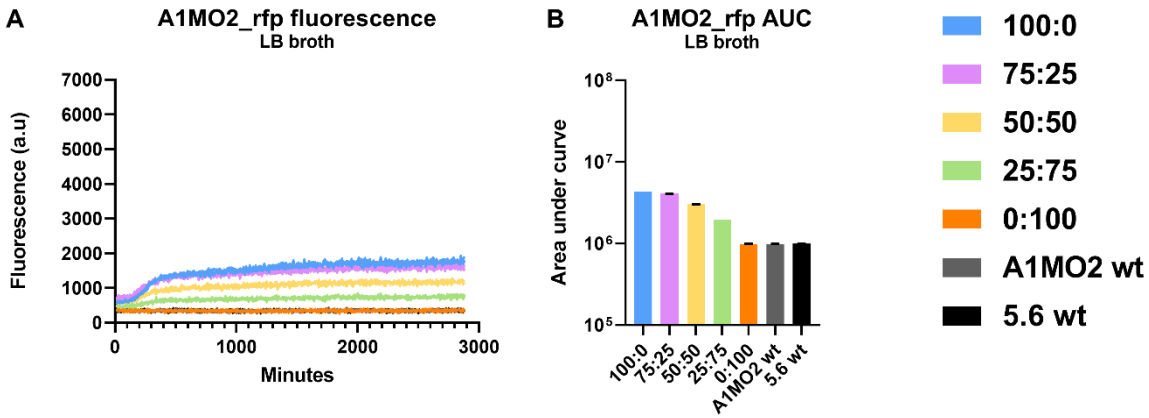


Figure 3.22. (A) The fluorescence intensity measurements (a.u) for A1MO2_rfp when cultured in LB broth over 48 hours alongside 5.6_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_rfp:5.6_gfp), and together in mixed ratios (75:25, 50:50, 25:75, A1MO2_rfp:5.6_gfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

The growth of 5.6_rfp exhibited a similar dynamic when competing against A1MO2_gfp. However, higher intensity measurements were recorded for 5.6_rfp when cultured separately and in mixed cultures with A1MO2_gfp. The highest measurements were recorded when 5.6_rfp was cultured separately (0:100), with an intensity reading of 2,315 a.u by 48 hpi (Fig. 3.23. A). Similar to the culturing of A1MO2_rfp, reducing the proportion of 5.6_rfp in the inoculant also corresponded with a reduction in the fluorescence intensity. When 5.6_rfp comprised the majority of the initial inoculant (25:75, A1MO2_gfp:5.6_rfp) it reached a fluorescence intensity of 2,115 a.u. by 48 hpi. The inoculation of the medium with both strains at an equal proportion (50:50, A1MO2_gfp:5.6_rfp) resulted yielded an intensity reading of 1,524 a.u by 48 hpi. Further reductions in the proportion of 5.6_rfp (75:25, A1MO2_gfp:5.6_rfp) resulted in an intensity reading of 958 a.u by 48 hpi. None of the non-fluorescent-tagged control strains showed any changes in fluorescence intensity, attaining measurements between 398 and 400 a.u by 48 hpi (Fig. 3.23. B). Much like A1MO2_rfp, the culturing of 5.6_rfp resulted in the onset of exponential growth by ~ 2.5 hpi (150 minutes), lasting until ~ 5.8 hpi (350 minutes). Following this 5.6_rfp entered into stationary growth, which lasted for the remainder of the assay until 48 hpi.

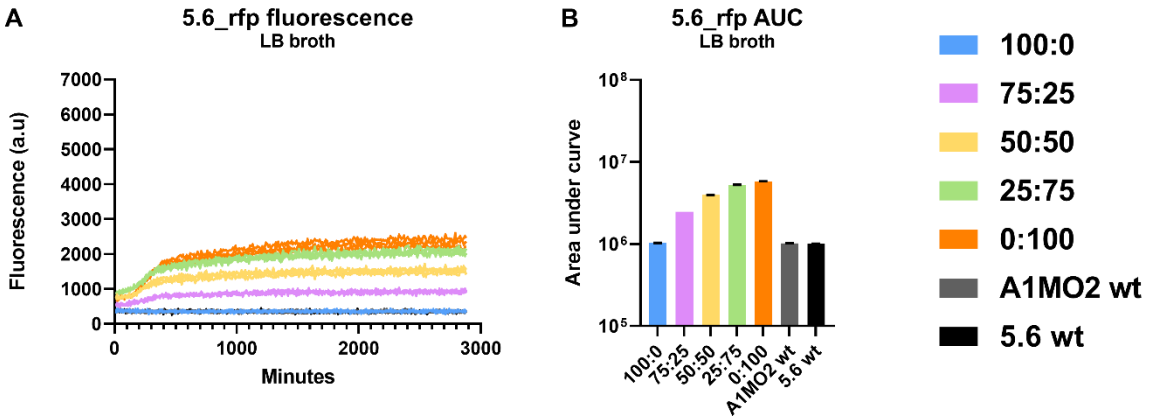


Figure 3.23. (A) The fluorescence intensity measurements (a.u) for 5.6_rfp when cultured in LB broth over 48 hours alongside A1MO2_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_gfp:5.6_rfp), and together in mixed ratios (75:25, 50:50, 25:75, A1MO2_gfp:5.6_rfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

3.2.8.b. Fluorescence intensity of A1MO2 and 5.6 fluorescence constructs in a 20 % glucose M9 medium

The culturing of A1MO2_rfp in the glucose medium resulted in distinct growth curves when compared to those produced when A1MO2_rfp was cultured in LB broth. This difference is seen clearly when A1MO2_rfp was cultured separately (100:0), resulting in a fluorescence intensity reading of 6,243 a.u by 48 hpi (Fig. 3.24. A). The inoculation of both A1MO2_rfp and 5.6_gfp at equal proportion (50:50) resulted in an intensity reading of 3,241 a.u by 48 hpi. Similar to previous results, no major changes in fluorescence intensity were recorded for the non-fluorescence-tagged control strains over the duration of the assay (Fig. 3.24. B). The fluorescence intensity measurements also showed that A1MO2_rfp entered into exponential growth by ~ 5 hpi (300 minutes), which lasted until ~ 13.3 hpi (800 minutes). However, instead of entering into stationary phase A1MO2_rfp continued to show increased fluorescence intensity, with another period of exponential growth occurring after ~ 23.3 hours (1,400 minutes), lasting until ~ 32.5 hpi (1950 minutes), exhibiting a diauxic shift. Following this second exponential growth phase A1MO2_rfp continued to increase in fluorescence intensity for the remainder of the assay until 48 hpi. As this second period of growth occurred at 24 hpi, it may reflect the cells in the medium utilising resourced from other dead and lysed cells within the culture.

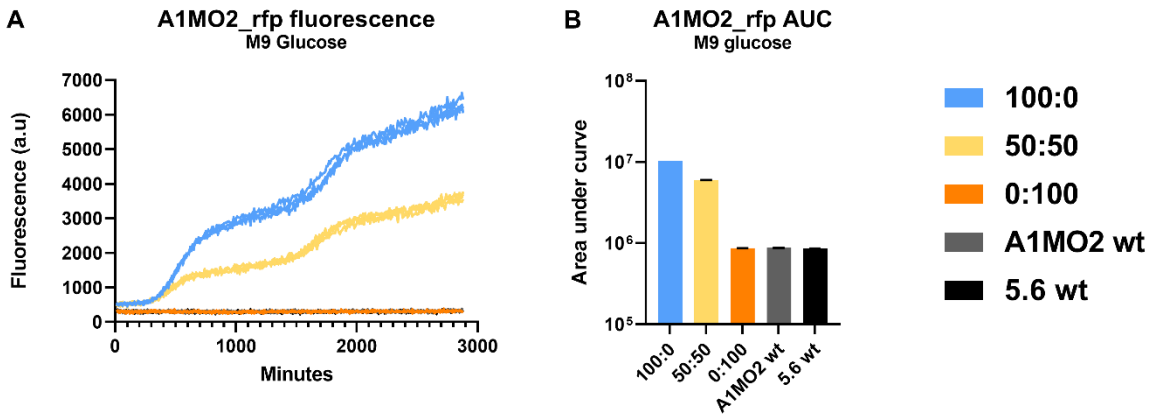


Figure 3.24. (A) The fluorescence intensity measurements (a.u) for A1MO2_rfp when cultured in a 20 % glucose M9 medium over 48 hours alongside 5.6_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_rfp:5.6_gfp), and together at an equal ratio (50:50, A1MO2_rfp:5.6_gfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

In contrast, the culturing of 5.6_rfp in the glucose medium yielded lower fluorescence intensity measurements than A1MO2_rfp, with an intensity reading of 3,422 a.u by 48 hpi when 5.6_rfp was cultured in the medium separately (0:100) (Fig. 3.25. A). Culturing both A1MO2_gfp and 5.6_rfp at equal proportions (50:50) caused a reduction in 5.6_rfp fluorescence intensity, with a reading of 1,871 a.u by 48 hpi. None of the control strains produced any major changes in fluorescence intensity of the duration of the assay (Fig. 3.25. B). Unlike A1MO2_rfp, the culturing of 5.6_rfp resulted in a single occurrence of exponential growth, occurring by ~ 4.1 hpi (250 minutes), lasting until ~ 11.6 hpi (700 minutes). Following this 5.6_rfp remained in stationary phase for the duration of the assay, up to 48 hpi.

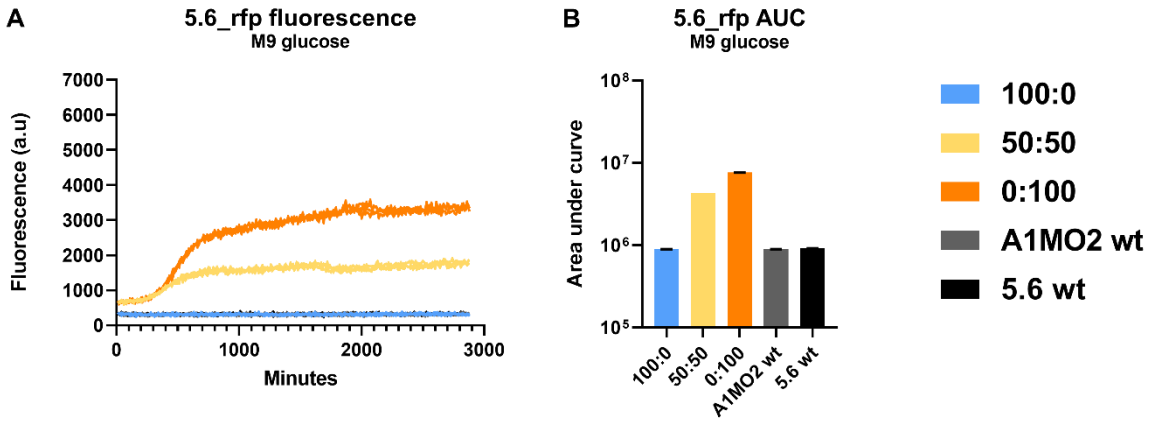


Figure 3.25. (A) The fluorescence intensity measurements (a.u) for 5.6_rfp when cultured in a 20 % glucose M9 medium over 48 hours alongside A1MO2_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_gfp:5.6_rfp), and together at an equal ratio (50:50, A1MO2_gfp:5.6_rfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

3.2.8.c. Fluorescence intensity of A1MO2 and 5.6 fluorescence constructs in a 20 % casamino acids M9 medium

The culturing of A1MO2_rfp and 5.6_gfp in the casamino acids medium resulted in a pattern of growth similar to when the strains were cultured in the glucose medium. The separate culturing of A1MO2_rfp (100:0) resulted in a fluorescence intensity reading of 6,352 a.u by 48 hpi, and culturing both A1MO2_rfp and 5.6_gfp at equal ratios (50:50) resulted in a comparatively lower fluorescence intensity of 3,241 a.u by 48 hpi (Fig 3.26. A). None of the control strains showed any increased in fluorescence intensity over the duration of the assay, finishing with readings between 337 and 345 a.u by 48 hpi (Fig. 3.26. B). As with the glucose medium two instances of increased growth were recorded over the duration of the assay. An initial period of exponential growth occurred at ~ 2.5 hpi (150 minutes), lasting until ~ 8.3 hpi (500 minutes), after which A1MO2_rfp entered into the stationary

phase. The second occurrence of exponential growth occurred at ~ 33.3 hpi (200 minutes), lasting for the remainder of the assay until 48 hpi. In the 50:50 culture this second phase of growth closer resembled linear growth rather than exponential. Similar to when the strains were cultured in the glucose medium, this may also reflect the cells in the medium using dead and lysed cells in the medium to fuel further growth.

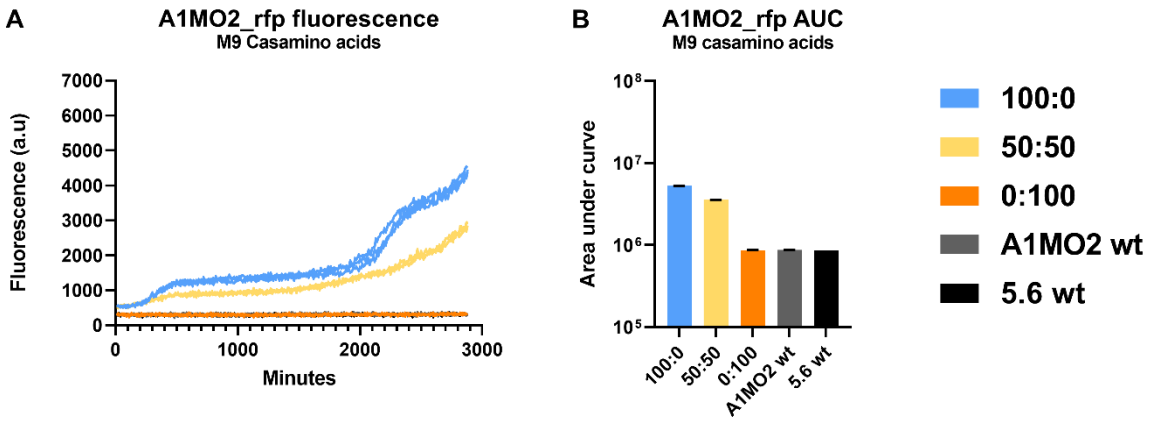


Figure 3.26. (A) The fluorescence intensity measurements (a.u) for A1MO2_rfp when cultured in a 20 % casamino acids M9 medium over 48 hours alongside 5.6_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_rfp:5.6_gfp), and together at an equal ratio (50:50, A1MO2_rfp:5.6_gfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

The culturing of 5.6_rfp within the casamino acids medium yielded lower fluorescence intensity readings when compared to A1MO2_rfp, with the separate culturing of 5.6_rfp producing an intensity reading of 5,416 by a.u 48 hpi (Fig. 3.27. A). The culturing of both A1MO2_gfp and 5.6_rfp at equal proportions in the inoculant (50:50) resulted in a lower fluorescence intensity for 5.6_rfp of 2,281 a.u at 48 hpi. Similar to other media, the control strains showed no major changes in fluorescence intensity, with readings between 342 and 350 a.u by 48 hpi (Fig. 3.27. B). The culturing of 5.6_rfp in the casamino acids medium also resulted in the occurrence of a diauxic shift. The first occurred at ~ 2.5 hpi (150 minutes), lasting until ~ 8.3 hpi (500 minutes). The second growth event occurred at ~ 12.5 hpi (750 minutes), lasting for the remainder of the assay until 48 hpi. The second growth phase closely resembled linear growth rather than exponential growth, especially in the 50:50 culture.

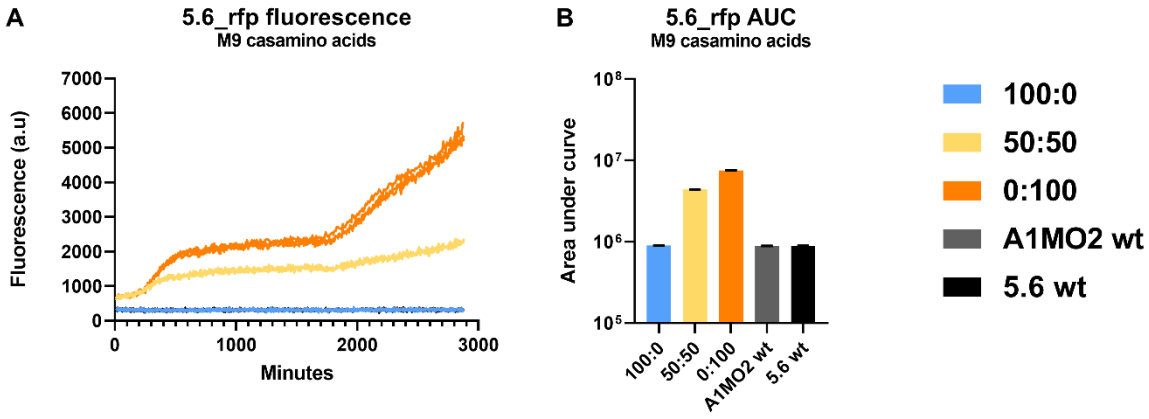


Figure 3.27. (A) The fluorescence intensity measurements (a.u) for 5.6_rfp when cultured in a 20 % casamino acids M9 medium over 48 hours alongside A1MO2_gfp when the strains were inoculated separately (100:0, 0:100, A1MO2_gfp:5.6_rfp), and together at an equal ratio (50:50, A1MO2_gfp:5.6_rfp) alongside wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures

3.3 Discussion

The data obtained during these assays showed that when the wild-type *Serratia* strains were inoculated separately in various types of nutrient-rich LB broth and restricted-nutrient M9 minimal media there was little difference between growth dynamics. However, when competing A1MO2 against 5.6 in LB broth cultures 5.6 was shown to consistently outcompete A1MO2. The increased competitiveness of 5.6 stemmed from a faster doubling rate than A1MO2 during the exponential growth period (occurring six to eight hours post-inoculation), and an increased persistence within aging LB broth cultures. Additionally, the outcome of competition between A1MO2 and 5.6 in the LB broth medium was not found to be dependent upon nutrient availability. This was seen when comparing the changes in the proportions of 5.6 cells isolated from the single LB broth cultures assessed over a seven-day period, to the continuously re-seeded LB broth cultures (Fig. 3.28). During these experiments a similarity in the increase of the proportion of 5.6 isolated from the mixed cultures over seven-days was observed, where in both the single-inoculated and re-seeded cultures 5.6 showed a strong competitive advantage over A1MO2 in each of the 90:10, 70:30 and 50:50 cultures (A1MO2:5.6). This might suggest that the competitive advantages displayed by 5.6 during these experiments are maintained regardless of the nutrient availability, as resources provided by the medium would diminish over the assay duration when compared to the continuously re-seeded cultures. Additionally, in the re-seeded cultures there would be a period of exponential growth following re-inoculation into the fresh medium, during which 5.6 would display a competitive advantage over A1MO2 through an increased growth rate.

One of the key components identified during the *In vitro* assessments was the calculation of a competition coefficient by way of a Lotka/Volterra-style competition model. Through the application of this model, the concept of competitive exclusion in the flasks can be explored further. During mixed inoculation, it was often seen that 5.6 had a positive competition coefficient, which, in comparison to the corresponding negative coefficient for A1MO2, would lead to increased competitive behaviour. Due to this difference, and the increased growth rate of 5.6, it would be more readily able to utilise the nutrients in the flask at a faster rate than A1MO2, leading to the exclusion of A1MO2 due to an inability to gather a sufficient amount of resources. The use of competitive exclusion to oust one bacterial species has been observed in animals using non-pathogenic probiotic cultures to prevent pathogens such as *Salmonella* or *E. coli* from establishing colonies within the intestine (Callaway et al., 2008), and thus a similar effect may be occurring within the larvae. However, whilst the competition coefficients correlate with the *in vitro* data, the high standard error of the associated coefficients indicates that more data is required to build more accurate models to quantify competition between multiple *Serratia* strains in a mixed inoculant.

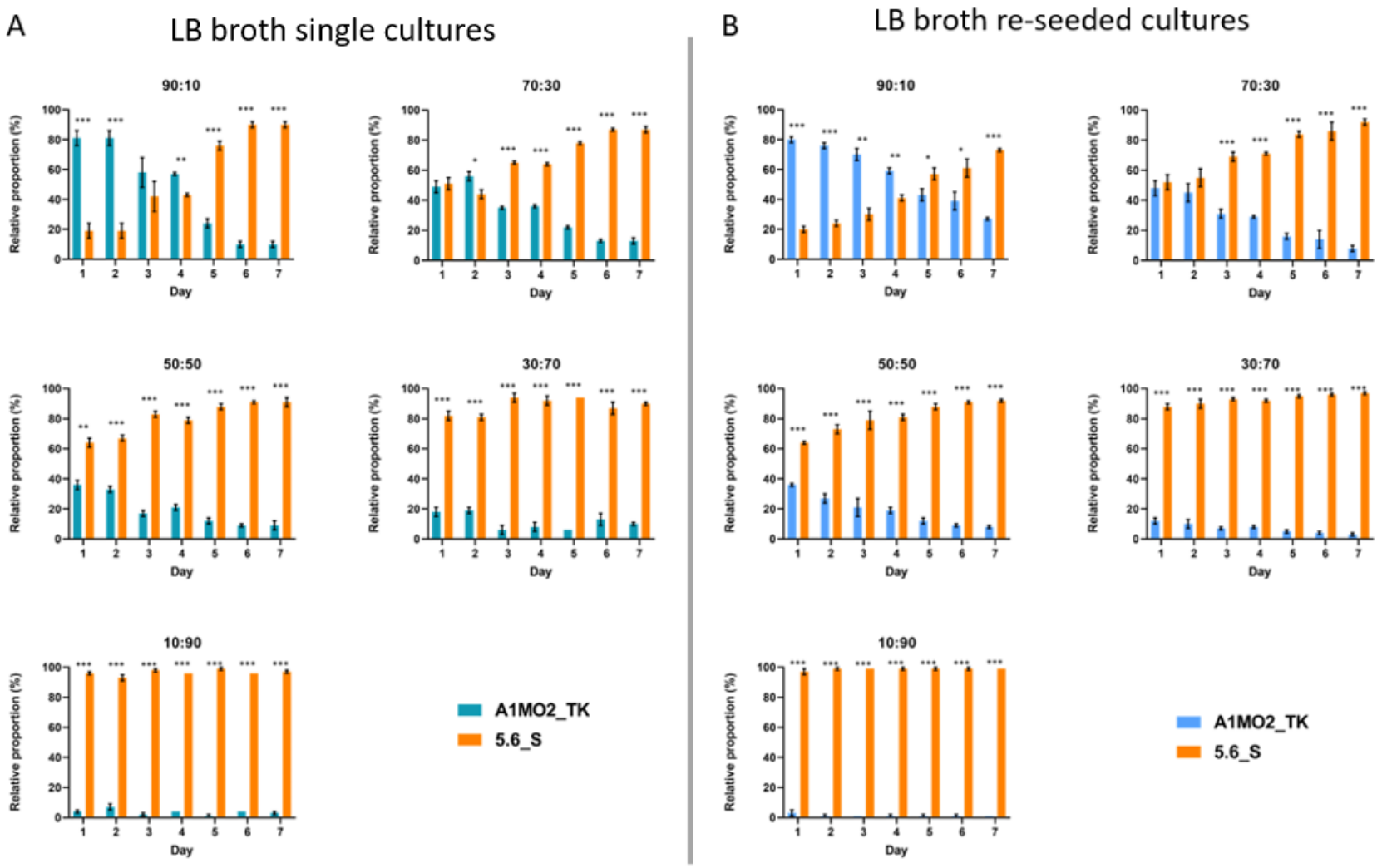


Figure 3.28. A comparison between the changes in the average relative proportion (n = 3) over a seven-day period of A1MO2_tk and 5.6_s isolated from either (A) LB broth cultures inoculated once and maintained for seven days, or (B) LB broth cultures that were re-seeded daily. Ratios represent the proportion of A1MO2_tk and 5.6_s in the initial inocula (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Another key finding obtained during these assays was the relationship between the proportion of the strain in the mixed inoculant and its fitness in competition. Many of the assays demonstrated that when the 5.6 comprised the lowest proportion of the inoculant it displayed increased competitive capabilities, relative to A1MO2, in the mixed inoculant. This was observed in many situations where 5.6 was grown in competition with A1MO2, and was also exhibited by A1MO2 when competing against 477. This is in accordance with previous studies which have found cheat strains to display a higher level of relative fitness when present in smaller proportions due to the increased amount of nutrients, or public goods which can be exploited (MacLean and Gudelj, 2006; Harrison et al., 2006; Ross-Gillespie et al., 2007). Interestingly, this did not manifest when A1MO2 comprised the minority of the mixed inoculant when competing against 5.6. Another key finding obtained during these assays was the relationship between the proportion of the strain in the mixed inoculant and its fitness in competition. Many of the assays demonstrated that when the 5.6 comprised the lowest proportion of the inoculant it displayed increased competitive capabilities, relative to A1MO2, in the mixed inoculant. This was observed in many situations where 5.6 was grown in competition with A1MO2, and was also exhibited by A1MO2 when competing against 477. This is in accordance with previous studies which have found cheat strains to display a higher level of relative fitness when present in smaller proportions due to the increased amount of nutrients, or public goods which can be exploited (MacLean and Gudelj, 2006; Harrison et al., 2006; Ross-Gillespie et al., 2007).

This outcome did not manifest when A1MO2 comprised the minority of the mixed inoculant when competing against 5.6. In addition, the effects of competition were not seen when A1MO2 and 5.6 were competing with each other in M9 media supplemented with either glucose or casamino acids carbon sources. This led to a distinct dynamic where both strains showed a similar level of persistence within the cultures over a seven-day period, with neither strain outcompeting the other (Fig. 3.29). The reduced cell density of the strains in this medium may have contributed to this outcome. Additionally, differences between the nutrient profiles of LB broth and the M9 media may have led to the differences in growth, with LB broth containing a wider variety of sugars than M9 medium, including sugars such as maltose and trehalose, which often have early utilisation by some bacteria. A similar outcome has been observed previously with *E. coli*, where a wild-type strain and a mutant strain with an advantage for growth in the stationary phase were able to coexist to maximise the long-term survival of the strains, rather than competing against each other (Keymer et al., 2008). However, whilst this coexistence between A1MO2 and 5.6 in the M9 media is evident from the data obtained, as the assay only covered seven days further interactions may be seen as time progresses. This was seen previously during competition between viral species, where viral particles coexisted under a constant state of evolutionary pressure, until one species eventually outcompeted the other (Solé et al., 1999).

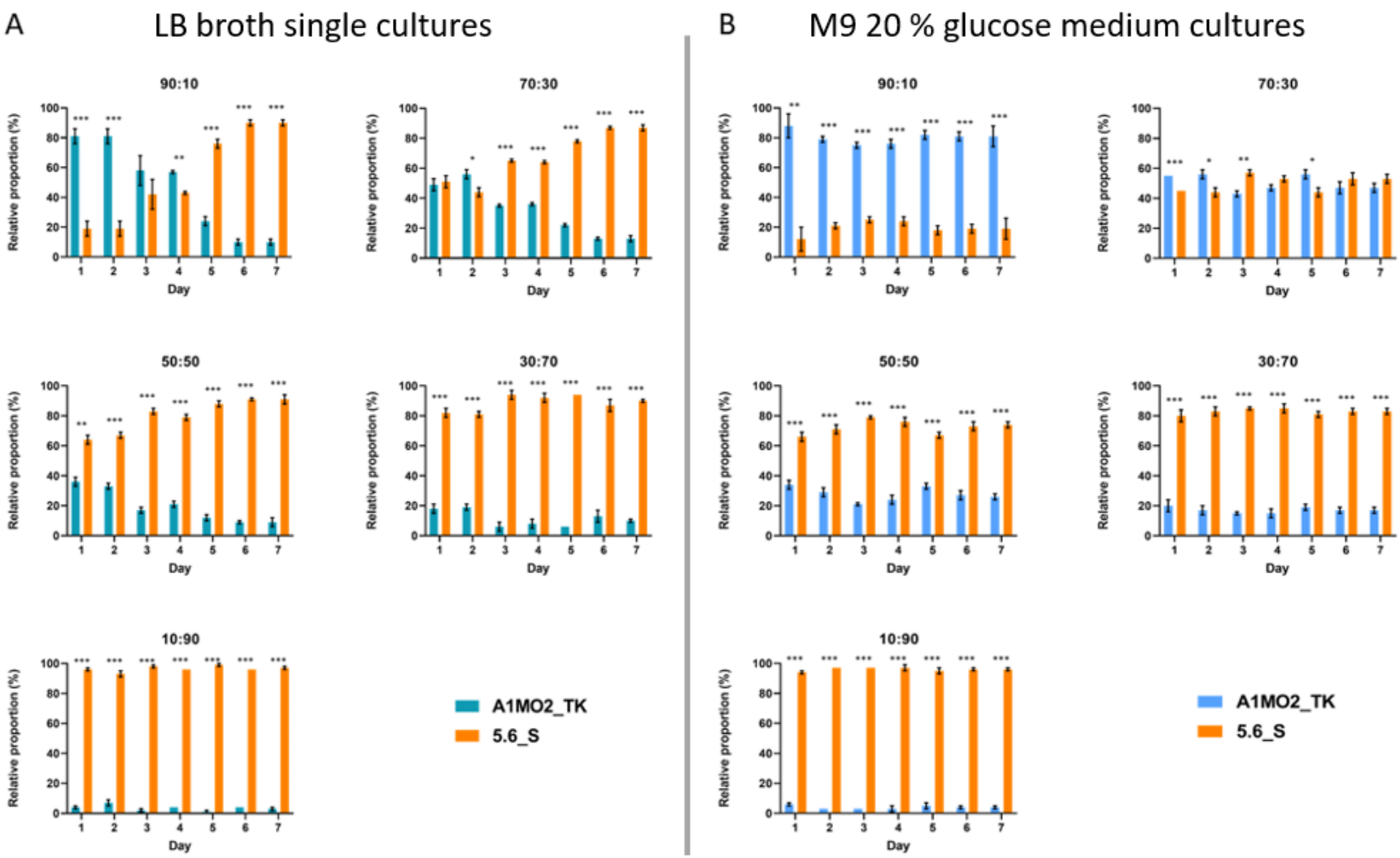


Figure 3.29. A comparison between the changes in the average relative proportion (n = 3) over a seven-day period of A1MO2_tk and 5.6_s isolated from either (A) LB broth cultures inoculated once and maintained for seven days, or (B) 20 % glucose M9 medium cultures inoculated once and maintained for seven days. Ratios represent the proportion of A1MO2_tk and 5.6_s in the initial inocula (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Further experimentation with the fluorescence-tagged strains showed that 5.6 attained higher fluorescence intensity readings than A1MO2 in both the LB broth and M9 casamino acids media, whereas A1MO2 achieved a higher intensity reading in the M9 glucose medium. It was anticipated that 5.6 would achieve higher fluorescence intensity measurements in each of the media types. It was also noted that both strains attained higher intensity measurements when cultured separately and together in competition in the M9 media than in the LB broth. It is unclear why a higher fluorescence was achieved in the M9 medium, as previous *in vitro* assessments conducted over seven days showed that fewer cells grew in the M9 medium compared to LB broth. Additionally, a second period of growth was recorded up to 24 hours post-inoculation for both A1MO2 and 5.6 in the M9 medium. Whilst this may have stemmed from the cells present using resources from dead and lysed cells to fuel growth, the use of fluorescence microscopy over a seven-day period showed that very little cell death occurred in the M9 medium. As such, the diauxic growth pattern observed may link to the increased expression of genes in the cells relating to the metabolism of other carbohydrate sources at those specific time-points, such as maltose and trehalose, allowing for further growth, which is explored further in Chapter 4. Furthermore, the AUC results showed that growing the strains in competition with each other had little effect on fluorescence intensity when compared to when the strains were grown independently. This suggested that whilst competition does occur in the first 48-hours post-inoculation, as indicated by the *in vitro* growth curves, it might not have a major impact on growth when the strains are cultured under optimal conditions.

In contrast to the interactions between A1MO2 and 5.6, the mixed inoculation of A1MO2 and other strains in LB broth, such as 477 and AGR96X, led to a different outcome, where A1MO2 was shown to be the more competitive strain based on changes in CFU counts. This is interesting, as it indicates that the competitive abilities of 5.6 may not be shared amongst other wild-type non-pathogenic *Serratia* species, reflective the isogenic nature of 5.6 to A1MO2. Furthermore, this might also underpin another difference between 5.6, a non-pathogenic strain isogenic to A1MO2 and 477, a natural non-pathogenic *S. entomophila* isolate. The differences between the outcome of growth between 5.6 and 477 and A1MO2 indicates that relatedness between *S. entomophila* species might not offset competition, as has been reported previously with other species (Hamilton, 1963; Crespi, 2001; Griffin et al., 2004; Diggle, et al., 2007). However, the fitting of linear regression models to the CFU data generated from mixed inoculation of A1MO2 and AGR96X showed a varying level of fitness in the cultures, where the minority strain in the inoculant had the greatest fitness relative to the majority strain (Ross-Gillespie et al., 2007).

Whilst these results clearly display the competitive capabilities of 5.6, and its ability to outcompete A1MO2 when both strains are inoculated together in a LB broth culture, further work is required to determine how competition might occur under other conditions, such as within the soil or the larvae. Firstly, a comparative assessment between gene expression for A1MO2 and 5.6 would help to define whether the fitness traits of 5.6 are caused by any genetic differences between the strains, or whether any other potential specialist traits for 5.6 can be identified. Through transcriptome assessments, the differences in gene expression between 5.6 and A1MO2 will be assessed; these results are presented in Chapter 4. Another key aspect to determine is whether competition between A1MO2 and 5.6 will have a similar outcome when the strains are inoculated into either the soil or the larval gut, as this will determine whether competition can impact the efficacy of A1MO2. These results are presented in Chapter 5 and Chapter 6, respectively.

Chapter 4

Chromosomal genetic differences between pathogenic and non-pathogenic *Serratia* spp.

4.1 Introduction

The data obtained during *in vitro* assessments of A1MO2 and 5.6 show that when the strains were inoculated and cultured separately there is very little difference between the recorded growth dynamics. However, when A1MO2 and 5.6 were inoculated together at mixed ratios 5.6 exhibited a faster doubling rate during exponential growth, and increased persistence within a LB broth culture monitored over seven days. One reason for this is that whilst self-limitation may hinder growth by a small amount, as discovered through the Lotka/Volterra-style models, the onset of competition can add further stress alongside self-competition, leading to a difference in growth rates that might not manifest when the strains are cultured separately. These differences may stem from genetic differences between the strains where the lack of pADAP in 5.6 results in the expression of genes associated with increased survival. If so, the increased expression of these genes would still incur a base metabolic cost, though these costs would be offset by the benefits of their expression (Lang et al., 2009). Furthermore, as 5.6 is an isogenic variant of A1MO2, heat-cured of pADAP, any differences in the expression of chromosomal genes between the strains should be apparent. These potential differences were explored with whole-genome shotgun RNA sequencing (RNAseq) using 5.6, and a construct 5.6 strain containing a conjugated pADAP (5.6+pADAP) to act as a proxy for A1MO2. The use of 5.6+pADAP also ensured that the only difference between the sequenced strains was the carriage of pADAP, and thus any differences in the expression of chromosomal genes would relate to the carriage of pADAP. Previous work has shown how plasmid carriage can influence the expression of chromosomal genes, including those related to metabolism (Shintani et al., 2009; Lang and Johnson, 2015; San Millan et al., 2018) and virulence (Buckner et al., 2018; Cheng et al., 2019).

The RNAseq data will be assessed through comparative transcriptomics, a method which has been used previously to identify the differences in gene expression between various microbial species, including between pathogenic and non-pathogenic strains of bacteria (Wurtzel et al., 2012) and fungi (Goa et al., 2011; Schuelke et al., 2017). Whilst the results from some of these comparative studies demonstrate a high degree of relatedness between pathogenic and non-pathogenic species, such as in *Listeria* spp. (Wurtzel et al., 2012), unique genetic clusters were still observed between strains. The identification of such clusters in a comparative analysis between 5.6 and 5.6+pADAP may identify mechanisms by which 5.6 is able to outcompete A1MO2 in a nutrient-rich LB broth culture.

To obtain samples for RNAseq, 5.6 and 5.6+pADAP were cultured separately in LB broth until the mid-exponential growth phase (eight to nine hours post-inoculation (hpi), approximating ~ 7 x 10^9 CFU/mL in an LB broth culture), with three replicates prepared from each culture (methods: Chapter 2, sections 2.3.11 to 2.3.14). This time-point was of interest as this was when 5.6 began to outcompete A1MO2 during previous *in vitro* assessments (Chapter 3). This culturing process was repeated for any strains with replicates which were not deemed suitable for sequencing due to a low RNA yeild. The genes encoded on pADAP in the 5.6+pADAP samples were included during the initial transcript analysis and are depicted and highlighted in Figures 4.2 and 4.3. However, during the analysis of differences between chromosomal gene expression between 5.6 and 5.6+pADAP, the pADAP expression profile was removed from the analysis. This ensured that only chromosomal gene expression differences would be compared between the strains. The identification of chromosomal operons with differing levels of expression between the strains would facilitate further *in vitro* assessment to determine the extent to which gene expression can influence the outcome of competition between the strains. The full RNAseq results for 5.6 and 5.6+pADAP are collated and presented in Appendix C.1.

4.2 Results

4.2.1 RNA sequencing of pathogenic and non-pathogenic *S. entomophila* strains

Performing a principal component (PC) analysis between the chromosomal gene expression data showed a clear degree of relatedness between the genes expressed within each of the strains (Fig. 4.1), with the 5.6 replicates showing a greater overall similarity than the 5.6+pADAP replicates. One consistent finding between the two strains was a similarity in PC1 variance between all three replicates, but a degree of separation for replicate c of both strains for the PC2 variance. Furthermore, similar CFU counts were recorded for the replicates during sampling at the mid-exponential growth phase, with CFU counts ranging between 7.00 x 10^9 to 7.60 x 10^9 CFU/mL. This included the replicate ‘c’ for each strain, both of which showed a high variance from other replicates despite a similar CFU count at the time of sampling. The differences observed in PC2 variance for replicates 5.6_c and 5.6+pADAP_c are likely batch effects, stemming from these samples being prepared on a different day to the other replicates for each strain. This was based on a need to replace the original replicates which were not deemed suitable for sequencing due to a low RNA yield. A higher CFU count was recorded for 5.6+pADAP replicate ‘b’, with a count of 1.14 x 10^{10} CFU/mL, though this difference did not lead to any noticeable changes in PC1 variance when compared to 5.6+pADAP replicate ‘a’.

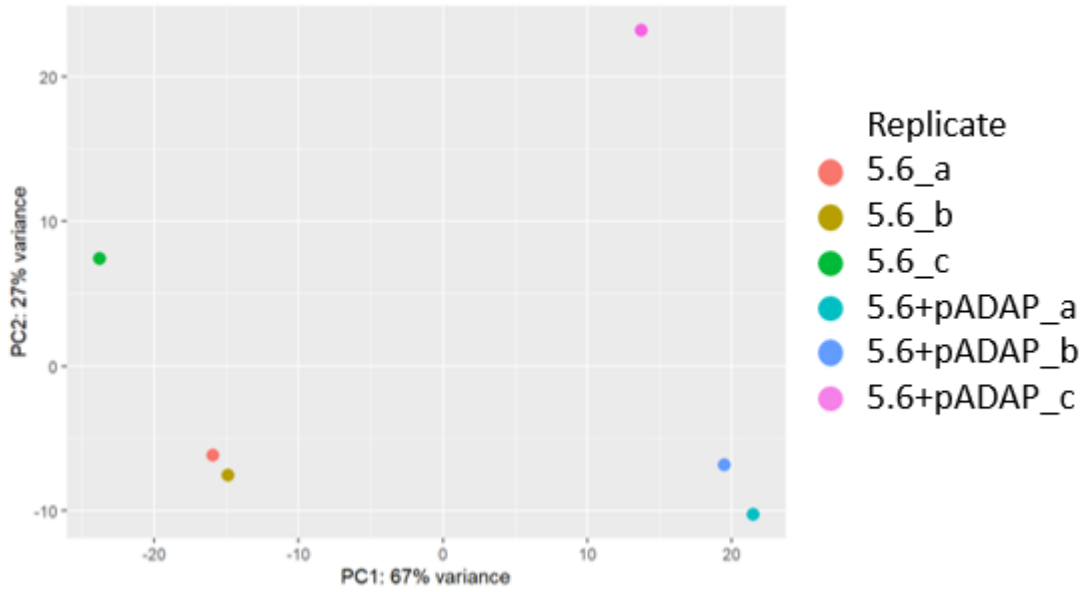


Figure 4.1. Principal component analysis plot comparing PC1 and PC2 variance for chromosomal gene expression in 5.6 and 5.6+pADAP replicates a, b , and c.

The correlation of gene expression between the two strains was then examined. These results show a high degree of similarity between chromosomal gene expression for both strains, with a strong significant positive correlation of 0.917 ($p < 0.0005$) (Fig. 4.2). However, as highlighted in Figure 4.2, a number of genes likely encoded on pADAP, and in the 5.6+pADAP replicates, are clearly visible. These genes are also represented in the lower quartiles for the strains, with $\log_{10}$ expression scores between 0.0 and 0.2 ($\log_{10}$ (FPM) on the Y axis).

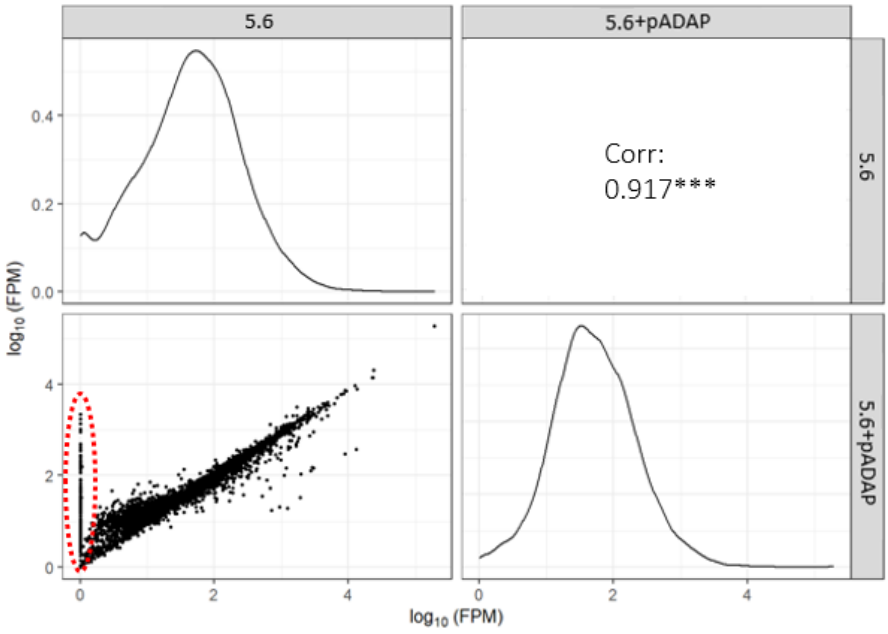


Figure 4.2. A scatter plot combining the distribution of gene expression in 5.6 and 5.6+pADAP replicates, alongside the correlation of gene expression in the strains. Genes likely encoded on pADAP are highlighted in red.

Further assessment of the RNAseq results allowed for the differentiation between genes encoded on the chromosome and those encoded on pADAP (Fig. 4.3). A total of 49 genes were identified with a $\log_2$ fold change greater than two, which, when cross referenced with the collated gene expression spreadsheet, were found to have a higher degree of expression in the 5.6+pADAP replicates. Similarly, a total of 71 genes were identified with a $\log_2$ fold change less than negative two, and whilst these genes were expressed by both strains there was a significantly higher level of expression in the 5.6 replicates ($p < 0.05$). The remaining 4,490 genes had a $\log_2$ fold change between two and negative two and no significant difference in expression between the strains, and thus were expressed at a similar level in both strains. A total of 124 genes were identified in the 5.6+pADAP replicates with no expression in 5.6, and thus were thought to be encoded on pADAP rather than the chromosome.

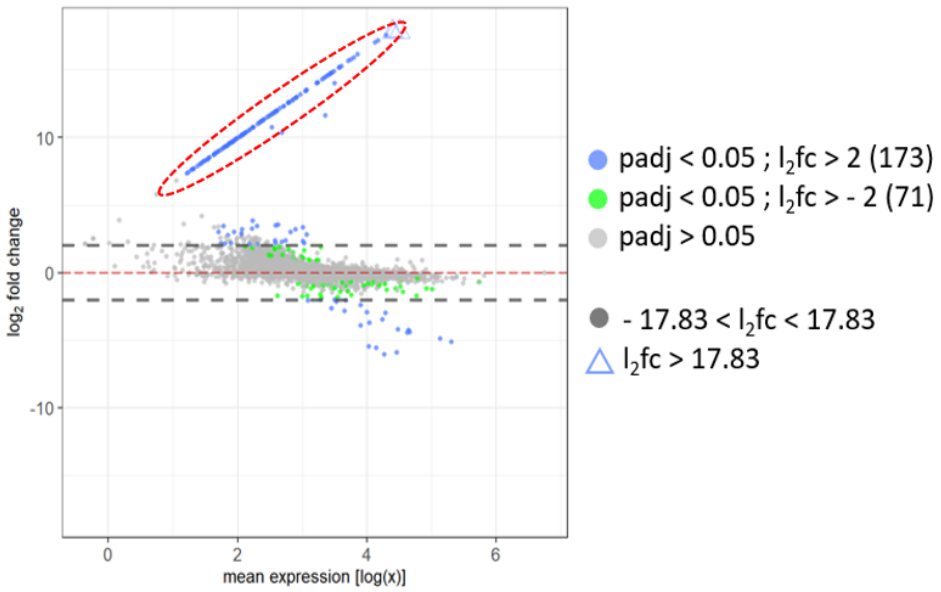


Figure 4.3. The differences in gene expression between 5.6 and 5.6+pADAP replicates based on mean log_2 fold change (l_2fc) scores: Blue = significantly higher expression in 5.6+pADAP replicates, green = significantly higher expression in 5.6 replicates, grey = no significant difference in expression between strains. Genes likely encoded on pADAP are highlighted in red.

4.2.2 Chromosomal genes with differing levels of expression in 5.6 and 5.6+pADAP replicates

The collated RNAseq gene expression spreadsheet (Appendix C.1) was colour-coded to differentiate between chromosomal genes based on their expression in the 5.6 and 5.6+pADAP replicates, with hypothetical products identified via nucleotide BLAST (BlastN) against the wild-type *S. entomophila* strain A1MO2 (method: Chapter 2, section 2.3.15). Operons with increased expression in 5.6 are colour-coded in green. Several chromosomal operons were identified within both strains and are presented in Tables 4.1 to 4.9. Furthermore, of the 71 genes classified as having significantly higher levels of expression in the 5.6 replicates ($p < 0.05$), 23 had a log_2 fold change score exceeding negative two, and 48 had a log_2 fold change score between zero and negative two. In total, 55 of these genes could be linked to a predicted product in the BlastN data. Of the 49 genes with a significantly higher level of expression in the 5.6+pADAP replicates ($p < 0.05$), 26 had a log_2 fold change score exceeding two, and 23 had a log_2 fold change score between zero and two. Of these genes, only 24 could be linked to a predicted product, with the remainder being listed with hypothetical products.

The first of the operons identified in the RNAseq data contained genes related to the production of adhesion-related fimbriae proteins, which were found to have an increased level of expression in the 5.6 replicates (Table 4.1). These gene products are used in cellular adhesion, facilitating attachment of the cell to nearby bacterial cells. The level of expression for the gene *smfa_1* was significantly higher in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.0005$), with an average expression score (n = 3) between the three 5.6 replicates of 81,053, compared to 28,917 in the 5.6+pADAP replicates. The genes *yfcQ_1*, *yfcQ_2*, and *yfcP* were also expressed at a significantly higher level in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.005$), with products related to fimbrial adhesion proteins. Only two of the translated gene products in this operon (Outer membrane usher protein PapC and Fimbrial chaperone YfcP) could be classified with the NCBI database and defined through the cluster of orthologous groups (COG) database as extracellular structures (W) (Table 4.1).

Table 4.1. The gene operon encoding for adhesion and fimbriae proteins (transcript IDs = 00269, 00271:00277) expressed at a higher level in 5.6 replicates than in 5.6+pADAP replicates. The genes are presented with the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned.

Transcript ID	Gene	5.6 expression score	5.6+pADAP expression score	COG	Predicted product[1]	Predicted function	Uniprot accession number
00269	*smfA_1*	81053	28917	-	Fimbria A protein	General cell adhesion	A0A0D8W4B3
00271	*papC*	5587	3485	W	Outer membrane usher protein	Involved in the export/assembly of pili subunits across outer membrane	P07110
00272	*yfcS*	2821	1806	W	Fimbrial chaperone	Contributes to cellular adhesion to various surfaces	P77599
00273	*lpfA*	2169	1410	-	Fimbrial protein	General cell adhesion	Q8VPB9
00274	*yfcQ_1*	1421	846	-	Uncharacterised fimbrial-like protein	Contributes to cellular adhesion to various surfaces	P76500
00275	*yfcQ_2*	1044	761	-	Uncharacterised fimbrial-like protein	Contributes to cellular adhesion to various surfaces	P76500
00276	*yfcP*	1726	904	-	Uncharacterised fimbrial-like protein	Contributes to cellular adhesion to various surfaces	P76500

[1] Predicted product for all genes obtained by BlastN.

The second operon identified in the RNAseq data contained genes related to the transport and metabolism of trehalose. These genes were found to have an increased level of expression in the 5.6 replicates (Table 4.2). Trehalose is a non-reducing disaccharide sugar comprised of two glucose molecules which can act as a carbon source for bacteria, and the genes related to its transport and metabolism, *treB* and *treC* (respectively), were found to have a significantly higher level of expression in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.0005$), with respective expression scores of 72,350 and 51,716 in 5.6. These genes are still expressed in the 5.6+pADAP replicates, but had much lower respective expression levels of 4,057 and 3,422. The expression of *treBC* is controlled and repressed by the regulator *treR*, which had a lower level of expression in the 5.6 replicates compared to 5.6+pADAP, with expression scores of 841 and 1195, respectively. Classifying the translated products of these genes to the COG database reveals that TreB and TreC belong to the carbohydrate metabolism and transport class (G), TreR belongs to transcription (K), and YrwO belongs to the prediction-only class (R).

Table 4.2. The gene operon encoding for trehalose metabolism and transport (transcript IDs = 01175:01178) expressed at a higher level in 5.6 replicates than in 5.6+pADAP replicates. The genes are presented with the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number.

Transcript ID	Gene	5.6 expression score	5.6+pADAP expression score	COG	Predicted product[1]	Predicted function	Uniprot accession number
01175	*ywrO*	567	211	R	General stress protein 14	Assist the growing cell with general stress management	P80871
01176	*treC*	51716	3422	G	Trehalose-6-phosphate hydrolase	Hydrolyses trehalose-6-phosphate to glucose and glucose-6-phosphate	P28904
01177	*treB*	72350	4057	G	Trehalose-specific E11BC component	Catalyses the phosphorylation of incoming sugar substrates, also involved in trehalose transport	P36672
01178	*treR*	841	1195	K	HTH-type transcriptional regulator	Repressor of the *treBC* operon, can also bind trehalose-6-phosphate and trehalose	P36673

[1] Predicted product for all genes identified by BlastN

The third operon identified contained genes related to the transport and binding of maltose, another disaccharide sugar formed from two glucose molecules, and the related genes were found to have an increased level of expression in the 5.6 replicates (Table 4.3). The genes *malG* and *malF* were expressed at significantly higher levels in the 5.6 replicates than the 5.6+pADAP replicates ($p < 0.005$), with expression scores of 2,621 and 2,823 in the 5.6 replicates, respectively, compared to 1,025 and 953 in the 5.6+pADAP replicates. These genes are involved in the formation of the *malFGK* complex with *malK*, facilitating the transport of maltose and maltodextrins across the cellular membrane (Mächtel et al., 2019). The genes *malK*, *malE*, *malM*, and *lamB* all showed increased levels of expression in the 5.6 replicates and their gene products are used to facilitate the transport of maltose and maltodextrins from the outer membrane to the periplasm (*lamB*) and their subsequent binding (*malE*). The function of *malM* is not fully understood, though it is thought to be associated with the cell wall outer membrane (Boos and Shuman, 1998). All COGs identified in this cluster belonged to the carbohydrate metabolism and transport class (G).

Table 4.3. The gene operon encoding for maltose metabolism and transport (transcript IDs = 01675:01677, 01679:01681) expressed at a higher level in 5.6 than in 5.6+pADAP. The genes are presented with the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned.

Transcript ID	Gene	5.6 expression score	5.6+pADAP expression score	COG	Predicted product[1]	Predicted function	Uniprot accession number
01675	*malG*	2621	1025	G	Maltose transport system permease protein	Responsible for the transport of maltose across cell membrane. Component of MalEFGK complex.	P68183
01676	*malF*	2823	953	G	Maltose transport system permease protein	Responsible for the transport of maltose across cell membrane. Component of MalEFGK complex.	P02916
01677	*malE*	11992	2775	G	Maltose transport system permease protein	Binds maltose and maltodextrins. Component of the MalEFGK complex.	P0AEX9
01679	*malK*	4356	872	G	Maltose transport system permease protein	Responsible for energy coupling to maltose transport system. Component of the MalEFGK complex.	P68187
01680	*lamB*	15646	2611	G	Maltoporin	Involved in the transport of maltose and maltodextrins.	P02943
01681	*malM*	4475	1392	-	Maltose operon periplasmic protein	Periplasmic protein of unknown function, associated with outer membrane.	P03841

[1] Predicted product for all genes identified by BlastN.

The fourth operon identified in the RNAseq data with a significantly higher level of expression in the 5.6 replicates than the 5.6+pADAP replicates ($p < 0.05$) encodes for genes with the translated products involved in various catalytic and transport processes within the cell (Table 4.4). In the bacterium *Bacillus subtilis*, the translated products of the genes *opuAA* and *opuAB* are involved with the transport of the osmolyte glycine betaine into the cytoplasm (Kempf and Bremer, 1995), where they can act as an osmoprotectant. The *opuAA* and *opuAB* genes had a level of expression significantly higher in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.05$), with respective expression scores of 23,924 and 18,393 in the 5.6 replicates, compared to 621 and 522 in the 5.6+pADAP replicates. Another gene with a significantly higher level of expression in the 5.6 replicates than in the 5.6+pADAP replicates is *proY_1* ($p < 0.05$), the translated product of which is related to the transport of proline, a proteinogenic amino acid, into the cytoplasmic membrane. This gene functions alongside other proline/glycine betaine transporter genes: *putP*, *proW*, *proV*, *proY*, and *proZ* (Deutch, 2018). Between these genes only *putP, proV,* and *proY* could be identified in the RNAseq results (respective transcript IDs of 00467, 04212, and 02596), with both *putP* and *proV* showing a similar level of expression in the replicates from both strains.

Additional genes related to amino acid formation in this operon are *hutH_1* and *hutH_2*, the translated products of which are used in the catabolism of L-histidine to N-formimino-L-glutamate. These genes also have a level of expression significantly higher in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.05$). The *hutU* gene is also involved in catabolic processes, catalysing the conversion of urocanate to 4-imidazolone-5-propionate, and possesses a high expression score of 342,789 in the 5.6 replicates, compared to 11,711 in 5.6+pADAP. The translated product of the final gene in this operon, *pcaK_2*, is used in the transport of 4-HBA and protocatechuate across the cellular membrane. This gene has the lowest level of expression in the cluster, with a score of 13,343 in the 5.6 replicates. However, the expression of *pcaK_2* is still significantly higher in 5.6 than in the 5.6+pADAP replicates ($p < 0.05$). All translated gene products which could be matched to a COG identity were matched to the amino acid metabolism and transport class (E).

Table 4.4. The gene operon encoding for various cellular catalytic functions (transcript IDs = 02592:02594, 02596:02599) expressed at a higher level in the 5.6 replicates than in the 5.6+pADAP replicates. The genes are presented with the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number.

Transcript ID	Gene	5.6 expression score	5.6+pADAP expression score	COG	Predicted product[1]	Predicted function	Uniprot accession number
02592	*hutH_1*	28131	3184	E	Histidine ammonia-lyase	Used in synthesises N-formimidoyl-L-glutamate from L-histidine	P21310
02593	*opuAA*	23924	621	E	Glycine betaine transport ATP binding protein	Component of a multicomponent binding transport system for glycine betaine. Likely related to energy coupling	P46920
02594	*opuAB*	18393	522	E	Glycine betaine transport permease protein	Component of a multicomponent binding transport system for glycine betaine. Likely related to substrate transport	P46921
02596	*proY_1*	49631	1013	E	Proline-specific permease	Transmembrane protein used in proline transport	A0A223UEC0
02597	*hutH_2*	230787	9182	E	Histidine ammonia-lyase	Used in synthesises N-formimidoyl-L-glutamate from L-histidine	P21310
02598	*hutU*	342789	11711	E	Urocanate hydratase	Catalyses the conversion of urocanate to 4-imidazolone-5-propionate	P25080
02599	*pcaK_2*	13343	1102	E	4-hydroxybenzoate transporter	Transports 4-hydroxybenzoate and protocatechuate across cell membrane	Q51955

[1] Predicted product for all genes identified by BlastN.

The final operon of genes identified as having an increased level of expression in the 5.6 replicates contained genes linked to various cellular catalytic processes, with all genes showing significantly higher level of expression in the 5.6 replicates than in the 5.6+pADAP replicates ($p < 0.05$ to 0.0005) (Table 4.5). The first gene contained within this operon, *cytR_4*, encodes for an HTH-type transcriptional repressor, negatively controlling the transcription of genes *deoCABD*, *udp*, and *cdd*. The translated products of these genes are linked to the catabolism of ribonucleotides to deoxyribonucleotides (Hirakawa et al., 2020), which are then used in further cellular processes, such as the control of replication rates (Mathews, 2014), and there was no significant difference in the expression of these genes between the replicates for each strain. Whilst *cytR_4* is located upstream of *Pa0142* (encoding 8-oxoguanine deaminase) and *ves* (encoding a putative cold-shock protein), the transcriptional repressor product of *cytR_4* does not regulate the activity of these genes. The translated products of *hutI* and *BK65_11970* are related to the formation process of L-glutamate with respective products of imidazolonepropionase and N-formylglutamate deformylase. The transcription of *hutI*, *BK654_11970*, and *dap* are regulated by the translated product of *yvoA*, which is located upstream of these genes. The gene products of this operon were also assigned to a variety of COG classes, including transcription (K), nucleotide transport and metabolism (F), and secondary metabolite biosynthesis (Q) (Table 4.5).

Table 4.5. The gene operon encoding for various cellular catalytic functions (transcript IDs = 04089:04095) expressed at a higher level in the 5.6 replicates than in the 5.6+pADAP replicates. The genes are presented with the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned.

Transcript ID	Gene	5.6 expression score	5.6+pADAP expression score	COG	Predicted product[1]	Predicted function	Uniprot accession number
04089	*cytR_4*	6856	1930	K	HTH-type transcriptional repressor	Regulation of transcription and DNA binding	A0A3N6CR30
04090	*ves*	7191	1175	-	Ves cold-shock protein	Cell response to cold	P76214
04091	*Pa0142*	31208	4534	F	8-oxoguanine deaminase	Deaminates 8-Oxoguanine to uric acid	Q9I6Z0
04092	*yvoA*	8076	2919	K	HTH-type transcriptional repressor	Involved in DNA binding transcription factor activity	M4L014
04093	*hutI*	79489	4423	Q	Imidazolonepropionase	Catalytic enzyme used in the formation of *N*-formimidoyl-L-glutamate	Q8U8Z6
04094	*BK654_11970*	75311	4551	-	N-formylglutamate deformylase	Catalytic enzyme used in the production of formate and L-glutamate	A0A423FXX5
04095	*dap*	4025	2239	-	D-aminopeptidase	Hydrolyses N-terminal residues in D-amino acid containing peptides	Q9ZBA9

[1] Predicted product identified for all genes by BlastN.

In addition to the operons with an increased level of expression on the chromosome of the 5.6 replicates, several operons of interest were identified in the 5.6+pADAP replicates sampled during the mid-exponential growth period. These operons are colour-coded in blue in the RNAseq spreadsheet (Appendix C.1). Unlike the genes identified in the 5.6 replicates, many of the chromosomal genes in the 5.6+pADAP operons could not be initially linked to a product following a BlastN assessment and protein prediction using the Phyre^2 protein homology engine (method: Chapter 2, section 2.3.16). Similarly, many of the translated products from the 5.6+pADAP chromosomal genes could not be linked to a COG function. However, BlastP searches using translated amino acid sequences were conducted to match the proteins to other hypothetical proteins in various bacterial species and showed some potential functions.

The first operon with significantly increased expression in the 5.6+pADAP replicates contained genes related to various enzymatic and catalytic processes ($p < 0.05$) (Table 4.6). The only gene initially identified in this operon with a nucleotide BLAST assessment was *xerD_2*, which is translated to produce a site-specific tyrosine recombinase, used in the cutting and re-joining of DNA molecules. The translated product also contributes to the stability of plasmids (Blakely et al., 1993). The remainder of the genes in this operon were predicted using the Phyre^2 engine, matching the homology of the translated protein to previously catalogued proteins from other species. The gene product with the highest confidence score of 95.8 % was TraR (matched to *E. coli*), a protein which acts as a global regulator of processes such as *in vitro* transcription (Gopalkrishnan et al., 2017). The Phyre^2 matches for *gstB* (matched to *E. coli*) and *aff4* (matched to *Homo sapiens*) both have lower confidence scores of 31.7 % and 19.1 % (respectively), and thus are treated as speculative. The gene *gstB* encodes for glutathione-S-transferase, a member of a family of metabolic isozyme used in the detoxification of electrophilic compounds (Vuilleumier, 1997). The speculative gene *aff4* encodes for a protein which comprises part of a complex used to increase the catalytic rate of RNA polymerase II transcription. However, due to the low Phyre^2 confidence and coverage score for this protein (19.1 and 29 %, respectively), and the protein being matched to the *Homo sapiens* genome, it was considered to be an inaccurate match. A subsequent assessment of the translated amino acid sequence for the speculative gene *aff4* via BlastP search provided a match to a hypothetical protein in the bacterium *Providencia rettgeri* (93 % coverage with 77 % positive matches), indicating that this potential product is found in other bacterial species. None of the translated predicted products in this cluster were linked to any COG classes.

Table 4.6. The gene operon encoding for various enzymatic and cellular catalytic functions (transcript IDs = 02964:02966, 02968:02969) expressed at a higher level in the 5.6+pADAP replicates than in the 5.6 replicates. The genes are presented with the Phyre[2] confidence percentage and amino acid coverage for the predicted product (%) and the matched organism (where applicable), the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned. NA Denotes data is not available.

Transcript ID	Gene	Phyre[2] confidence (%)	Coverage (%) (Phyre[2])	Matched organism (Phyre[2])	5.6 expression score	5.6+pADAP expression score	COG	Predicted product	Predicted function	Uniprot accession number
02964	*gstB*	31.7	68	*E. coli*	69	282	-	GST-like protein	Catalytic enzyme used in dehalogenation of bromoacetate	P0ACA7
02965	NA[1]	NA[2]	NA[2]	NA[2]	30	95	-	tRNA (ARG)	Transfer ribonucleic acid	NA[2]
02966	*xerD_2*	NA[2]	NA[2]	*E. coli*	1059	2341	-	Tyrosine recombinase	Site-specific tyrosine recombinase, contributes to plasmid stability	A0A0F7JBQ4
02968	*traR*	95.8	81	*E. coli*	50	223	-	TraR	Zinc ion binding	P41065
02969	*aff4*	19.1	29	*Homo sapiens*[3]	27	139	-	AF4/FMR2 family member 4	Used to increase rate of RNA polymerase II transcription	Q9UHB7

[1] Gene was not identified during initial nucleotide BLAST.
[2] No Uniprot match identified.
[3] BlastP of translated amino acid sequenced matched to *Providencia rettgeri* hypothetical protein.

The genes in the second 5.6+pADAP chromosome operon were not identified during the initial nucleotide BLAST assessment (hypothetical proteins), and the prospective gene products were predicted using the amino acid sequences and the Phyre^2 engine. The potential genes and their products, related to various catalytic processes, are listed in Table 4.7. Each of the amino acid sequences was matched to a tertiary product, with confidence ratings between 73 and 41 %, indicating a moderate-to-high degree of accuracy in the matches. Two of the products identified from the Phyre^2 results were transferases matched to *Pseudomonas putida* (*cymD*) and *E. coli* (*agaB*), which are used in various catalytic processes (Qian et al., 2012).The product with the lowest confidence score is Tsa1 (matched to *Saccharomyces cerevisiae*), which is speculated to be involved in the production of tryptophan synthase, used in the biosynthesis of tryptophan (Iraqui et al., 2009). A subsequent investigation of the translated amino acid sequence of this potential gene with a BlastP search provided a match to a hypothetical protein found in *Serratia plymuthica* (100 % coverage, 74 % positive matches). The final product in this operon identified from the Phyre^2 search was an aminoacyl tRNA synthetase (matched to *Brucella abortus*), which is used in translation processes (Carter, 2017). None of these prospective products or the associated genes were able to be defined by COG classes.

Table 4.7. The gene operon encoding for various catalytic proteins (transcript IDs = 04318:04321) expressed at a higher level in the 5.6+pADAP replicates than in the 5.6 replicates. The genes are presented with the Phyre[2] confidence percentage and amino acid coverage for the predicted product (%) and matched organism (where applicable), the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned.

Transcript ID	Gene	Phyre[2] confidence (%)	Coverage (%) (Phyre[2])	Matched organism (Phyre[2])	5.6 expression score	5.6+pADAP expression score	COG	Predicted product	Predicted function	Uniprot accession number
04318	*bab2*	73.2	23	*Brucella abortus*	252	985	-	Aminoacyl tRNA synthetase	Anticodon-binding domain of class I aminoacyl-tRNA synthetase	Q2YJF0
04319	*cymD*	58.2	37	*Pseudomonas putida*	157	636	-	Transferase	Catalytic enzyme which reverses N-prenylation of tryptophan	O33458
04320	*tsa1*	41.3	54	*Saccharomyces cerevisiae*[1]	70	337	-	Peroxiredoxin TSA1	Protects the cell against oxidative stress	P34760
04321	*agaB*	71.1	12	*E. coli*	128	666	-	Transferase	Catalytic enzyme used in N-acetylgalactosamine transport	P42909

[1] BlastP of translated amino acid sequenced matched to *Serratia plymuthica* hypothetical protein.

The third operon with an increased level of expression in the 5.6+pADAP replicates contained genes related to various catalytic processes (Table 4.8). Of the genes in this cluster only *ssb_2* was identified during the initial nucleotide BLAST and encodes for a single-stranded DNA binding protein which is used in DNA replication, recombination, and repair. This gene was also matched to a COG class for replication, recombination, and repair (L). The translated products from the remainder of genes in this operon were predicted in the Phyre^2 engine based on similarities in protein folds with other species. The products predicted using the Phyre^2 engine all had confidence scores exceeding 50 %, with three above 99 %, which, in conjunction with high coverage scores, indicated the products were likely to be accurate. The products with the highest confidence scores were IpxD, RecA, and NadE, with confidence scores of 100, 99.7, and 99.2 %, respectively. The RecA and NadE predicted products were both matched to *E. coli*, whilst IpxD was matched to *Candidatus Photodesmus blepharus*. These proteins are linked to several catalytic functions: IpxD is used in the biosynthesis of lipid A via the N-acetylastion of UDP-3-O-acylglucosamine, producing a phosphorylated glycolipid which anchors the lipopolysaccharide to the outer membrane of the cell.

RecA catalyses the hydrolysis of ATP in the presence of single-stranded DNA, and NadE catalyses the ATP-dependent amidation of deamido-NAD to form nicotinamide adenine dinucleotide, which is used further as a cofactor in multiple metabolic reactions. The products with lower Phyre^2 scores of 66 and 43.8 %, respectively, and therefore less likely to be accurate, are CcmH and Tfa1, and were matched with *E. coli* and the yeast species *Saccharomyces cerevisiae*, respectively. Further assessment of the translated amino acid sequences for both Tfa1 and CcmH with a BlastP search provided matches to hypothetical proteins in *Serratia liquefaciens* (100 % coverage, 95 % positive matches) and *S. marcescens* (98 % coverage, 92 % positive matches), respectively. CcmH is part of an operon encoding eight *ccm* genes (*ccmABCDEFGH*), with the translated products being involved with the maturation of C-type cytochromes. The Tfa1 product acts as a large subunit of transcription initiation factor IIE, part of the RNA polymerase transcription initiation complex.

Table 4.8. The gene operon encoding for various catalytic proteins (transcript IDs = 04669:04671, 04673, 04676:04677) expressed at a higher level in the 5.6+pADAP replicates than in the 5.6 replicates. The genes are presented with the Phyre[2] confidence percentage and amino acid coverage for the predicted product (%) and species (where applicable), the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned.

Transcript ID	Gene	Phyre[2] confidence (%)	Coverage (%) (Phyre[2])	Matched organism (Phyre[2])	5.6 expression score	5.6+pADAP expression score	COG	Predicted product	Predicted function	Uniprot accession number
04669	*tfa1*	56.2	51	*Saccharomyces cerevisiae*[2]	149	862	-	Transcription initiation factor	The initiation of transcription with RNA polymerase II	P36100
04670	*ccmH*	66.0	53	*E. coli*[3]	171	740	-	Cytochrome c-type biogenesis protein	Possibly used in biogenesis of c-type cytochromes	P0ABM9
04671	*nadE*	100.0	57	*E. coli*	757	3318	-	NH(3)-dependent NAD(+) synthetase	Catalytic enzyme used in the formation of NAD	P18843
04673	*ipxD*	100.0	80	*Candidatus Photodesmus blepharus*	352	1878	-	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	Catalytic enzyme used in biosynthesis of lipid A	A0A084CNY4
04676	*ssb_2*	NA[1]	NA[1]	*Acinetobacter venetianus*	313	1837	L	Single stranded DNA binding protein	Used in binding of single-stranded DNA	A0A150I0Q8
04677	*recA*	99.4	71	*E. coli*	136	1242	-	Protein RecA	Catalyses DNA homologous pairing and strand exchange	P0A7G6

[1] Gene was not identified during initial nucleotide BLAST.
[2] BlastP of translated amino acid sequenced matched to *Serratia liquefaciens* hypothetical protein.
[3] BlastP of translated amino acid sequenced matched to *Serratia marcescens* hypothetical protein.

The final chromosomal operon identified with an increased level of expression in the 5.6+pADAP samples, relative to 5.6, contains genes related to transcription and cellular apoptosis (Table 4.9). All of the predicted products in the Phyre[2] results have a confidence score less than 35 %, suggesting that the products predicted using the amino acid sequences might not be accurate, and thus are treated as speculative. The first of the products identified was matched to a *Homo sapiens* RNA polymerase III component used in the synthesis of small RNAs. Whilst the assessed amino acid sequence has a low confidence score, the sequence assessed had a coverage of 94 % when compared to the matched template. A subsequent assessment of the translated amino acid sequence with a BlastP search provided a match to a hypothetical protein found in the bacterium *Yersinia mollaretii* (75 % coverage, 77 % positive matches). The next predicted product, Est1e, was matched to the bacterium *Butyrivbrio proteoclasticus*, with the product and esterase being used in hydrolysis reactions. However, the amino acid sequence assessed has both a low confidence score and low coverage with the match template. The final predicted product in this operon, Bak_2, was matched to the freshwater triclad *Schimdtea mediterranea*, and is purported to be involved in apoptotic processes. However, as previously with Est1e, the matched product has a low confidence and coverage score when compared to the matched template. An assessment of the translated amino acid sequence for Bak_2 with a BlastP search provided a match to a hypothetical protein found in *Serratia ureilytica* (100 % coverage, 98 % positive matches). None of the products and associated genes were linked to a COG class.

Table 4.9. The gene operon encoding for RNA binding and apoptotic proteins (transcript IDs = 04723:04726) expressed at a higher level in the 5.6+pADAP replicates than in the 5.6 replicates. The genes are presented with the Phyre[2] confidence percentage and amino acid coverage for the predicted product (%) and matched species (where applicable), the average level of expression in the strains (n = 3), the cluster of orthologous groups (COG) classification, any corresponding products identified with a nucleotide BLAST search, the predicted function, and Uniprot accession number. - Denotes no COG class could be assigned

Transcript ID	Gene	Phyre[2] confidence (%)	Coverage (%) (Phyre[2])	Matched organism (Phyre[2])	5.6 expression score	5.6+pADAP expression score	COG	Predicted product	Predicted function	Uniprot accession number
04723	*polr3F*	23.8	94	*Homo sapiens*[1]	10	47	-	RNA polymerase III component	RNA polymerase III component used in transcription	POLR3F
04724	*est1e*	34.2	22	*Butyrivibrio proteoclasticus*	31	172	-	Est1e	Hydrolase activity	D2YW37
04725	*bak_2l*	25.3	40	*Schimdtea mediterrane* [2]	21	146	-	Apoptosis protein	Regulation of apoptotic processes	Q1RPT5

[1] BlastP of translated amino acid sequenced matched to *Yersinia mollaretti* hypothetical protein.
[2] BlastP of translated amino acid sequenced matched to *Serratia ureilytica* hypothetical protein.

4.2.3 The impact of mixed inoculation with tagged *Serratia* spp. using a maltose carbon source

Following the identification of the increased expression of genes relating to maltose and trehalose transport and metabolism in the 5.6 replicates several *in vitro* competition experiments were conducted using an M9 20 % maltose medium (method: Chapter 2, section 2.3.1). These competition experiments were conducted using the tagged A1MO2_tk and 5.6_s constructs and assessed over a 48-hour period (method: Chapter 2, section 2.3.4). This would determine whether these carbon sources, and the corresponding increase in the expression of related genes for 5.6 during the mid-exponential growth period (corresponding with the RNAseq sample assessment at 10^9 CFU/mL in LB broth at eight to nine hours post-inoculation), would have any impact on growth dynamics or the outcome of competition between A1MO2 and 5.6. During these experiments A1MO2_tk was used in place of 5.6+pADAP to determine how competition between A1MO2 and 5.6 would occur in a maltose environment.

4.2.3.a. Mixed inoculation of tagged A1MO2_tk and 5.6_s in a 20 % maltose medium over 48 hours

Prior to an assessment of A1MO2_tk and 5.6_s in competition, both A1MO2_tk and 5.6_s were inoculated separately into the maltose medium to characterise growth in the absence of competition (Fig. 4.4). Within the 48-hour period of growth assessment there were only two instances of significant difference identified between the A1MO2_tk and 5.6_s CFU counts. Both strains displayed similar dynamics over the first four hpi during the initial lag phase. A significant difference in cell numbers occurred after six hpi during the beginning of the exponential growth phase, in which A1MO2_tk had a significantly higher CFU count than 5.6_s ($p < 0.05$). CFU counts of ~ 5 x 10^7 CFU/mL were recorded for the strains between eight and ten hpi, the time-point at which the initial RNAseq samples were assessed, with no significant differences between these counts. This suggested that the increased expression of genes observed at the same point in the growth cycle of the 5.6 RNAseq samples did not have a significant impact when the strains were cultured separately. Furthermore, CFU counts for the strains between eight to ten hpi were between 10^7 and 10^8 CFU/mL, one log lower than the original RNAseq samples at the same time (10^9 CFU/mL), however, as highlighted during previous *in vitro* assessments (Chapter 3), the CFU counts for A1MO2 and 5.6 in the M9 medium were often one log CFU/mL lower than LB broth cultures. However, whilst the M9 medium cultures yielded CFU counts one log CFU lower than LB broth, the cultures still reached the stationary growth phase. From this it can be assumed that the limited nutrient availability of the M9 medium alters the growth potential of the bacterium.

The exponential growth phase continued until 24 hpi, with both strains attaining similar CFU counts. A significant difference in cell counts was recorded also during the stationary phase at 36 hpi, in which 5.6_s had a significantly higher CFU count than A1MO2_tk ($p < 0.05$). However, by the end of the assay at 48 hpi the CFU counts did not differ between the strains. These results suggest that when inoculated separately the lack of competition between the strains may not reveal any fitness advantages associated with the increased expression of the maltose operon.

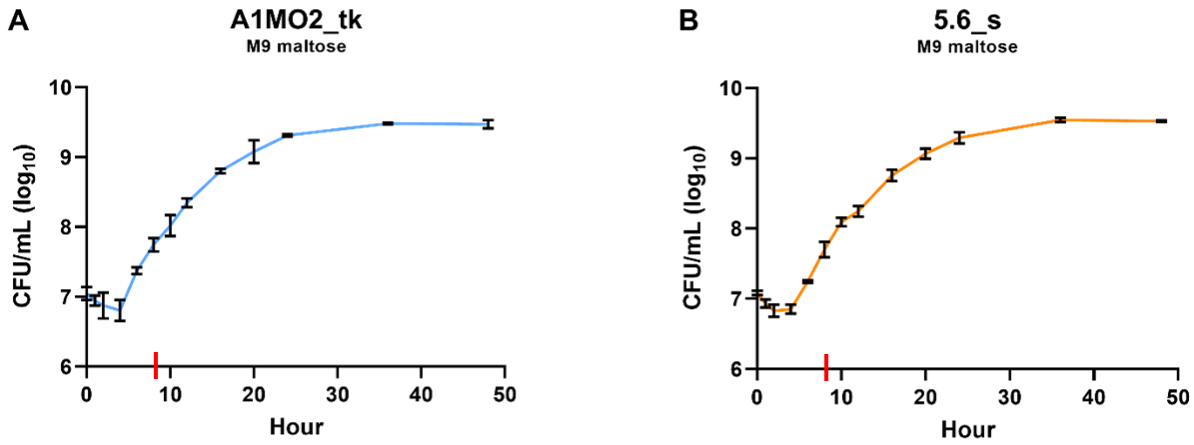


Figure 4.4. Average log_{10} CFU/mL counts (n = 3) for (A) A1MO2_tk and (B) 5.6_s when inoculated separately in a 20 % maltose M9 medium and cultured over 48 hours. The medium was inoculated at a rate of 5.40 x 10^8 CFU/mL (A1MO2_tk) and 6.70 x 10^8 CFU/mL (5.6_s). Results are presented with standard deviation error bars. The red line on the X-axis denotes the time at which the initial RNAseq samples were assessed, aligning with an increased expression of the maltose operon in 5.6 samples (approximately 10^8 CFU/mL ~ 8 hours post-inoculation).

The mixed inoculation of A1MO2_tk and 5.6_s at an equal ratio (50:50, A1MO2_tk:5.6_s) resulted in similar average CFU counts (n = 3) at zero hpi of 5.57 x 10^6 and 4.87 x 10^6 CFU/mL, respectively (Fig. 4.5. A). However, during the initial lag phase from the time of inoculation to six hpi, significant differences were noted at four and six hpi during which A1MO2_tk had significantly higher CFU counts than 5.6_s ($p < 0.05$). However, during the exponential growth period the CFU count of 5.6_s increased at a faster rate than A1MO2_tk, resulting in 5.6_s having significantly higher CFU counts than A1MO2_tk between ten and 20 hpi ($p < 0.05$ to < 0.005). Using the CFU counts obtained between six and ten hpi a faster doubling time was calculated for 5.6_s (T_d = 22.6 mins, R^2 = 0.99) than for A1MO2_tk (T_d = 28.6 mins, R^2 = 0.92), resulting in these differences. This corresponded with CFU counts of 3.33 x 10^7 CFU/mL for A1MO2_tk at ten hpi, and 5.73 x 10^7 CFU/mL for 5.6_s. This linked back to the initial RNAseq data, which showed an increased expression for the maltose operon in the 5.6 replicates approximately nine hpi. These CFU counts (10^6 to 10^7 CFU/mL at eight and ten hpi, respectively) are also similar to the original RNAseq samples assessed at the same time-point in the LB broth medium

(10^9 CFU/mL). This similarity accounts for an expected reduction of cells in the M9 medium as opposed to LB broth (often one log CFU/mL difference between media types), and a further CFU reduction between the strains due to competition, which is consistent with previous competition assays conducted in M9 medium. The significant difference between the strains persisted into the stationary phase until 36 hpi, during which 5.6_s maintained significantly higher CFU counts than A1MO2_tk ($p < 0.05$). However, by 48 hpi there was no longer a significant difference between the strains with final CFU counts of 1.36 x 10^9 CFU/mL for A1MO2_tk and 2.07 x 10^9 CFU/mL for 5.6_s. This indicated that, whilst 5.6_s had a fitness advantage during the exponential growth phase, the advantage dissipated during the latter part of the assay.

These dynamics are also reflected in the changes in relative proportion of each strain recovered from the maltose medium over the assay duration (Fig. 4.5. B). Following inoculation at an equal ratio (53:47, A1MO2_tk:5.6_s), the proportion of A1MO2_tk cells isolated increased during the initial lag phase to 64:36 by six hpi, indicating an initial increased competitiveness of A1MO2_tk following inoculation. However, following the onset of exponential growth, the proportion of 5.6_s increased to 37:63 after ten hpi, which was maintained until 48 hpi, where there was little difference (± 6 %). This indicated that 5.6 had a competitive advantage over A1MO2 during mixed culturing in a maltose-based medium which may stem from the increased expression of the genes identified the maltose operon in the RNAseq data, as that is the time where these genes were shown to have a higher level of expression in 5.6 when compared to 5.6+pADAP (an equivalent of A1MO2).

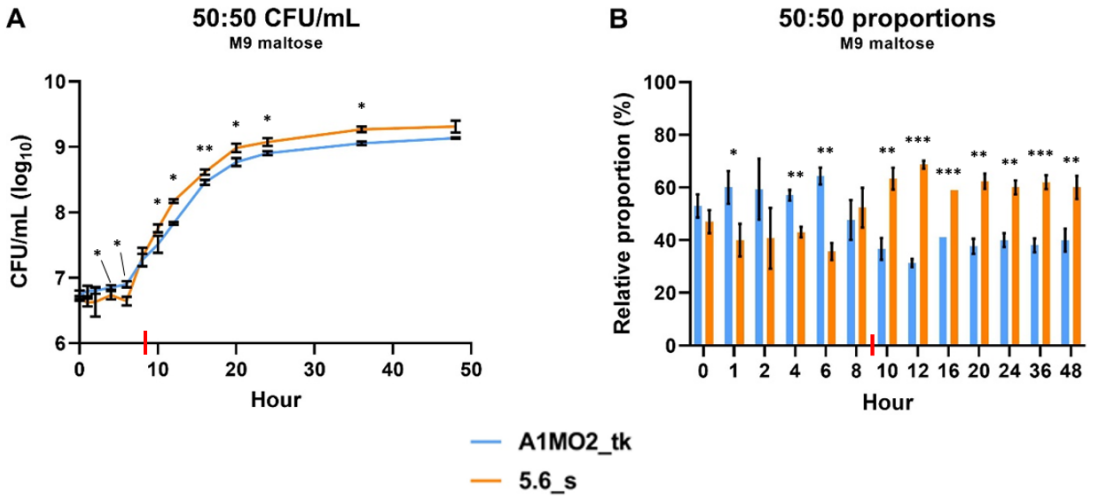


Figure 4.5. (A) Average log_{10} CFU/mL counts (n = 3) for tagged *Serratia* strains A1MO2_tk and 5.6_s when inoculated together in a 20 % maltose medium over 48 hours at a 50:50 ratio (A1MO2_tk:5.6_s). The strains were inoculated at rates of 3.67 x 10^8 CFU/mL (A1MO2_tk) and 3.80 x 10^8 CFU/mL (5.6_s). (B) The changes in average relative proportion (%) (n = 3) of cells isolated from the culture over 48 hours. Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. The red line on the X-axis denotes the time at which RNAseq samples were assessed, aligning with an increased expression of the maltose operon in 5.6 samples (10^7 CFU/mL ~ 8 to 9 hours post-inoculation).

4.2.4 Assessing persistence of tagged *Serratia* spp. at mixed ratios using a maltose carbon source

Further investigation was conducted on how competition between A1MO2 and 5.6 might occur in a maltose medium over seven days (method: Chapter 2, section 2.3.5). The separate inoculation of A1MO2_tk and 5.6_s in the maltose medium led to a unique dynamic in which A1MO2_tk increased in CFU over the duration of the assay, whereas 5.6_s continually decreased (Fig. 4.6. A - B). This initially indicated that despite the increased expression of maltose-related genes by 5.6_s, it had a lower persistence in the medium than A1MO2_tk. Furthermore, 5.6_s initially maintained significantly higher CFU counts than A1MO2_tk over the first two days of the assay ($p < 0.0005$), beginning with CFU counts of 1.63 x 10^8 CFU/mL for A1MO2_tk and 1.37 x 10^9 CFU/mL for 5.6_s. By three dpi A1MO2_tk attained and maintained significantly higher CFU counts than 5.6_s for the remainder of the presistence assay ($p < 0.005$ to 0.0005), finishing with CFU counts of 5.11 x 10^8 CFU/mL for A1MO2_tk and 5.45 x 10^7 CFU/mL for 5.6_s by seven dpi. Fitting the CFU data to multiple regression models revealed that only the 5.6_s exhibited signifiant CFU changes per day ($p < 0.005$), whereas A1MO2_tk did not (Table 4.10).

In contrast, the use of a mixed ratio inoculant resulted in a clear difference between the dynamics of the strains over the seven-day assay, leading to a clear competitive dominance of 5.6_s in the cultures (Fig. 4.6. C - G). This was evident from the difference between the CFU counts for the strains one dpi, in which 5.6 attained a significantly higher average CFU counts between one to two log values higher

than A1MO2_tk in each of the mixed ratio inocula, even when initially being inoculated at a lower proportion ($p < 0.005$ to 0.0005). Over the first three days of the assay both A1MO2_tk and 5.6_s decreased in CFU. However, whilst 5.6_s continuously decreased over the remainder of the assay duration until seven dpi, A1MO2_tk maintained its CFU count three dpi, after which it showed slight changes until the end of the assay. Despite these changes in dynamics there was still a significant difference between the CFU counts of A1MO2_tk and 5.6_s on day seven of the assay, with 5.6_s maintaining significantly higher CFU counts than A1MO2_tk in each of the mixed ratio treatments ($p <$ 0.005 to 0.0005). These results show that whilst 5.6_s dominated in competition within A1MO2_tk in the maltose medium, primarily within the first 24 hours post-inoculation, and continued to maintain significantly higher CFU counts than A1MO2_tk over the seven-day assay duration, an increase in persistence was noted for A1MO2_tk. The increase in persistence for A1MO2_tk, as evident in Figure 4.6, begins to occur three dpi in each of the mixed ratio treatments where the A1MO2_tk CFU counts stabilise and was maintained until the end of the assay at seven dpi. In contrast, the CFU counts for 5.6 continued to steadily decrease over the remainder of the assay until seven dpi.

Whilst this does show that 5.6_s exhibits an initial competitive advantage in the medium as evident from the higher CFU counts and having a significantly higher AUC than A1MO2_tk ($p < 0.05$), a change of dynamics is seen as the experiement progressed. This is also depicted in the multiple regression models fitted to the mixed inocula data (Table 4.10); in each of the 90:10, 70:30, 50:50, and 30:70 treatments (A1MO2_tk:5.6_s) the daily CFU change for 5.6_s is significant ($p < 0.05$), whilst A1MO2_tk does not change by a significant amount.

When assessing the CFU data using the Lotka/Volterra-stlye copmetition models described in Chapter 3, Section 3.2.7, 5.6_s exhibited weak positive competition coefficients (between 0.03 and 0.09) I neach of the mixed ratio treatments, indicating that it benefitted from the presence of A1MO2_tk in the culture. In contrast, A1MO2_tk had weak negative coefficients in each of the 90:10, 70:30, and 50:50 treatments (A1MO2_tk:5.6_s), and weak positive coefficients in the 30:70 and 10:90 treatments. This indicates that whilst A1MO2_tk was hindered when it comprised a majority of the inoculant, it benefitted from the presence of 5.6_s when it copmrised less of the initial ratio.

A repetition of this assay conducted at a later date using the same experimental approach with fresh inocula and maltose medium resulted in the same outcome, where 5.6_s outcompeted A1MO2_tk in each of the mixed ratio treatments prior to the first sampling at one dpi. The results for the second repetition are avaliable in Appendix C.2.

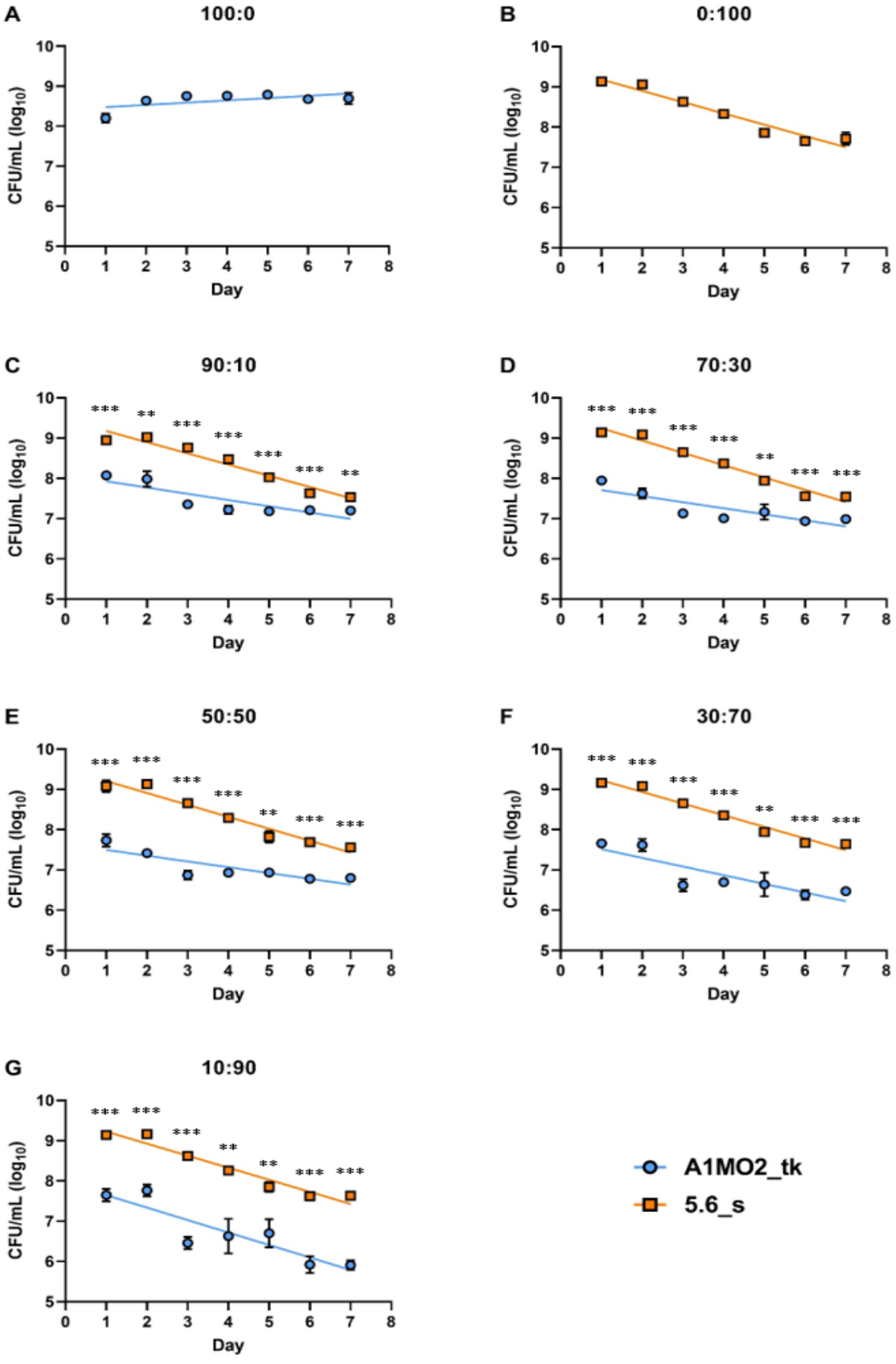


Figure 4.6. Average $\log_{10}$ CFU/mL counts (n = 3) for A1MO2_tk and 5.6_s when inoculated in a 20 % maltose M9 medium and cultured over seven days. Strains were inoculated separately (A) 100:0 and (B) 0:100, and together in varying ratios: (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, (G) 10:90 (A1MO2_tk:5.6_s). The inocula were produced from overnight cultures with rates of 2.38 x 10^9 CFU/mL (A1MO2_tk) and 2.02 x 10^9 CFU/mL (5.6_s). Results are presented alongside linear regression lines and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Full CFU data is available in Appendix C.2.

Table 4.10. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 5.6_s cultured in 20% M9 maltose medium following inoculation at various ratios (A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.95	0.057	0.881	0.952	54.07	0.001
0:100	5.6_s		-3.653	0.365	0.001		
90:10	A1MO2_tk	0.93	-1.378	0.968	0.227	29.44	0.002
	5.6_s		-2.521	0.607	0.014		
70:30	A1MO2_tk	0.96	-0.908	0.698	0.263	76.05	0.001
	5.6_s		-2.826	0.437	0.003		
50:50	A1MO2_tk	0.95	-0.398	0.671	0.586	53.25	0.001
	5.6_s		-3.147	0.446	0.002		
30:70	A1MO2_tk	0.96	-0.502	0.723	0.526	69.31	0.001
	5.6_s		-3.174	0.502	0.003		
10:90	A1MO2_tk	0.96	-2.034	0.970	0.104	79.04	0.001
	5.6_s		-1.799	0.820	0.093		

The initial dominance of 5.6 following inoculation in the maltose medium is also evident in the changes of proportion for each strain isolated from the culture (Fig. 4.7. A - E). The proportions show how within one dpi 5.6_s constituted the majority of cells being isolated from each of the mixed ratio treatments. The most notable occurrence of this was in the 90:10 treatment (A1MO2_tk:5.6_s), where by one dpi the recorded proportion was 12:88, which showed how 5.6_s was able to outcompete A1MO2_tk when it comprised the minority proportion of the initial inoculant, with significantly more cells being isolated than A1MO2_tk ($p < 0.0005$). An increase in the proportion of A1MO2_tk cells isolated from the cultures was seen five dpi in the 90:10, 70:30, and 50:50 cultures (A1MO2_tk:5.6_s). This corresponded with the maintenance of CFU for A1MO2_tk, as depicted above in Figure 4.6. As such, the increase in the proportion of A1MO2_tk cells isolated from the mixed cultures does not indicate the occurrence of new growth within the medium, rather a stable persistence in the medium relative to 5.6_s, which could suggest an increase in expression for the maltose operon in A1MO2_tk at a later point than 5.6_s.

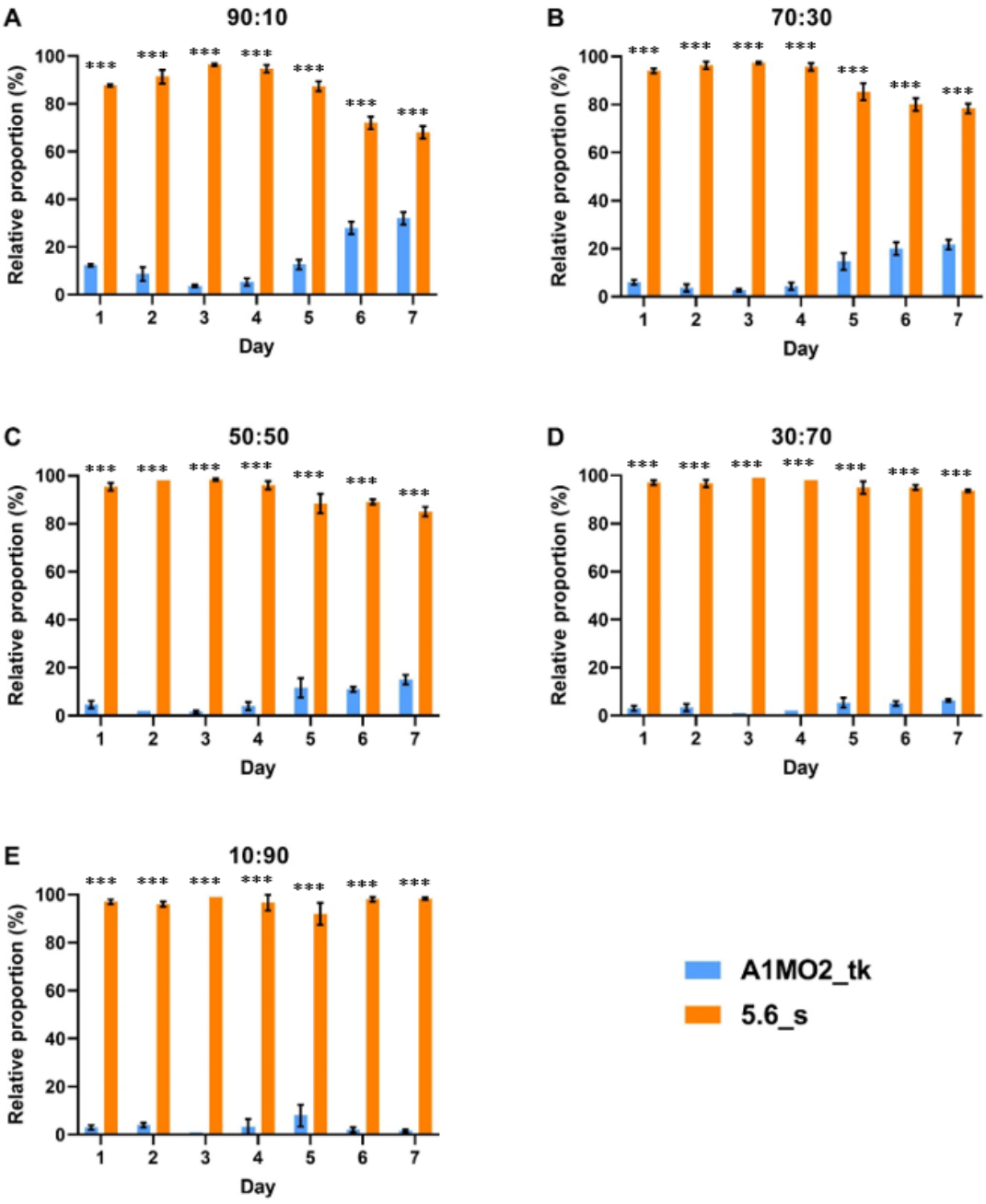


Figure 4.7. Changes in the average relative proportion (%) (n = 3) for A1MO2_tk and 5.6_s cells isolated from 20 % maltose M9 mixed cultures when inoculated at various ratios: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

4.2.4.a. Re-inoculation of A1MO2_tk and 5.6_s in aged M9 20 % maltose medium

During the assessment of competition between A1MO2_tk and 5.6_s in the maltose medium over a seven-day period it was clear that 5.6 was able to outcompete A1MO2 within one dpi in each of the mixed ratio treatments, leading to significantly higher CFUs for 5.6_s than A1MO2_tk. This is depicted above in Figures 4.6 and 4.7, where a significantly higher number of 5.6_s cells were also isolated from the mixed cultures than A1MO2_tk. However, it was also noted that the CFU counts for A1MO2_tk stabilised three dpi whilst the CFU counts for 5.6_s continued to steadily decrease from 10^9 to 10^7 CFU/mL, with this being most pronounced in the 90:10, 70:30, and 50:50 cultures (A1MO2_tk:5.6_s). Further experimentation was conducted in which freshly prepared mixed inocula for A1MO2 were used to re-inoculate a filter-sterilised six-day old M9 maltose culture in which a similar competition assay had been conducted to determine the growth of the strains in the re-inoculated medium, and whether the persistence noted for A1MO2 during the seven-day assay would be observed again in the re-inoculated medium. The cells in medium were pelleted via centrifugation, and the medium was filter sterilised prior to inoculation with the new cultures (method: Chapter 2, section 2.3.17). The re-inoculated cultures were assessed over a further five days, with the strains inoculated both separately and at ratios of 90:10, 70:30, and 50:50 cultures (A1MO2_tk:5.6_s).

In each of the separately inoculated cultures in the spent medium (100:0 and 0:100, A1MO2_tk:_5.6_s), both A1MO2_tk and 5.6_s showed a similar level of growth over the duration of the assay, with no significant differences in CFU counts being recorded for either strain when comparing CFU counts at day one to day five (Fig. 4.8. A - B). However, prior to the first recorded CFU measurements in the spent medium at one dpi 5.6_s attained a CFU count significantly higher than A1MO2_tk ($p < 0.0005$). This indicated that during the first 24 hours post-inoculation the increased expression of the maltose operon may have assisted 5.6_s in an increased rate of replication during the mid-to-late exponential growth phase, when the increased expression of the maltose operon was noted in the RNAseq data. The growth dynamics for A1MO2_tk when inoculated independently in the spent maltose medium resemble those recorded previously when the strain was inoculated into the fresh maltose medium (Section 4.2.4.a), with CFU counts approximately one log CFU/mL lower in the spent maltose medium than the fresh maltose medium. In contrast, during the previous seven-day assay in the fresh maltose medium 5.6_s showed a continual decrease in CFU, whereas in the spent maltose medium 5.6_s sustained CFU counts of 10^7 CFU/mL over the five-day assay duration. Fitting the data to multiple regression models indicated that the daily CFU changes for the strains were non-significant (Table 4.11), though A1MO2_tk was just outside of the level of significance ($p = 0.06$). Furthermore, the AUC measurement for 5.6_s was significantly higher than that of A1MO2_tk ($p < 0.05$).

In the spent maltose medium mixed strain cultures, it is evident that competition between A1MO2_tk and 5.6_s occurred within the first 24 hours post-inoculation, with 5.6_s showing an increased competitive fitness. This is seen at one dpi in both the 90:10 and 70:30 cultures (A1MO2_tk:5.6_s) (Fig. 4.8. C - D), where there was no significant difference between the CFU counts for the strains, despite 5.6_s constituting the minority strain in the mixed inoculant. Similarly, in the 50:50 culture, where both strains were inoculated at equal ratios (Fig. 4.8. E), 5.6_s had a CFU count significantly higher than A1MO2_tk by one dpi ($p < 0.0005$), indicating the occurrence of competition prior to sampling at 24 hpi. Over the first three days of the assay in the spent maltose medium both strains showed a similar rate of growth in the mixed cultures, with no major changes occurring in the trends for either strain. However, between three and four dpi the CFU counts of 5.6_s in the 90:10 and 70:30 mixed cultures saw a significant increase in comparison to A1MO2_tk ($p < 0.005$), which continued to increase at the same rate. Whilst there was no longer a significant difference between the CFU counts of A1MO2_tk and 5.6_s in the 90:10 culture by five dpi, in both the 70:30 and 50:50 the CFU count for 5.6_s was still significantly higher than A1MO2_tk by five dpi ($p < 0.005$ and 0.05, respectively). This indicates that 5.6_s was able to both attain the CFU counts significantly higher than those of A1MO2_tk and was also able to maintain these higher counts within the medium. Fitting multiple regression models to the data indicated that neither of the strains in any of the mixed ratio inocula treatments changed in CFU by a significant amount each day (Table 4.11), though A1MO2_tk was just above the level of significance in the 90:10 treatment (A1MO2_tk:5.6_s) ($p = 0.07$). Additionally, the 90:10 treatment was the only mixed ratio treatment in which 5.6_s had an AUC measurement significantly higher than AM1O2_tk ($p < 0.05$).

During the previous assessments with a fresh maltose medium conducted over a seven-day period (Section 4.2.4.a) the CFU counts of A1MO2_tk were seen to stabilise at three dpi, when the strain reached a CFU count in the medium of 10^6 to 10^7 CFU/mL. This was similar to the counts in the spent maltose medium, which also showed a stable CFU count around 10^7 CFU/mL for A1MO2_tk with slight growth over the five-day assay. In contrast, during the previous seven-day assay 5.6_s showed a continued decrease in CFU from 10^9 to 10^7 CFU/mL, whereas in the spent maltose medium it increased from 10^7 to 10^8 CFU/mL.

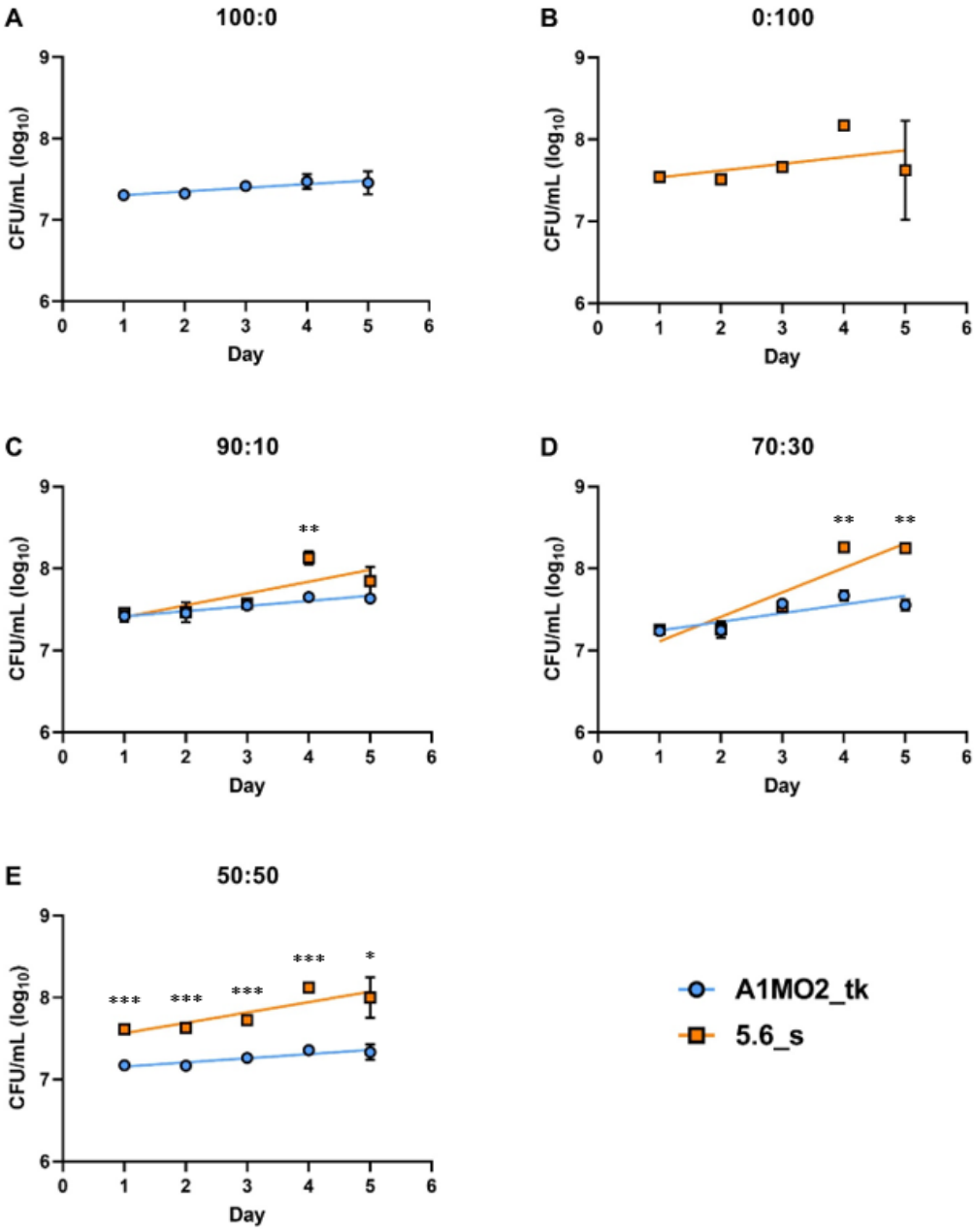


Figure 4.8. Average log_{10} CFU/mL counts (n = 3) for A1MO2_tk and 5.6_s when inoculated into a spent six-day old 20 % maltose M9 medium and cultured over five days. The strains were inoculated separately (A) 100:0 and (B) 0:100, and together in varying ratios: (C) 90:10, (D) 70:30, (E) 50:50 (A1MO2_tk:5.6_s). The inocula were produced from overnight cultures with rates of 1.98 x 10^9 CFU/mL (A1MO2_tk) and 2.17 x 10^9 CFU/mL (5.6_s). Results are presented alongside linear regression lines and standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Table 4.11. The multiple linear regression model parameters and one-way ANOVA data derived from log_{10} CFU data for A1MO2_tk and 5.6_s cultured in six-day old 20% M9 maltose medium following inoculation at various ratios (A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with four degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.89	23.994	6.375	0.064	17.72	0.053
0:100	5.6_s		-2.244	2.004	0.379		
90:10	A1MO2_tk	0.90	21.912	6.302	0.074	18.69	0.051
	5.6_s		-2.883	2.251	0.329		
70:30	A1MO2_tk	0.75	1.552	3.823	0.724	6.94	0.126
	5.6_s		2.366	1.537	0.264		
50:50	A1MO2_tk	0.75	18.029	16.452	0.387	6.88	0.127
	5.6_s		-0.784	6.328	0.913		

The changes in the proportion of each strain isolated from the medium in the mixed inoculation culture also provides evidence for the increased competitive fitness of 5.6_s in the maltose medium in each of the mixed ratio treatments. This is evident in the initial cell proportions recorded at one dpi in the 90:10 and 70:30 treatments (A1MO2_tk:5.6_s) (Fig. 4.9. A - B), where despite being inoculated at a lower ratio an equal number of 5.6_s and A1MO2_tk cells were isolated from the culture. This balance was then maintained until three dpi, indicating a coexistence between the strains. However, between three and four dpi the proportion of 5.6_s increased, with significantly more of the cells isolated from the culture being 5.6_s, as opposed to A1MO2_tk ($p < 0.005$ to 0.0005). This significant difference between the strains was maintained until the end of the assay at five dpi. The occurrence of competition in the 50:50 inoculant (A1MO2_tk:5.6_s) was also evident in the proportion changes (Fig. 4.9. C). By one dpi significantly more of the cells isolated from the 50:50 culture were 5.6_s as opposed to A1MO2_tk ($p < 0.0005$), indicating 5.6_s had outcompeted A1MO2_tk within the first 24 hours post-inoculation. Over the remainder of the assay a significantly higher proportion of the cells isolated were 5.6_s, indicating that it was able to maintain this higher proportion of cells in the medium.

With the co-existence of the strains in the re-inoculated maltose medium observed over the assay duration, the results obtained resembled the growth dynamics recorded over seven days in the M9 glucose medium (Chapter 3, section 3.2.6) in which the A1MO2 and 5.6 were also able to co-exist. However, the trends in both the 90:10 and 70:30 treatments (A1MO2:5.6) show an increase in growth for 5.6 at four dpi, which was not recorded during previous assessments, and may reflect 5.6 utilising resources from dead cells in the medium for growth.

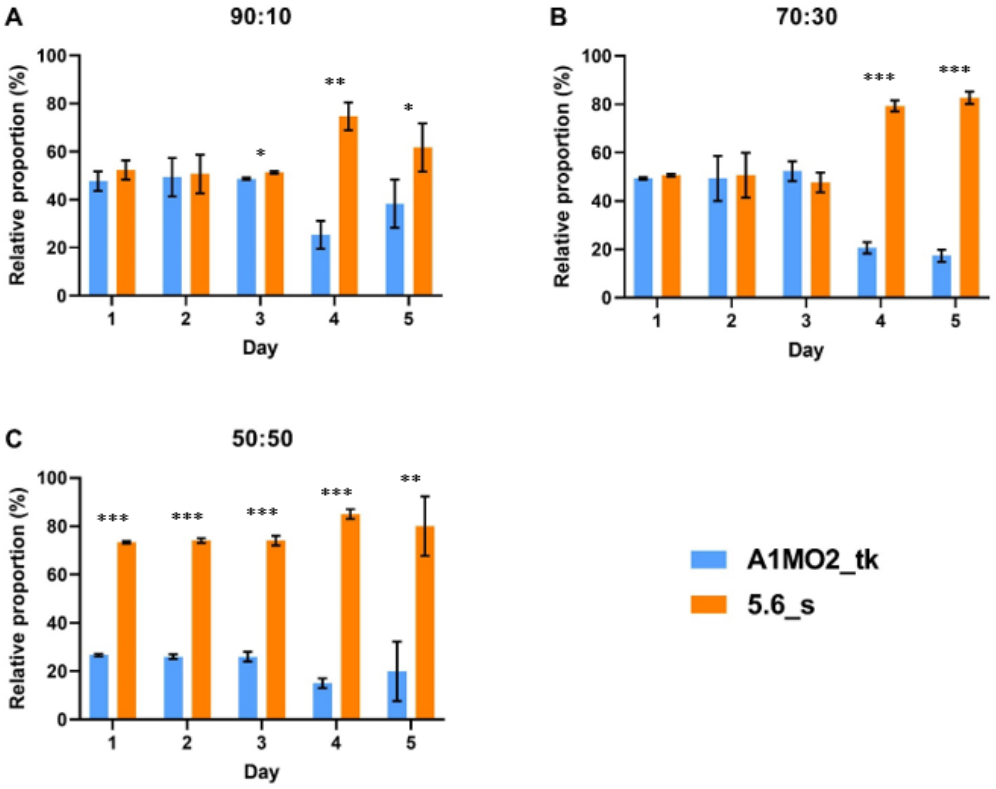


Figure 4.9. Changes in the average relative proportion (%) (n = 3) for A1MO2_tk and 5.6_s cells isolated from spent six-day old 20 % maltose M9 mixed cultures when inoculated at various ratios: (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

4.2.5 The impact of mixed inoculation with tagged *Serratia* spp. using a trehalose carbon source

Alongside the increased expression of a gene operon related to maltose metabolism and transport, the RNAseq data also highlighted an increased expression of genes with products relating to the transport and metabolism of trehalose in the 5.6 replicates as opposed to 5.6+pADAP (Table 4.2). Similar to the maltose operon, further experimentation was conducted competing A1MO2_tk and 5.6_s using a M9 medium with a 20 % trehalose carbon source to determine if this would have any outcome on competition over a 48-hour period (method: Chapter 2, section 2.3.4).

The separate inoculation of A1MO2_tk and 5.6_s led to the generation of similar growth curves over a 48-hour period. Similar average CFU counts (n = 3) were recorded following inoculation of the medium at zero hpi, with counts of 1.31 x 10^7 CFU/mL for A1MO2_tk and 1.46 x 10^7 CFU/mL for 5.6_s (Fig. 4.10). During the initial lag phase, lasting until four hpi, the CFU count of 5.6_s decreased, resulting in a significant difference between the strains at two and four hpi, in which the CFU count for A1MO2_tk was significantly higher than 5.6_s ($p < 0.05$). Additionally, both strains exhibited an overall decrease in CFU following inoculation, forming a dip in the growth curves, as pictured in Figure 4.10. This may

suggest the acclimatisation of the strains in the medium, where the cells present were competing with each other for available nutrients. Both strains showed a similar pattern of growth during the exponential growth phase, with no occurrences of significant differences in CFU counts being identified between four and 12 hpi. The CFU counts generated during the exponential growth period also show a similar doubling time for both A1MO2_tk (T_d = 31 mins, R^2 = 0.99) and 5.6_s (T_d = 27.6 mins, R^2 = 0.99) between six and eight hpi. Similar CFU counts were also recorded between the strains during the stationary phase, finishing with final CFU counts of 3.03 x 10^9 CFU/mL for A1MO2_tk, and 3.44 x 10^9 CFU/mL for 5.6_s at 48 hpi. This indicated, that when inoculated separately, both A1MO2_tk and 5.6_s showed a similar pattern of growth in the trehalose medium. This, alongside the lack of any significant difference during the exponential growth phase, suggested that the increased expression of the trehalose operon in 5.6 at approximately nine hpi did not cause any changes in growth rates when inoculated separately.

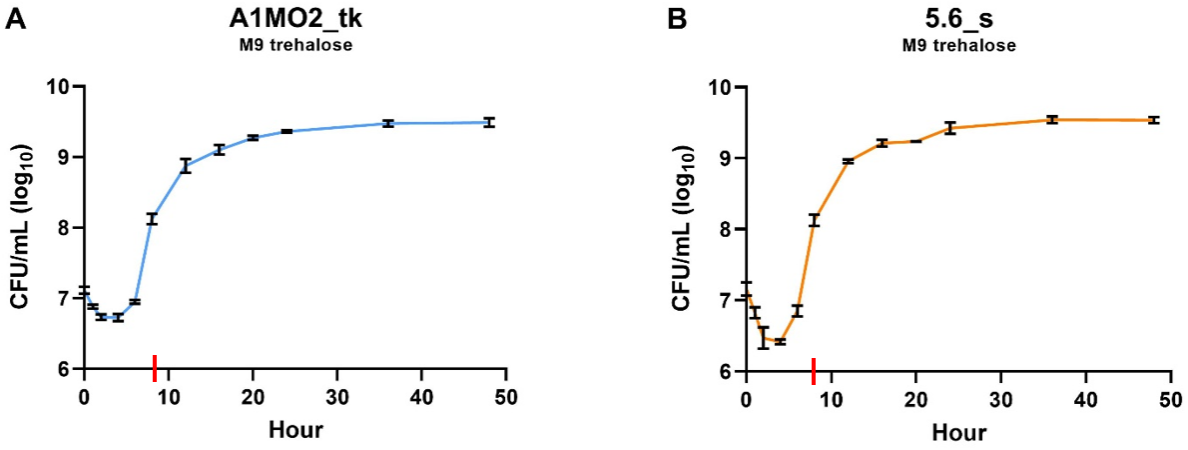


Figure 4.10. Average log_{10} CFU/mL counts (n = 3) for (A) A1MO2_tk and (B) 5.6_s when inoculated separately in a 20 % trehalose M9 medium and cultured over 48 hours. The medium was inoculated at a rate of 5.77 x 10^8 CFU/mL (A1MO2_tk) and 6.77 x 10^8 CFU/mL (5.6_s). Results are presented with standard deviation error bars. The red line on the X-axis denotes the time at which the initial RNAseq samples were assessed , aligning with an increased expression of the trehalose operon in 5.6 samples (10^8 CFU/mL ~ 8 to 9 hours post-inoculation).

In contrast, the mixed inoculation of the trehalose medium with A1MO2_tk and 5.6_s at a 50:50 ratio (A1MO2_tk:5.6_s) resulted in the occurrence of competition between the strains. Following the inoculation of the medium both strains had attained similar average CFU counts (n = 3) of 4.43 x 10^6 CFU/mL for A1MO2_tk and 6.80 x 10^6 CFU/mL for 5.6_s (Fig. 4.11. A), with both strains maintaining similar counts at two hpi. By four hpi the CFU counts for 5.6_s decreased, resulting in a significant difference between the strains in which A1MO2_tk had a significantly higher CFU count than 5.6_s

between four and six hpi ($p < 0.05$ to 0.005). This suggested an initial increase in fitness within the medium. However, following the onset of exponential growth between six and eight hpi there was a lack of significant difference between the strains, suggesting that both strains were growing at the same rate. CFU counts of 5.63×10^7 and 7.47×10^7 CFU/mL were recorded for A1MO2_tk and 5.6_s, respectively, at eight hpi, when the trehalose genes were at an increased level of expression in the 5.6 RNAseq samples. Whilst there was no significant difference between the CFU counts of the strains at the start of the stationary phase at 12 hpi, by 16 hpi 5.6_s had a significantly higher CFU count than A1MO2_tk ($p < 0.05$). Additionally, whilst there were no significant differences between the CFU counts between eight and ten hpi, higher counts were recorded for 5.6_s, which coincide with the time at which the increased expression of the trehalose operon was recorded during the RNAseq data. By 48 hpi there was still a significant difference between the strains, in which 5.6_s had a significantly higher CFU count than A1MO2_tk ($p < 0.05$), with final CFU counts of 1.45×10^9 CFU/mL for A1MO2_tk and 2.41×10^9 CFU/mL for 5.6_s. Whilst both strains exhibited similar doubling rates during the exponential growth phase, the significant difference between the strains during the exponential phase suggested a competitive advantage for 5.6_s.

This is also reflected in the changes of relative proportion for the cells isolated from the culture over the duration of the assay. These changes show an initial increased in the proportion of A1MO2_tk in the culture during the lag phase (Fig. 4.11. B). This may suggest an increased expression of the trehalose operon at this time in A1MO2_tk, or it may indicate that A1MO2_tk was more able to adapt to the medium following inoculation, as was seen when the strains were independently cultured. However, following the onset of exponential growth after eight hpi the proportion of 5.6_s cells isolated increases, which links to the increased expression of the trehalose-related genes in 5.6 at the mid-exponential growth phase. This also causes significantly more 5.6_s cells to be isolated from the medium than A1MO2_tk by the end of the exponential growth phase ($p < 0.0005$), with this significance being maintained over the remainder of the assay until 48 hpi.

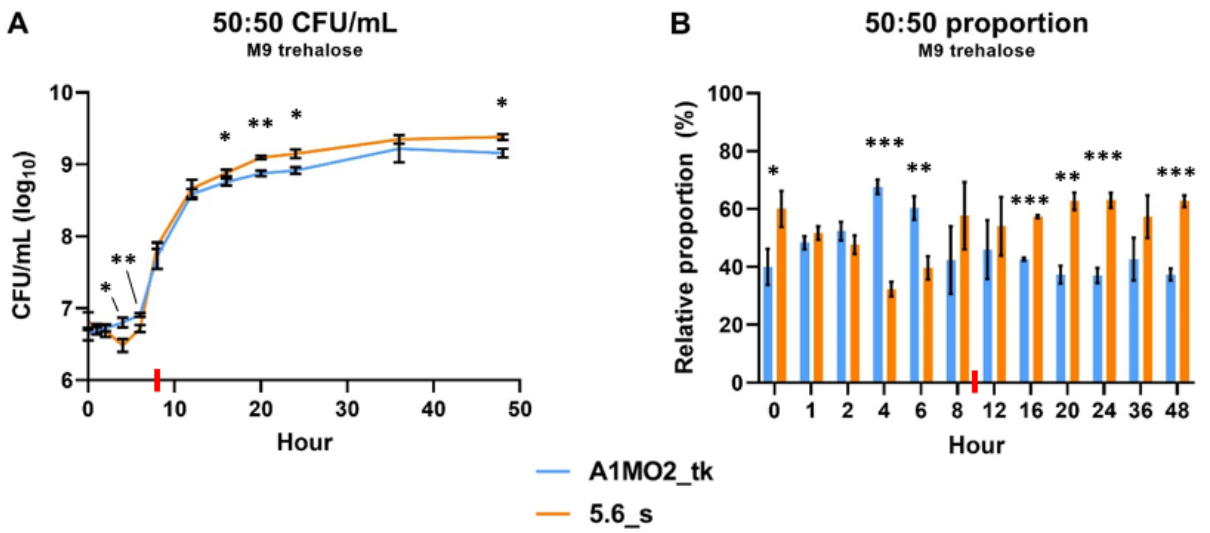


Figure 4.11. (A) Average log_{10} CFU/mL counts (n = 3) for tagged *Serratia* strains A1MO2_tk and 5.6_s when inoculated together in a 20 % trehalose medium over 48 hours at a 50:50 ratio (A1MO2_tk:5.6_s). The strains were inoculated at rates of 2.57 x 10^8 CFU/mL (A1MO2_tk) and 3.37 x 10^8 CFU/mL (5.6_s). (B) The changes in average relative proportion (%) (n = 3) of cells isolated from the culture over 48 hours. Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. The red line on the X-axis denotes the time at which RNAseq samples were assessed, aligning with an increased expression of the trehalose operon in 5.6 samples (10^8 CFU/mL between eight to nine hours post-inoculation).

4.2.6 Mixed inoculation with tagged *Serratia* spp. using a glycine betaine carbon source

Whilst a gene operon with increased expression relating to the increased transport of glycine betaine was identified in the 5.6 replicates of the RNAseq data (Table 4.4), subsequent investigation with a growth curve conducted over a 48-hour period in an M9 20 % glycine betaine medium did not yield any growth data. When A1MO2_tk and 5.6_s were inoculated into the medium, both separately and together at a 50:50 ratio (A1MO2_tk:5.6_s), no measurable growth was recorded when sampled at regular intervals over a 48 hour-period (method: Chapter 2, section 2.3.4). This indicated that this N-trimethylated amino acid did not facilitate the growth of the strains.

4.2.7 Characterising growth of fluorescence tagged *Serratia* spp. in a maltose medium

Due to the competitive dominance of 5.6 during competition with A1MO2 in the maltose medium over seven days, and an increased expression of the maltose operon in 5.6 during the exponential growth period, further experimentation was conducted using fluorescence-tagged *Serratia* constructs (A1MO2_rfp and 5.6_rfp) (method: Chapter 2, section 2.3.8). This would provide a clear picture of how

the increased expression of the maltose genes might relate to the growth of the strains during competition. The 20 % maltose medium was inoculated either separately with either A1MO2_rfp (100:0) or 5.6_rfp (0:100), or with a mixed ratio of the strains: 75:25, 50:50, 25:75 (method: Chapter 2, section 2.3.10). As in the previous iteration of this experiment (Chapter 3, section 3.2.8), the red-fluorescence construct strains were competed against gfp variant constructs (A1MO2_gfp or 5.6_gfp) using red-light, due to previous issues with autofluorescence of the strains upon exposure to green-light.

When A1MO2_rfp was inoculated into the maltose medium it was possible to see an increase in fluorescence intensity as the proportion of A1MO2_tk in the inoculant increased. However, the increases in A1MO2_rfp intensity had less of a correlation with the inoculant proportion than in previous fluorescence intensity assays (Chapter 3). The highest levels of fluorescence intensity were recorded when A1MO2_rfp was inoculated separately in an independent culture (100:0), reaching an average intensity measurement of 4,851 a.u by 48 hpi (n = 3) (Fig. 4.12. A). Reducing the proportion of A1MO2_rfp in the inoculant (75:25, A1MO2_rfp:5.6_gfp) resulted in a fluorescence reading of 3,264 a.u at 48 hpi, and an intensity of 2,264 a.u was reached by 48 hpi when both strains were inoculated at equal proportions (50:50, A1MO2_rfp:5.6_gfp). The weakest intensity measurements were recorded when A1MO2_rfp comprised a lower proportion of the inoculant (25:75, A1MO2_rfp:5.6_gfp), with an intensity of 672 a.u at 48 hpi. This score is lower than what was anticipated for this inoculant, indicating that A1MO2_rfp may have been outcompeted by 5.6_gfp during this assay. Following inoculation A1MO2_rfp entered the exponential growth phase at ~ 8.3 hpi (500 minutes), which was maintained until ~ 16.6 hours (1,000 minutes). Over the duration of the assay neither the wild-type A1MO2 and 5.6 control strains (A1MO2 wt and 5.6 wt), nor the 5.6_gfp control (0:100) showed any significant changes in fluorescence intensity (Fig. 4.12. B).

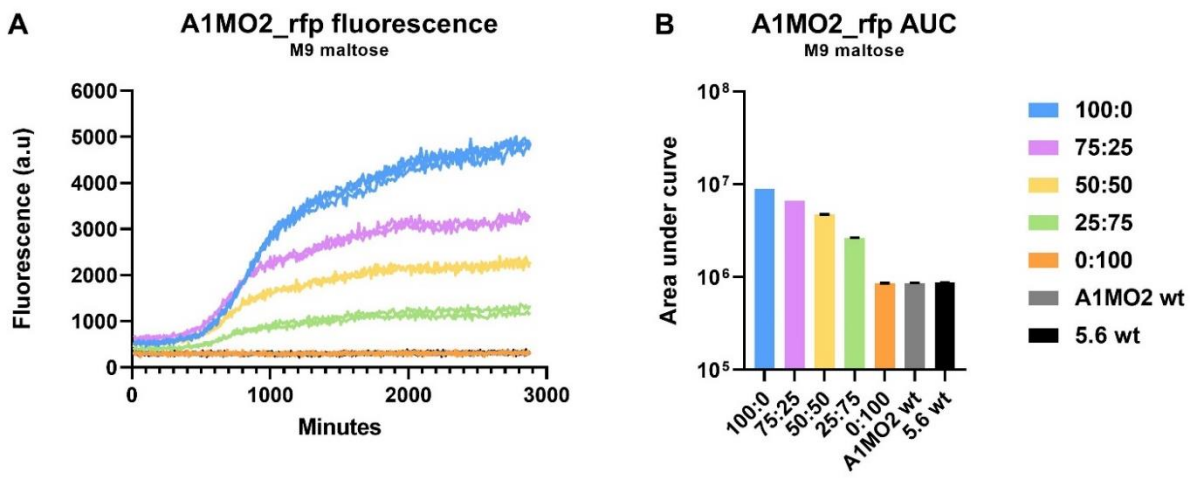


Figure 4.12. (A) The fluorescence intensity measurements (a.u) for A1MO2_rfp and 5.6_gfp when cultured in a 20 % maltose M9 medium over 48 hours when the strains were inoculated separately (100:0, 0:100, A1MO2_rfp:5.6_gfp), and together in mixed ratios (75:25, 50:50, 25:75, A1MO2_rfp:5.6_gfp) alongside untagged wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

The inoculation of 5.6_rfp into the maltose medium resulted in a similar outcome as A1MO2_rfp (depicted above), where an increase in the proportion of 5.6_rfp in the mixed cultures led to corresponding increases in fluorescence intensity. However, based on the maximum fluorescence intensity measurements, it is clear that 5.6_rfp achieved a higher fluorescence intensity than A1MO2_rfp in the maltose medium which also suggests more growth of 5.6_rfp in the mixed cultures, likely owing to the increased expression of the maltose operon in 5.6 at eight hpi. Furthermore, the highest overall level of 5.6_rfp fluorescence intensity were recorded in the 27:75 mixed ratio culture (A1MO2_gfp:5.6_rfp), with an average intensity measurement (n = 3) of 4,597 a.u at 48 hpi, with one replicate achieving a peak intensity of 5,094 a.u at 42 hpi (Fig. 4.13. A). This result initially suggested that under the conditions assessed 5.6_rfp grew better when in competition with A1MO2_tk than when it was inoculated separately due to the increased growth exhibited between 16 and 33 hpi. However, the area under the curve measurements (Fig. 4.31. B) show that the overall growth for 5.6_rfp in the 25:75 inoculant was similar to when 5.6_rfp was cultured separately, with a similar outcome being observed during the culturing of A1MO2_tk and 5.6_s in the maltose medium over seven days.

The separate inoculation of 5.6_rfp (0:100) resulted in a similar fluorescence measurement of 4,280 a.u at 48 hpi, with the fluorescence measurement decreasing in the remaining treatments as the proportion of A1MO2_gfp increased, with a measurement of 2,965 and 1,618 a.u in the 50:50 and 75:25 inocula (A1MO2_gfp:5.6_rfp), respectively. During the assay 5.6_rfp entered exponential growth at ~ 8.3 hpi (500 minutes), which coincides with the increased expression of the maltose genes in the RNAseq data. A second period of growth was recorded at ~ 28.3 hpi (1,700 minutes), which may also suggest another period of increased expression for the genes. No significant changes in fluorescence intensity were recorded for the wild-type control strains nor the A1MO2_gfp control (Fig. 4.13. B).

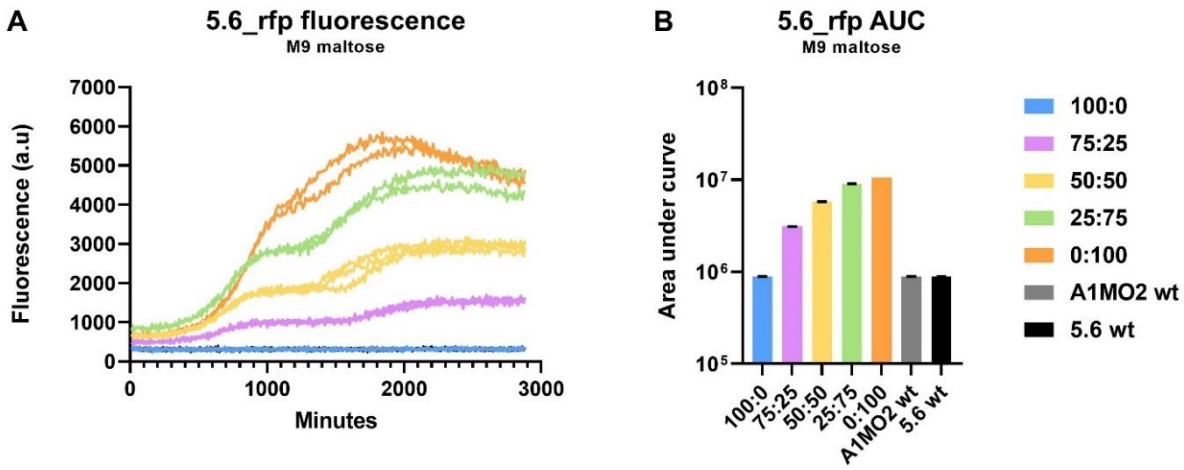


Figure 4.13. (A) The fluorescence intensity measurements (a.u) for A1MO2_gfp and 5.6_rfp when cultured in a 20 % maltose M9 medium over 48 hours when the strains were inoculated separately (100:0, 0:100, A1MO2_gfp:5.6_rfp), and together in mixed ratios (75:25, 50:50, 25:75, A1MO2_gfp:5.6_rfp) alongside untagged wild-type control strains (wt), with three replicates plotted for each inoculant. (B) The area under the curve (AUC) of the fluorescence intensity measurements for the cultures.

4.3 Discussion

The results presented in this chapter indicate that there are distinct differences in the chromosome gene expression profiles of 5.6, and a pathogenic variant of 5.6 containing a conjugated pADAP (5.6+pADAP), indicating that the carriage of pADAP leads to an alteration in gene expression. The most notable differences in gene expression relate to the increased expression of gene operons used in the transport and metabolism of specific carbohydrates, such as maltose and trehalose, on the chromosome of 5.6. An initial assessment through the independent inoculation of A1MO2 and 5.6 into an M9 medium containing either a maltose or trehalose carbon source did not indicate any major differences in growth patterns between the strains. This was likely due to the absence of competition between the strains and any obvious beneficial competitive effects of the increased gene expression would not be seen until both strains were competing against each other for resources.

The mixed inoculation of both A1MO2 and 5.6 into an M9 medium using either maltose or trehalose as the supplemental carbon source revealed that the increased gene expression did confer a competitive advantage for 5.6 when cultured in these media types both over a 48-hour period during the exponential growth phase. The increase in competitiveness for 5.6 during the exponential growth phase also coincided with the increased expression of the gene operons seen in the RNAseq data, which used samples of each strain obtained during the exponential growth period, with CFU counts approaching 10^9 to 10^{10} CFU/mL (~ eight hours post-inoculation). A clear increase in competitive capabilities for 5.6 was also demonstrated during a seven-day competition assay in an M9 maltose culture, where 5.6 outcompeted A1MO2 within the first 24 hours post-inoculation, leading to significantly higher CFU counts over the duration of the assay in each of the mixed ratio treatments.

An assessment of the RNAseq data also revealed the increased expression of the genes *xerD_2* and *traR* in both the 5.6+pADAP and 5.6 replicates, though the amino acid sequences of the proteins do not share a similarity to the Int2 and TraR pADAP-encoded proteins. The chromosomal TraR protein is also found in the *E. coli* conjugative F plasmid, and shares homology with other predicted proteins encoded by conjugative plasmids and bacteriophages. However, there is also evidence for the use of TraR in transcription (Gopalkrishnan et al., 2017). Similarly, *xerD_2*, a structural orthologue also encoded on pADAP (Sitter et al., 2021), is expressed in both strains, with an increased level of expression in the 5.6+pADAP replicates. A XerDC domain is found on pADAP containing an open reading frame, designated Int2, which facilitates the recombination of dimerised plasmids at the XerDC site to form monomers (Castillo et al., 2017; Cameranesi et al., 2018). The expression of these genes in the 5.6 replicates can only relate to the absence of pADAP from 5.6, a plasmid-cured variant of A1MO2. It is possible that the regulation of pADAP orthologues may lead to a suppression of the equivalent genes encoded on the chromosome, enabling control of their replication. Further

investigation with a natural non-pathogenic isolate, such as *S. entomophila* plasmid-free strain 477, would determine whether similar expression of these genes is seen in a strain which does not carry pADAP.

Many of the genes identified with an increased level of expression in the 5.6+pADAP (A1MO2 equivalent strain) RNAseq replicates are used in various catalytic processes, including speculative products used in housekeeping functions such as apoptosis regulation, or catalytic enzymes used in the formation of substrates such as lipid A. Furthermore, in comparison to the genes identified in the 5.6 replicates, the genes with an increased level of expression in the 5.6+pADAP replicates had a much lower overall level of expression. This can be seen when comparing the expression scores of genes listed in Tables 4.1 - 4.5 (increased expression in 5.6), which have expression scores between 500 and 80,000, against the expression scores of genes listed in Tables 4.6 - 4.9 (increased expression in 5.6+pADAP), which have scores between 10 and 3,000. This suggests that the scores with an increased expression in the 5.6+pADAP replicates, relative to 5.6, might not have such a large impact on competition. Additionally, it was noted that the autofluorescence recorded during the previous fluorescence intensity assays in Chapter 3, assessing growth of non-fluorescence tagged wild-type A1MO2 and 5.6 control strains in both LB and M9 media, was likely caused by the excitation of flavin compounds within the cell (Surre et al., 2018). With respect to *Serratia*, the genes responsible for flavin mononucleotide and flavin adenine dinucleotide production, *ribA*, *ribB*, *ribC*, and *ribE* (Fischer and Bacher, 2008), were all identified in the RNAseq data for the 5.6+pADAP and 5.6 replicates.

This increase in competitive behaviour for 5.6 was also highlighted during the fluorescence intensity assay, where 5.6_rfp gave higher intensity readings and higher AUC measurements when inoculated into a maltose medium, which also suggested further growth during the stationary phase. However, the genes identified relating to glycine betaine transport did not appear to confer a competitive advantage for 5.6 or A1MO2 when inoculated into the M9 medium in both mixed and separate cultures. As glycine betaine functions as an osmoprotectant it may confer a fitness advantage for 5.6 in conditions of high osmolarity, which might be encountered in situations such as the drying of soil (Kemph and Bremer, 1998).

Based on the use of 5.6+pADAP and 5.6, the differences in gene expression between the strains identified in the RNAseq data can only be attributed to the carriage of pADAP, as this was the only difference between the strains assessed. Previous investigations with RNAseq with a variety of bacterial species have shown how the plasmid carriage altered the metabolic pathways of the cell, resulting in differences in gene expression between plasmid-bearing and plasmid-free *Salmonella* species (Lang and Johnson, 2015). A similar outcome has been observed for *P. aeruginosa* strain PAO1, with the carriage of plasmids impacting genes relating to translation efficiency and replication (San

Millan et al., 2018). More specifically towards the alteration in carbohydrate metabolism displayed in 5.6, the conjugation of a plasmid into *Klebsiella pneumoniae* was shown to impact the expression of 16 genes related to carbohydrate and amino acid metabolism and transport (Buckner et al., 2018).

Whilst these differences in gene expression, specifically the expression of maltose, were shown to impact competition when the strains were cultured *in vitro* in liquid media, it is unclear as the effect they would have either *in vivo* or in the soil. Maltose can be formed during the breakdown of starch and glycogens, and thus might be encountered by *Serratia* spp. in the rhizosphere from decomposing plant matter, providing the required sugars to the bacterium to enable an increase of fitness (Lu et al., 2006; Pfister and Zeeman, 2016). Furthermore, both white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*), common pastoral plants in New Zealand, contain starches, which can be metabolised into maltose by amylase in the pre-caecal portion of the *C. giveni* midgut area (Biggs and McGregor, 1996). The decomposition of plant matter in the rhizosphere would also lead to the release of starches and maltose near plant roots, where grass grubs and *Serratia* in the soil are typically found. Based on the *in vitro* culturing results, the presence of maltose in this environment would confer at fitness advantage to 5.6 during the exponential growth stages due to the increased expression of the maltose genes.

In contrast, trehalose is commonly found circulating in the insect haemolymph (Simpson and Chapman, 2003; Thompson, 2003), and would not be encountered until the final stages of amber disease infection where the bacteria invade the weakened gut to enter the haemocoel. Therefore, whilst the presence of trehalose might not impact competition prior to larval death, an increased expression of trehalose-related genes in 5.6 might assist in the proliferation of the strain within the cadaver. Additionally, trehalose can be found at low levels in plants, where it is believed to act as a signal metabolite (Lunn et al., 2014). Whilst the maltose content in the gut of *C. giveni* larvae has not been defined, a study on the gut contents of the parasitic wasp *Diadegma insulare* found little-to-no maltose or trehalose within the gut, whilst an abundance of glucose was noted (Wackers et al., 2006). However, maltose metabolism has been shown to increase the fitness of the pathogenic *E. coli* strain EDL933 during colonisation of the intestine *in vivo* using a mouse model (Jones et al., 2008). The key finding obtained from the RNAseq of 5.6+pADAP and 5.6 was that there are distinct differences between the expression of certain chromosomal genes between the strains, where these differences likely stemmed from the carriage of pADAP. Furthermore, these differences in gene expression were found to contribute to the competitive abilities of the strains in a liquid medium, with 5.6 outcompeting A1MO2 when inoculated into both maltose and trehalose media. This suggests that the carriage of pADAP by A1MO2 may be detrimental to the fitness of the A1MO2 under specific conditions, leading to it being outcompeted by non-pathogenic strains which do not bear the burden of pADAP carriage.

Chapter 5

Competition between *Serratia* spp. in natural soil microcosms

5.1 Introduction

Characterisation of the growth of *Serratia* spp. in liquid culture showed that when A1MO2 and 5.6 were inoculated at varied ratios, 5.6 was able to consistently outcompete A1MO2, showing increased persistence and a competitive advantage during the exponential growth phase. The next step was to assess whether a similar outcome was observed when the strains were inoculated into natural soil. The persistence of *Serratia* following mixed strain inoculation into the soil is of interest because *Serratia* spp. are non-spore forming bacteria. Thus, their ability to persist in the soil in the absence of grass grub larvae may be limited, whereas residing within the grass grub may protect the bacteria from adverse environmental conditions and competition from other microbial species in the rhizosphere (Driks, 2002). The persistence of *Serratia* within the soil has been investigated previously with some *S. entomophila* biopesticide products, such as Invade®, which was applied to the soil as a liquid drench. Subsequent analysis following the application of a liquid drench to the field found that the number of viable cells decreased over a period of 145 days (O'Callaghan and Gerard, 2005), potentially limiting its effectiveness towards larvae. However, as previously mentioned, it might also be possible that *Serratia* cells are able to avoid competition in the soil with other microbial species by persisting within larval cadavers.

By inoculating both A1MO2 and 5.6 into the soil using mixed ratio inocula of the previously constructed tagged *Serratia* strains, A1MO2_tk and 5.6_s (Chapter 2, section 2.2.16), it will be possible to determine whether competition between the strains has any impact on persistence of the strains in the soil. A model developed by Godfray et al. (1999) predicted the capability for saprotrophic reproduction in pathogenic *Serratia* strains in the soil. However, the pathogenic *Serratia* spp. must compete for these resources with non-pathogenic *Serratia* spp., which are hypothesised to be better competitors due to the lack of pADAP. Therefore, carriage of pADAP would incur a fitness cost on pathogenic *Serratia* spp., potentially reducing its persistence within the soil. The mixed inoculation of *Serratia* spp. also provided an opportunity to assess pADAP horizontal gene transfer (HGT) between A1MO2 and 5.6 in the soil. The horizontal transfer of genes has been observed previously between the pathogenic *B. thuringiensis* subsp. *kurstaki* and other indigenous *Bacillus* spp. in the soil (Donnarumma et al., 2010). Furthermore, previous work using *Serratia* has demonstrated that the horizontal transfer of pADAP between *Serratia* spp. can occur in soil microcosms, where the addition of grass grub larvae to the microcosms increased the rate at which HGT occurred (Dodd, 2003) when compared to soil-only microcosms. However, as these strains were raised and selected for nalidixic acid resistance this may

have affected the validity of the results due to the chance for mutations in enzymes used for DNA replication. The use of natural soil also allowed for the inclusion of additional factors which may impact the strain dynamics, such as other naturally occurring bacterial and fungal species resident within the soil. This background microbial community could include other competitor or cheat species present in the rhizosphere (Denison et al., 2003). Furthermore, the use of a Wakanui silt loam soil as the basis of microcosm experiments presented in this chapter simulated the soil profile which would typically be present in the Canterbury region. This is a region of interest due to the high incidence of grass grub in Canterbury pastures (Jackson, 1990), and an estimated combined area of 1.7 million hectares of land used either as grassland or tussock for grazing for sheep and beef (Stats NZ, 2021).

5.2 Results

5.2.1 The persistence of wild-type *Serratia* strains in soil microcosms

An initial assay was conducted using microcosms in which natural soil samples were inoculated separately with wild-type, untagged *Serratia* strains A1MO2, 5.6, and AGR96X, to characterise the dynamics of the strains in the soil over an eight-week period (method: Chapter 2, section 2.4.5). The soil used in the microcosms was a Wakanui silt loam, a type of soil found in Canterbury, and was assessed using an enumeration method and CTA medium to ensure the absence of any *Serratia* spp. prior to the preparation of microcosms (method: Chapter 2, section 2.4.5). The strains were inoculated into the microcosms at a rate of 5×10^5 CFU/g, equivalent to the field rate used for the liquid *Serratia* biopesticide product Invade (O'Callaghan and Gerard, 2005).

The microcosms were first assessed one-week post-inoculation (wpi), with average CFU counts of 2.29×10^6 CFU/g for A1MO2, 1.89×10^6 CFU/g for 5.6, and 2.09×10^6 CFU/g for AGR96X, with no significant differences between the CFU counts of the strains (Fig. 5.1). Over the eight-week assay A1MO2 and AGR96X populations only gradually decreased, with no significant differences between the CFU counts for each strain at one wpi and eight wpi, indicating a high persistence of the strains under the conditions assessed. By eight wpi CFU counts of 9.20×10^5 and 1.15×10^6 CFU/g were recorded for A1MO2 and AGR96X, respectively. A decrease in CFU was also recorded for 5.6 over the assay duration, though a significant difference between time-points was only noted between the CFU counts for 5.6 at one and eight wpi ($p < 0.05$), with a final count of 3.87×10^5 CFU/g at eight wpi. Despite this change for 5.6 specifically, there were no significant differences between the CFU counts of the three strains at the end of the assay at eight wpi. These trends were reflected in the linear regression lines fitted to the CFU data, where 5.6 showed the largest decrease in CFU changes, followed by AGR96X and then

A1MO2 (Table 5.1). Under the conditions assessed, the lack of a significant difference between the CFU counts of the strains at eight wpi denotes a similar level of persistence within the soil.

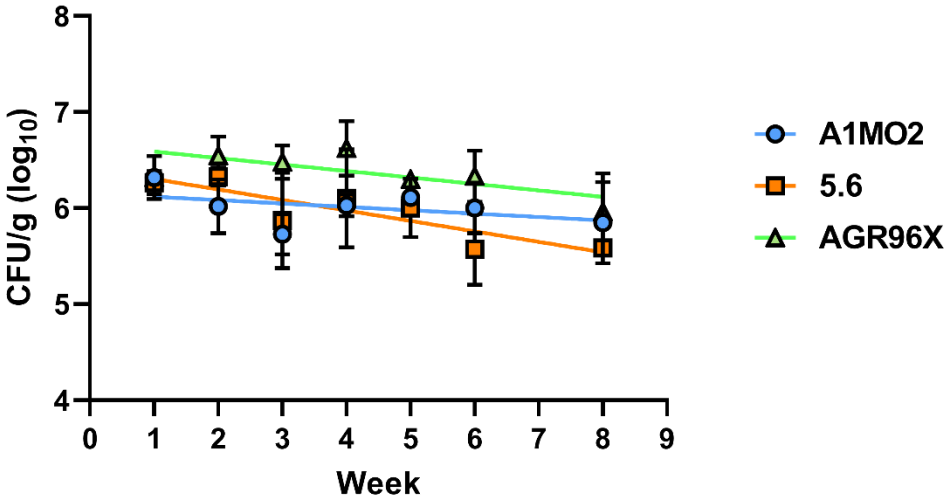


Figure 5.1. The changes in average log_{10} CFU/mL (n = 3) for A1MO2, 5.6, and AGR96X when inoculated separately in soil microcosms at a rate of 5 x 10^5 CFU/g and assessed over eight weeks. CFU data is presented with standard deviation error bars and fitted linear regression lines for each of the strains.

Table 5.1. The simple linear regression models for changes in log_{10} CFU/g per week for A1MO2, 5.6, and AGR96X, showing the regression equation modelling CFU (*y*) by week (*x*), the error of the slopes, the adjusted R^2 value and model significance (*p*). Models were calculated to 19 degrees of freedom.

Strain	Regression equation	Slope error	R^2	*p*
A1MO2	$y = -0.035*x + 6.15$	0.026	0.09	0.199
5.6	$y = -0.109*x + 6.41$	0.031	0.39	0.002
AGR96X	$y = -0.067*x + 6.66$	0.027	0.27	0.024

5.2.2 Validating pathogenicity of tagged *Serratia* strains compared to wild-type *Serratia* strains

Whilst previous comparisons showed no difference between the dynamics of the growth for the antibiotic tagged *Serratia* strains and their wild-type counterparts in liquid culture (Chapter 3, section 3.2.2), a bioassay was conducted to ensure that the constructs were still able to cause amber disease when used in microcosm and bioassay experiments. Using a standard bioassay method (method: Chapter 2, section 2.5.2), healthy larvae (n = 6) were challenged via ingestion of carrot cube pieces rolled across bacterial lawns to produce a dose rate exceeding ~ 1 x 10^8 CFU/mL and assessed every three days over a nine-day period.

At nine dpi all six larvae (100 %) challenged with wild-type A1MO2 displayed characteristic amber disease symptoms such as the amber colouration from gut clearance and feeding cessation (Fig. 5.2). Similarly, challenge with the A1MO2_tk construct strain resulted in five larvae (83 %) exhibiting amber disease symptoms at nine dpi, with one larva dying prior to the third day of the nine-day bioassay. Challenge with both wild-type 5.6 and the 5.6_s construct did not cause disease, with five of the challenged larvae (83 %) remaining healthy at nine dpi, though a single larva in each of the treatments died prior to the conclusion of the bioassay.

Challenge of the larvae with AGR96X resulted in a mixed outcome, with two larvae (33 %) remaining healthy at nine dpi, two larvae becoming diseased, and two larvae dying. The two diseased larvae in this group developed a brown discolouration, characteristic of AGR96X infection, with the two deceased larvae changing to a blackened colour. Challenge with the AGR96X_s construct resulted in five larvae (83 %) becoming diseased at nine dpi with brown colouration, with one larva remaining healthy. Five of the larvae (83 %) in the uninoculated control group remained healthy and feeding at nine dpi, with one larva becoming diseased prior to the third day of the bioassay. A low mortality rate of 12 % was recorded over the duration of the bioassay, with no death in the uninoculated control group, and a single death in the larvae challenged with wild-type 5.6 and 5.6_s; two deaths were recorded in the larvae challenged with AGR96X. These results show that the virulence characteristics of the construct strains are similar to those of the wild-type counterparts, indicating that the insertion of the antibiotic cassettes had not impacted pathogenicity.

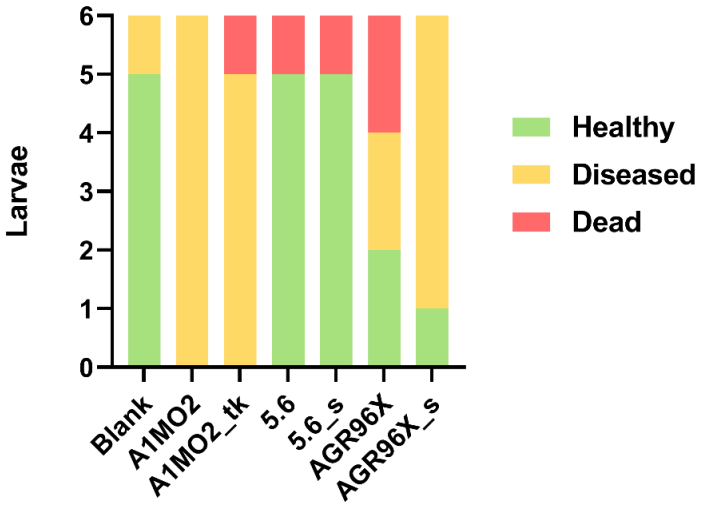


Figure 5.2. The conditions of larvae (n = 6) after nine days following challenge with either wild-type *Serratia* strains A1MO2, 5.6, and AGR96X, or their antibiotic-tagged counterparts of A1MO2_tk, 5.6_s, and AGR96X_s at a dose rate exceeding 1 x 10^8 CFU/mL. Blank control larvae were uninoculated. Disease classification was based on amber discolouration of the larvae and a lack of feeding behaviour.

5.2.3 The persistence of tagged *Serratia* spp. inoculated at mixed ratios in soil microcosms in the absence of grass grub larvae

The assessment of competition via the mixed inoculation of a LB broth growth medium using the tagged *Serratia* strains A1MO2_tk and 5.6_s demonstrated an increased persistence for 5.6_s in the mixed cultures (Chapter 3, section 3.2.4.a). This increase in persistence for 5.6_s was exhibited in all mixed ratio treatments. As such, the inoculation of natural soil microcosms with these tagged strains was conducted using mixed inocula which predominantly contained A1MO2_tk (90:10, 70:30, and 50:50, A1MO2_tk:5.6_s). Alongside this, A1MO2_tk and 5.6_s were also inoculated independently in separate treatments (100:0, and 0:100, A1MO2_tk:5.6_s, respectively). The inocula were prepared from overnight cultures to provide a single dose at a rate of 5 x 10^5 CFU/g (method: Chapter 2, section 2.4.3).

The independent inoculation of soil with A1MO2_tk (100:0) and 5.6_s (0:100) resulted in a similar number of viable cells being isolated from the microcosms one dpi, with average CFU counts (n = 3) of 5.80 x 10^5 CFU/g for A1MO2_tk and 5.07 x 10^5 CFU/g for 5.6_s (Fig. 5.3. A - B). Both strains decreased in CFU at a steady rate over the duration of the 56-day assay, decreasing to final counts of 1.60 x 10^4 CFU/g for A1MO2_tk and 9.00 x 10^4 CFU/g for 5.6_s at 56 dpi. A lack of significant difference between these final CFU counts suggested a similar degree of persistence for the strains in the microcosms, which was confirmed from the multiple regression models fitted to $\log_{10}$ transformed CFU data (Table 5.2), which showed no significant difference between the rates of CFU change for the strains when inoculated separately.

The inoculation of soil microcosms with the mixed inocula resulted in a similar outcome, where the CFU counts for both strains decreased at a steady rate over the assay duration, with no significant difference in the rate of CFU change between A1MO2_tk and 5.6_s in each of the mixed ratio treatments, and no difference between the AUC measurements (Fig. 5.3. C - E). The results suggest a co-existence between the strains in the soil over the assay duration. Additionally, no instances of pADAP horizontal transfer from A1MO2_tk to 5.6_s were detected under the conditions assessed during this assay. Whilst no differences were recorded when assessing the raw CFU counts, the use of multiple regression models indicated that the CFU changes for A1MO2_tk in the 50:50 treatment (A1MO2_tk:5.6_s) were significant ($p < 0.05$) (Table 5.2).

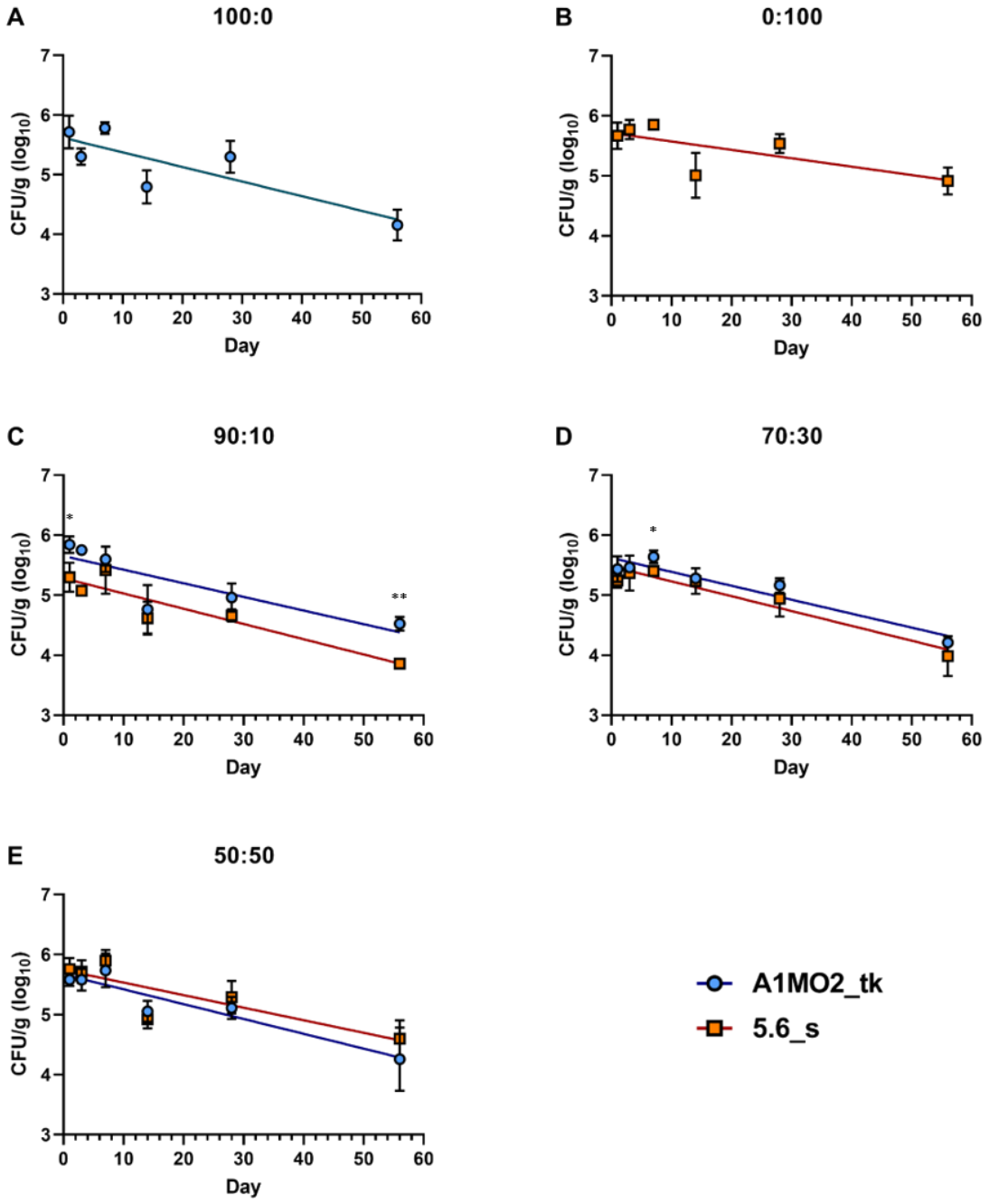


Figure 5.3. The changes in average log_{10} CFU/g (n = 3) over 56 days or A1MO2_tk and 5.6_s when (A, B) inoculated separately in soil microcosms and together at mixed ratios of (C) 90:10, (D) 70:30, and (E) 50:50 (A1MO2_tk:5.6_S) at an initial rate of 5 x 10^5 CFU/g. CFU data are presented with standard error bars and fitted linear regression lines for the strains. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. The CFU data is available in Appendix D.1.

Table 5.2. The multple linear regression models for changes in log_{10} CFU/g per week for A1MO2_tk (100:0) and 5.6_s (0:100) when inoculated in soil separately and together in mixed ratios (90:10, 70:30, 50:50, A1MO2_tk:5.6_s). Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with five degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.52	-36.367	26.960	0.270	3.68	0.156
0:100	5.6_s		12.838	41.193	0.776		
90:10	A1MO2_tk	0.78	-0.825	19.195	0.968	9.66	0.049
	5.6_s		-33.349	18.762	0.174		
70:30	A1MO2_tk	0.88	0.325	44.581	0.995	19.71	0.019
	5.6_s		-37.927	42.330	0.436		
50:50	A1MO2_tk	0.92	-67.258	16.791	0.028	31.29	0.010
	5.6_s		33.836	17.637	0.151		

During the assay it was noted that both A1MO2_tk and 5.6_s in the mixed cultures exhibited a similar level of persistence within the soil microcosms. This is also reflected in the changes of proportions for the strains isolated from the soil samples (Fig. 5.4). Despite fluctuations in proportion during the assay, when comparing the cells isolated at one dpi against the cells isolated at 56 dpi in the mixed ratio treatments there is little difference (± 5 %). The proportions also suggest the potential occurrence of competition between the strains prior to sampling at one dpi, due to the difference from the inoculated ratios. This is most clear in the 50:50 treatment (Fig. 5.4. C), with an initial proportion of 39:61 (A1MO2_tk:5.6_s) at one dpi, when the strains were inoculated at a 50:50 proportion.

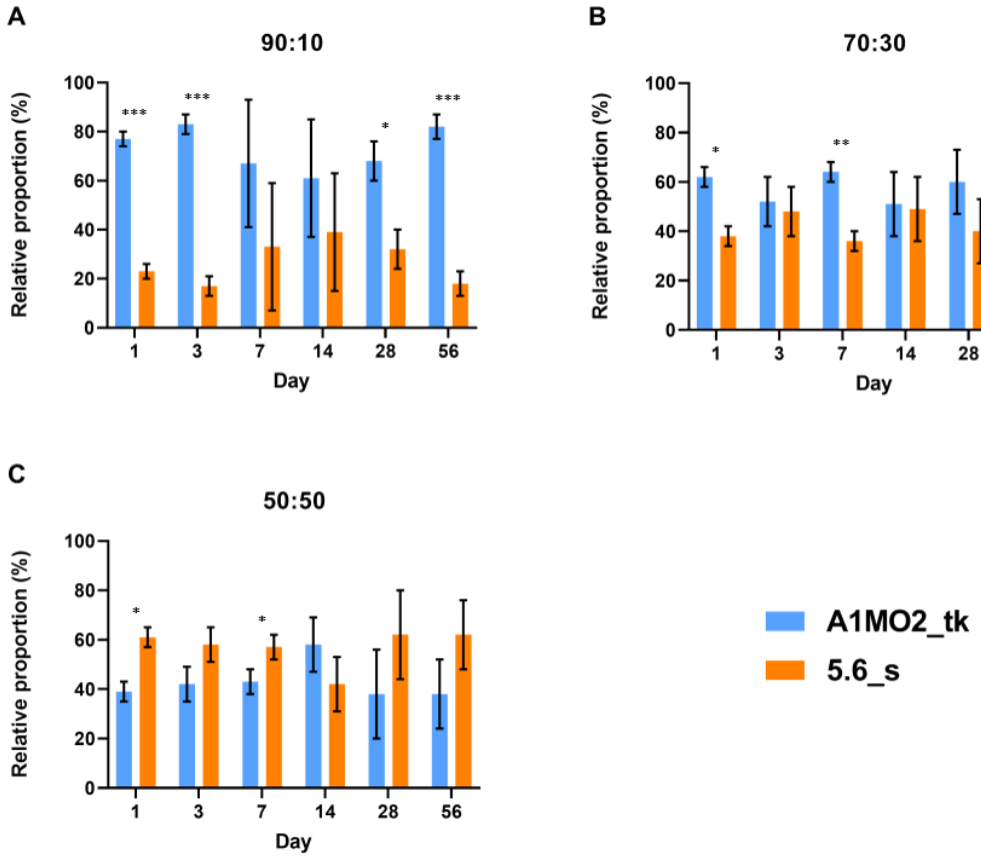


Figure 5.4. Changes in average proportion (%) (n = 3) for A1MO2_tk and 5.6_s cells isolated from soil microcosms over 56 days when inoculated at (A) 90:10, (B) 70:30, and (C) 50:50 ratios (A1MO2_tk:5.6_s) at an initial rate of 5 x 10^5 CFU/g. Data is presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

5.2.4 The persistence of tagged *Serratia* spp. inoculated at mixed ratios in soil microcosms containing a single grass grub larva

Further assessments were conducted using the mixed ratio A1MO2_tk and 5.6_s treatments to inoculate soil microcosms containing a single healthy grass grub larva. This would determine whether the presence of the larva in the soil has any impact on the dynamics of the strains in the soil. The soil microcosms were prepared using the same method as the soil-only microcosms (Section 5.2.3), using inocula produced from the same overnight cultures at the same rate (5 x 10^5 CFU/g). The same treatments were used, to assess the persistence of A1MO2_tk and 5.6_s when inoculated independently (100:0 and 0:100, respectively), and both strains together focussing on A1MO2_tk (90:10, 70:30, 50:50, A1MO2_tk:5.6_s). The larvae used were screened via an assessment of feeding behaviour to ensure the lack of any latent amber disease infections (method: Chapter 2, section 2.5.2), with a single healthy larva being added to each microcosm. Fresh carrot cube pieces were also added to each of the microcosms every three days, alongside the addition of 500 µL of water.

The separate inoculation of soil microcosms with A1MO2_tk (100:0) and 5.6_s (0:100) resulted in a continuous and steady decrease in CFU in the soil over the 56-day assay duration, as was seen previously in the soil-only microcosms, with no significant difference between the strains in the rate of CFU decrease. A similar number of cells were isolated in each of the treatments after one dpi, with an average CFU count (n = 5) of 7.24 x 10^5 CFU/g for A1MO2_tk and 1.06 x 10^6 CFU/g for 5.6_s, with no significant differences between the strains (Fig. 5.5. A - B). By the end of the assay at 56 dpi the CFU counts had decreased to 3.05 x 10^4 CFU/g for A1MO2_tk, and 2.65 x 10^4 CFU/g for 5.6_s. The multiple regression models derived from the CFU data show a similar rate of daily CFU decreases for both A1MO2_tk and 5.6_s when inoculated separately (Table 5.3), indicating a similar persistence for the strains in the absence of competition.

The inoculation of soil with the mixed ratio treatments resulted in a similar trend, in which both strains appear to decrease in CFU at a similar rate over the 56-day duration of the assay. Furthermore, despite A1MO2_tk attaining significantly higher CFU counts than 5.6_s post-inoculation ($p < 0.05$) in the 90:10 and 70:30 treatments (A1MO2_tk:5.6_s), due to the ratios at which the strains were inoculated, by the end of the assay at 56 dpi there was no longer a significant difference in CFU counts (Fig. 5.5 C - D). This suggested that whilst a similar persistence was recorded for both strains, the rate of CFU change for 5.6_s was slightly lower in the 90:10 treatment, which is indicated in Table 5.3. However, further comparisons between the rates of CFU change in the 70:30 treatment showed a significantly slower rate of CFU decrease for 5.6_s when compared to A1MO2_tk ($p < 0.05$). Similarly, in the 50:50 treatment, despite no significant differences being identified between the CFU counts over the duration of the assay, the rate of CFU change between the strains was close to being statistically significant ($p = 0.06$), suggesting that over an extended duration any differences in the rate of change would become more apparent. When fitted to multiple regression models the daily CFU change for A1MO2_tk was found to be significant in each of the mixed ratio treatments ($p < 0.05$) (Table 5.3), whereas 5.6_s was not, indicating a higher persistence of 5.6_s. Additionally, in the 90:10 treatment, A1MO2_tk had a significantly higher AUC measurement than 5.6_s ($p < 0.05$), though given the proportions of the strain in the inoculant this was to be expected.

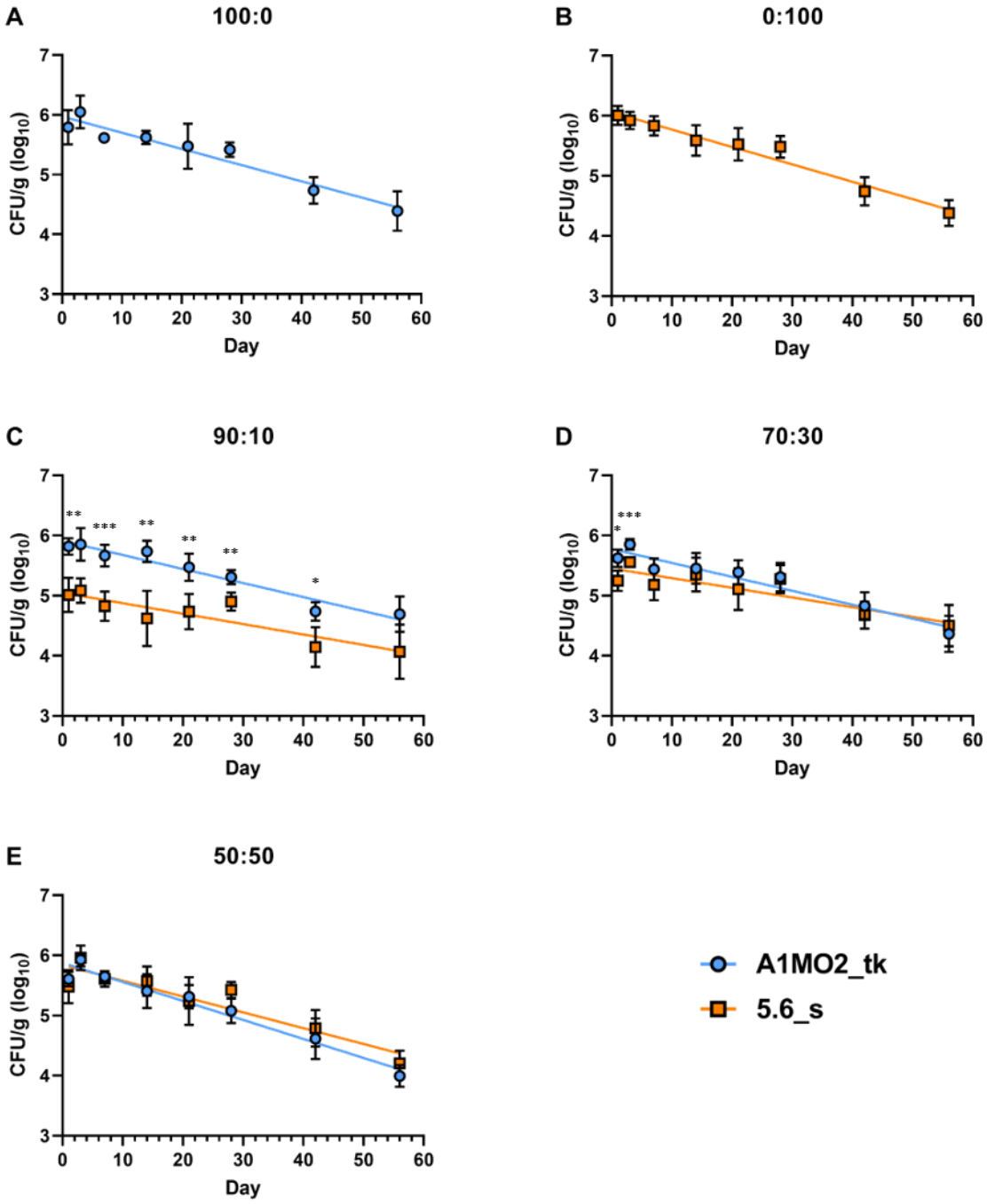


Figure 5.5. The changes in average log_{10} CFU/g (n = 5) over 56 days or A1MO2_tk and 5.6_s when (A, B) inoculated separately in soil microcosms and together at mixed ratios of (C) 90:10, (D) 70:30, and (E) 50:50 (A1MO2_tk:5.6_S) at an initial rate of 5 x 10^5 CFU/g. Each microcosm contained a single healthy grass grub larva. CFU data are presented with standard error bars and fitted linear regression lines for the strains. * p < 0.05, ** p < 0.005, *** p < 0.0005. The CFU data is available in Appendix D.2.

Table 5.3. The multiple linear regression models for changes in log_{10} CFU/g per week for A1MO2_tk (100:0) and 5.6_s (0:100) when inoculated separately in microcosms containing a single healthy grass grub larva, and together in mixed ratios (90:10, 70:30, 50:50, A1MO2_tk:5.6_s) in microcosms containing larvae. Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with seven degrees of freedom

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.95	-4.351	14.465	0.776	71.04	0.001
0:100	5.6_s		-29.161	13.779	0.088		
90:10	A1MO2_tk	0.93	-36.591	9.918	0.014	44.38	0.001
	5.6_s		-5.840	12.160	0.651		
70:30	A1MO2_tk	0.90	-55.623	17.026	0.022	34.19	0.001
	5.6_s		21.948	22.736	0.379		
50:50	A1MO2_tk	0.86	-46.663	18.107	0.050	22.98	0.003
	5.6_s		26.789	19.723	0.232		

A comparison between the relative proportion of the cells isolated from the microcosms containing a single grass grub larva in each of the mixed ratio treatments indicates an increased competitive ability of 5.6_s relative to A1MO2_tk. This was most obvious in the 70:30 and 50:50 treatments (A1MO2_tk:5.6_s). In the 90:10 mixed ratio treatment (A1MO2_tk:5.6_s) (Fig. 5.6. A) there was very little change in the proportions of bacteria over the 56-day assay duration, with 5.6_s showing a slight increase at 56 dpi. However, as previously mentioned, for this treatment specifically there was less difference between the trends depicted by the linear regression equations. In both the 70:30 and 50:50 treatments (Fig. 5.6. B - C) the proportion of 5.6_s cells isolated from the soil increased over the assay duration. This also suggests that the presence of the larva in the soil may have contributed to the increased competitiveness of 5.6_s, as an increase in the proportion of 5.6_s was not recorded in the soil-only microcosms, despite the treatments being prepared from the same inocula. For instance, despite the cells starting with a proportion of 55:46 (A1MO2_tk:5.6_s) one dpi in the 50:50 treatment, by 56 dpi the proportions had changed to 32:68, favouring 5.6_s.

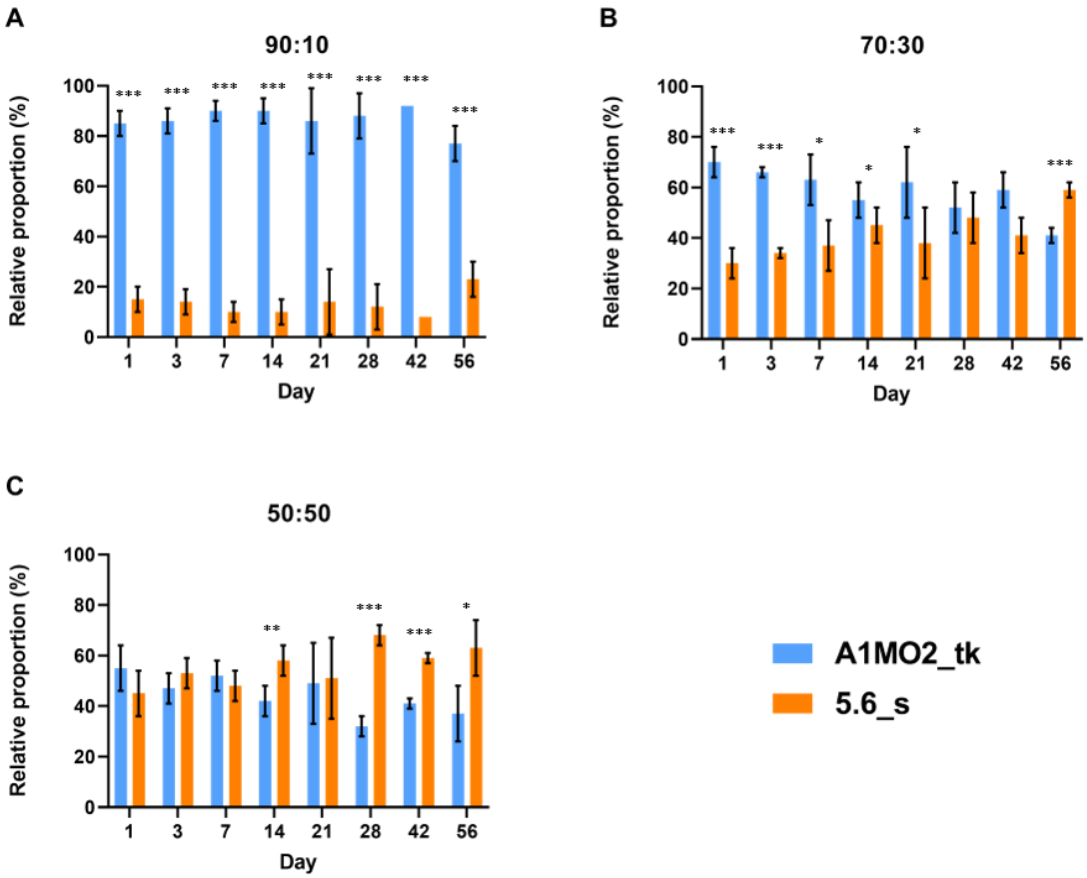


Figure 5.6. Changes in average proportion (%) (n = 3) for A1MO2_tk and 5.6_s cells isolated from soil microcosms containing a single grass grub larva over 56 days when inoculated at (A) 90:10, (B) 70:30, and (C) 50:50 ratios (A1MO2_tk:5.6_s) at an initial rate of 5 x 10^5 CFU/g. Data is presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Upon the conclusion of the 56-assay period the larvae contained in the microcosms sampled at 56 dpi were macerated and assessed to determine whether any of the A1MO2_tk or 5.6_s cells used to inoculate the soil were present within the macerate samples. However, the plating of the whole-macerate samples from three larvae in each treatment group (15 total) on both LB at CTA media did not produce any colonies on the agar. Despite the macerate samples being diluted at a low rate (plating 50 µL of a 1:10 dilution) any cells in the macerate samples may have been below detection limits (~ 10^2 CFU/mL). A similar factor was encountered during *in vivo* assessments, where the direct inoculation of larvae did not yield countable cells, likely to being below detection limits (Chapter 6, section 6.2.4). However, some of the larvae assessed (those in the 5.6-only control group, and one larva in each of the 50:50 and 70:30 groups) still exhibited a dark gut, suggesting the larvae were still healthy and therefore not infected, thus unlikely to harbour any of the inoculated strains. Furthermore, over the 56-day assay duration, six of the microcosm replicates out of the 200 total replicates assessed did not yield any viable cells on either LB agar infused with antibiotics (Spec, Tet, or Kan) and CTA media, which

may have also been due to any viable cells being below detection limits. These replicates are highlighted in the supplemental CFU data (Appendix D.2). Horizontal transfer of pADAP between A1MO2_tk and 5.6_s in these microcosms was not detected under the conditions assessed during the direct plating of soil samples on LB agar containing the appropriate antibiotics for transconjugant cells (kanamycin and spectinomycin).

5.2.5 Detection of *Serratia* cells released from larval cadavers into the soil

Another aspect of this project was the release of pathogenic and non-pathogenic *Serratia* cells into the soil following death and decomposition of larvae infected with amber disease. Evidence of this would suggest a potential long-term survival method for *Serratia* cells, escaping competition with other microorganisms in the rhizosphere through persistence within the larval cadaver. However, despite multiple attempts to isolate *Serratia* cells from the soil (methods: Chapter 2, section 2.4.6) following death of larvae three months after being dosed with an inoculant of 5 x 10^5 CFU/mL, no inoculated tagged *Serratia* cells were isolated. Although non-*Serratia* bacteria species were present within the microcosms, indicated by biofilm formation on top of the liquid medium in the tubes used to contain the soil samples, neither these species nor the inoculated *Serratia* spp. were isolated following plating on CTA (semi-selective) and LB agar containing antibiotics appropriate for the tagged *Serratia* strains (Spec, Tet, and Kan). The lack of detection might suggest that the strains were below the detection limits of the assay (10^3 CFU/g). Furthermore, the potential loss of the antibiotic markers in the *Serratia* strains might have prevented growth on the antibiotic-infused LB agar plates. The microcosms assessed had a moisture content of ~ 15 % (w/v) following incubation at 15 °C.

5.3 Discussion

The results obtained during these assays showed that when inoculated into soil using a liquid drench equivalent to a recommended field-level rate of 5 x 10^5 CFU/g of air-dried soil, wild-type A1MO2, 5.6, and AGR96X populations all decreased at a similar rate. This decrease in CFUs over time conforms with what is already known about the dynamics of the strains in the soil from previous work (O'Callaghan and Gerard, 2005). It was initially hypothesised that 5.6, as a non-pathogenic *Serratia* strain, would show an increased persistence within the soil, as the non-pathogenic *Serratia* strains were thought to be better competitors due to a lack of pADAP (Godfrey et al., 1999). However, this initial lack of difference in persistence between wild-type 5.6 and A1MO2 in the first soil assessment may have stemmed from 5.6 being a heat-cured pADAP-free variant of A1MO2, and thus might carry different adaptations compared to other natural non-pathogenic *Serratia* strains, such as 477. Therefore, further testing would be required to determine the differences in persistence between wild-type A1MO2 and a naturally occurring non-pathogenic *Serratia* strain such as 477 in the soil. Similar to the RNA sequencing assessments, the soil *in vitro* experiments were conducted primarily between A1MO2 and 5.6. This is because some non-pathogenic *Serratia* strains, such as 477 or 626, are found in certain regions, and thus may only represent competition in a field scenario, whilst the use of 5.6 allows for further exploration of the nature of competition between isogenic strains such as A1MO2 and 5.6.

During the assessment of *Serratia* competition dynamics using the single mixed ratio inocula, in Sections 5.2.3 and 5.2.4, it was found that mixed inoculation of both A1MO2 and 5.6 in soil-only microcosms resulted in a similar level of persistence for the strains, similar to the earlier growth of the wild-type strains when grown separately. However, the addition of a single grass grub larva to the microcosms resulted in an increase in competitiveness for 5.6, as indicated by the increases in the proportion of 5.6 during the latter stages of the assay. Additionally, the rate of CFU decrease for 5.6 in the 70:30 treatment (A1MO2:5.6) was found to be significantly slower than A1MO2, also suggesting an increased persistence of 5.6 within the soil when grown alongside A1MO2 in the presence of larva. The increased persistence of 5.6 was shown to occur later in the assay in the 50:50 treatment, between 21 and 28 dpi, though it is unclear how the larvae contributed to this dynamic. A comparison between the proportions of A1MO2 and 5.6 in the soil-only and microcosms containing a healthy larva is depicted in Figure 5.7.

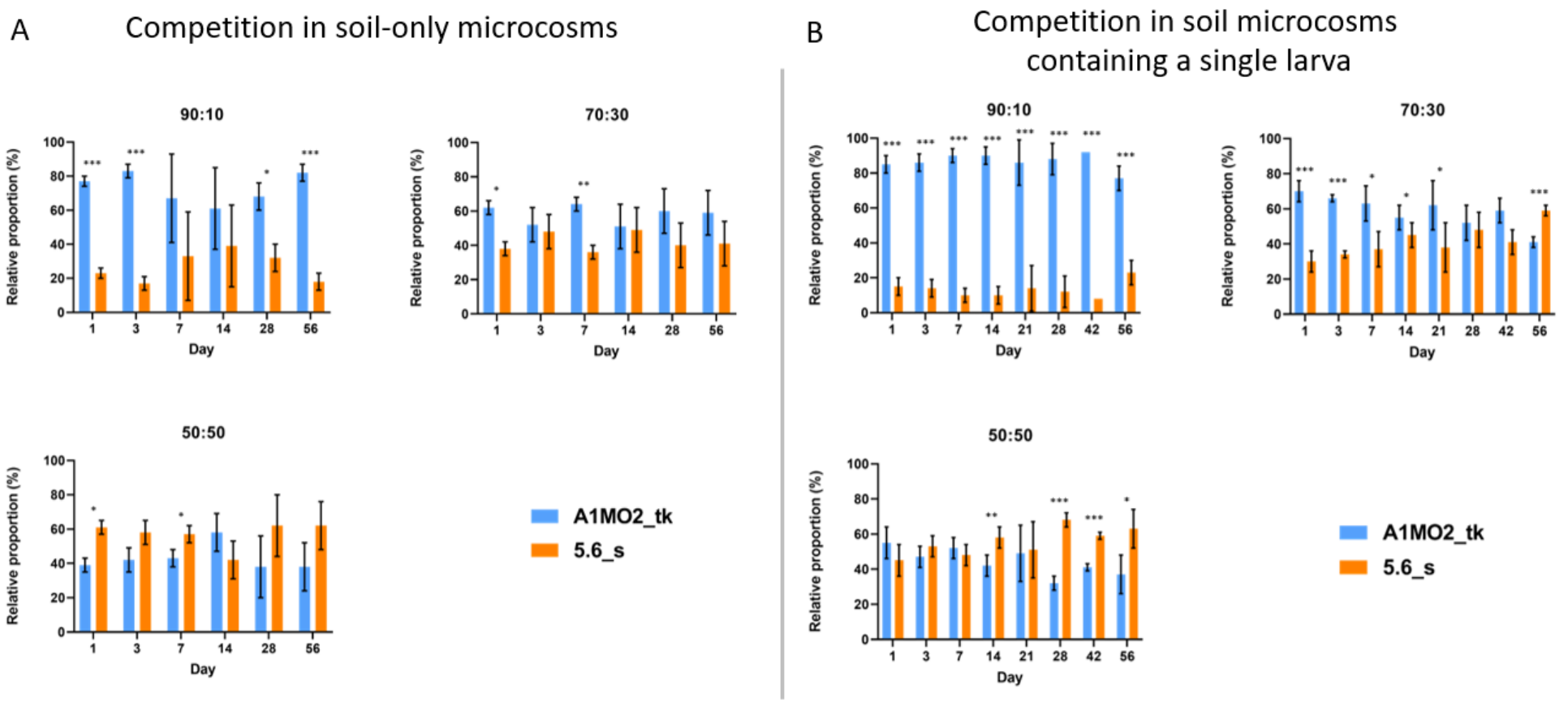


Figure 5.7. A comparison between the changes in the average relative proportion over a 56-day period of A1MO2_tk and 5.6_s isolated from either (A) 20 g soil-only microcosms (n = 3) or (B) 20 g soil microcosms containing a single healthy grass grub larva (n = 5). Ratios represent the proportion of A1MO2_tk and 5.6_s in the initial inocula (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

There were six total instances in which the tagged *Serratia* cells inoculated into the soil microcosms were not isolated during the whole-sampling of the soil. This may have been due to the number of cells within the soil being too low for the detection limits of the assays (~ 10^{2-3} CFU/g). The microcosms from which cells were not isolated were sampled between 28 and 56 dpi, where the CFU counts had decreased to a range between ~ 10^5 and 10^3 CFU/g. Similarly, following the conclusion of the microcosm experiment involving the use of grass grub larvae, no viable *Serratia* cells were isolated when whole-larval macerate samples were spread across both LB agar (with Spec, Tet, or Kan), or CTA medium at a low dilution (50 µL of a 1:10 serial dilution). This may have stemmed from the behaviour of the larvae in the microcosms. It was noted that some of the larvae would burrow to the bottom of the microcosm and stay there through the duration of the experiment, not feeding on the fresh carrot cube pieces added every three days. In such an event the larvae might not have a sufficient dose of the pathogen inoculant applied to the top of the soil as a liquid drench, and thus would have remained healthy; this may have also allowed the larvae to enter a pre-pupa stage. Whilst no pupae were recovered from the soil during the experiment, when in the pre-pupa stage the larvae take on an amber discolouration due to a build-up of fat, and do not feed as much as a healthy larvae, giving the impression of amber disease occurrence.

No horizontal transfer (HGT) of pADAP between A1MO2 and 5.6 was detected in these assays. Previous work by Dodd (2003) has shown that whilst HGT did not occur at a detectable level in natural soil under the conditions tested, the inoculation of soil containing a single larva with a 10^4 CFU/g dose did enable HGT, yielding a maximum of 1 x 10^2 CFU/g transconjugant cells, with the detection of transconjugant cells occurring at seven and 21 dpi. These results also found that the rate of transconjugant cells detection was close to the detection limit for the assay. As such, based on the absence of detected HGT in the soil microcosms in this study it is plausible that it occurred at a rate below detection limits when the samples were plated on LB agar containing the appropriate antibiotics. Furthermore, the previous iterations of *Serratia* HGT detection in soil microcosms by Dodd (2003) were conducted using inocula at a rate of 10^7 CFU/g of soil, in comparison to the 10^5 CFU/g of soil as used in this study, where an increased dose rate would be more favourable to cell-to-cell contact.

The results obtained indicated that when in the soil in the presence of grass grub larvae, 5.6 can display increased competitive capabilities when compared to A1MO2, which aligns with a previous model of *Serratia* interactions by Godfray et al. (1999). This suggested an increased competitiveness for the plasmid-free *Serratia* strain in the soil, particularly with 5.6, a strain that was constructed to be a plasmid-free variant of a pathogenic *Serratia* strain. The interactions between these species, and between A1MO2 and 477, a naturally occurring non-pathogenic *S. entomophila* isolate, are explored further using a pot trial, with the results presented in Chapter 7.

Chapter 6

Competition between *Serratia* spp. within larvae

6.1 Introduction

Following the assessment of interactions between pathogenic and non-pathogenic *Serratia* strains in both liquid growth media and natural soil microcosms, the next aspect to be explored was the interactions between the strains within the larvae. The results generated during previous inoculation experiments showed that 5.6 had a competitive advantage when inoculated alongside A1MO2, primarily in a faster doubling time during exponential growth (Chapter 3, section 3.2.3.a) and increased persistence over a seven-day period (Chapter 3, section 3.2.4.a). As such, an investigation of these interactions within the larvae will determine whether these dynamics could influence the progression of amber disease infections within the larvae.

The metabolic burdens associated with virulence factor production are well documented, but it has also been speculated that competition may select for reduced pathogenicity by discouraging the production of virulence factors (Brown et al., 2002). Such an outcome might apply to A1MO2 and its production of Afp and Sep toxins, the loss of which would reduce the efficacy of the strain as a pathogen. This was determined through bioassays by challenging larvae with mixed ratio inocula consisting of tagged A1MO2 and 5.6 construct strains (Chapter 2, section 2.2.16), to determine whether the presence of the non-pathogenic *S. entomophila* strain 5.6 reduces amber disease onset and disease rates. Previous work by Raymond et al. (2007) using mixed strains of pathogenic and non-pathogenic *Bacillus* spp. inocula to challenge diamondback moth larvae (*Plutella xylostella*) showed that the pathogenic isolates were outcompeted *in vivo* where the non-pathogenic isolates displayed a higher growth rate.

In addition to interactions within larvae, *in vivo* experimentation allows for comparisons to be made regarding the impact of pre-dosing larvae with non-pathogenic *Serratia* strains, and how this might impact the efficacy of A1MO2, potentially reducing disease rates. Variable pathogenicity has also been reported in other wild-type pathogenic *Serratia* isolates, such as *S. proteamaculans* isolate 143, which only causes disease in 60 to 70 % of inoculated larvae, regardless of the inocula amount (Glare et al., 1993b). This reduction in efficacy may potentially be the result of competition between isolate 143 and other microbial species within the larvae.

An investigation into competition within larvae also provided an opportunity to detect the occurrence and rate of horizontal transfer of pADAP between A1MO2 and 5.6 *in vivo*. HGT has been documented *in vivo* previously with multiple bacterial species (van Reenen and Dicks, 2010; Stecher et al., 2012;

McCarthy et al., 2014), including between *Serratia* spp. (Dodd, 2003), and can sometimes be advantageous for bacterial fitness through the horizontal transfer of traits such as antibiotic resistance (Ochman et al., 2000). Additionally, an assessment of HGT using tagged *S. entomophila* and *S. proteamaculans* strains will determine the rate at which *in vivo* pADAP transfer can occur between different *Serratia* species.

6.2 Results

6.2.1 The impact of mixed ratio inoculation with A1MO2 and 5.6 on amber disease efficacy

An initial bioassay was conducted to determine how the cell density of the A1MO2 and 5.6 in a mixed inoculant would impact on amber disease progression and disease rates. Using a standard bioassay method (method: Chapter 2, section 2.5.2) larvae were challenged with both separate inocula for A1MO2 (100:0) and 5.6 (0:100), and mixed ratio inocula (90:10, 70:30, 50:50, 30:70, 10:90, A1MO2:5.6) (Chapter 2, section 2.5.3). The 5 µL inocula were prepared from overnight cultures to produce a rate of 6.76 x 10^6 CFU/mL for A1MO2 and 6.31 x 10^6 CFU/mL for 5.6. An additional control group of uninoculated larvae (0:0) were also assessed.

Challenging larvae (n = 12) using the 100 % A1MO2 single strain inoculant (100:0) resulted in expected amber disease progression, with disease first occurring four dpi in two larvae (17 %) (Fig. 6.1). These diseased larvae displayed both an amber discolouration in the gut and a lack of feeding behaviour. Over the bioassay duration the number of diseased larvae increased, with seven larvae (83 %) showing symptoms of amber disease at the end of the bioassay 12 dpi. A similar progression in disease was recorded in the larvae challenged with the 90:10 inoculant, with disease first emerging in two larvae (20 %) at four dpi, progressing to seven diseased larvae (70 %) at 12 dpi. However, a reduction in the rate of disease was noted in the larvae challenged with the remaining mixed ratio inocula.

A lower incidence of disease was seen as the proportion of A1MO2 within the inoculant decreased. In addition, a delay in the onset of amber disease was also noted as the proportion of 5.6_s in the inoculant increased (Fig. 6.1); this was evident when comparing disease rates and onset between the 70:30, 50:50, and 30:70 inocula to the 100:0 and 90:10 inocula. Following challenge with the 70:30 inocula amber disease was first recorded four dpi in two larvae (18 %), however, challenge with the 50:50 inoculant resulted in disease in only one larva (9 %) at five dpi, and challenge with the 30:70 inoculant first resulted in disease in three larvae (27 %) at seven dpi. By the end of the bioassay a disease incidence between 20 and 33 % was observed for these inocula, compared to the 70 to 80 % in the 100:90 and 90:10 inocula. The lowest amount of disease was recorded in the larvae challenged

with 10:90 inoculant, with disease first occurring at seven dpi in one larva (9 %), and no further larvae becoming infected by 12 dpi.

The challenge of larvae with the 100 % 5.6 single strain inoculant (0:100) also resulted in a single larva (9 %) becoming diseased at seven dpi, with no additional larvae showing signs of disease for the remainder of the bioassay. All of the uninoculated control larvae remained healthy over the duration of the bioassay. An overall mortality rate of 15 % was recorded for all larvae used in this bioassay amongst the different treatments (Fig. 6.2), with a single death occurring in the uninoculated control larvae, and two deaths in the larvae challenged with the 0:100 inoculant. The remainder of the deaths were distributed between the larvae challenged with the 100:0, 90:10, 70:30, and 50:50 inocula.

When comparing disease incidence in the mixed ratio treatments against the A1MO2-only group (100:0) at day 14 of the bioassay a significant reduction in disease is noted in the larvae challenged with the 10:90 and the 50:50 treatments (A1MO2:5.6) ($p < 0.05$) (Table 6.1). A significant reduction in amber disease incidence was also noted between the A1MO2-only and 5.6-only groups ($p < 0.05$), which was expected.

The lower dosage of A1MO2 in the 10:90 treatment group, alongside the lower rate of disease (8 %), might suggest a typical dose-dependent response in which disease incidence decreased, and time until symptoms emerged increased, relative to the dose of A1MO2. Previous work (Jackson et al., 2001; Vaughan, 2021) places the minimum rate of a pathogenic *Serratia* spp. required to cause disease in 50 % of challenged larvae between 10^4 and 10^5 CFU/mL. During this bioassay the minimum rate at which A1MO2 was used was 6 x 10^4 CFU/mL (in the 10:90 treatment), which, based on the dose-response curve by Jackson et al. (2001) would have caused disease in approximately 50 % of challenged larvae. Using the same response curve, the rate of A1MO2 used in the A1MO2-only treatment (100:0), approximately 4 x 10^5 CFU/mL, would have been sufficient to cause disease in 60 % of challenged larvae, with a disease occurrence of 83 % being noted in the treatment. Furthermore, in a preliminary version of the pre-inoculation bioassay (Section 6.2.3), a 5 µL dose of A1MO2 at a rate of ~ 5 x 10^4 CFU/mL was sufficient to cause disease within 75 % of challenged larvae.

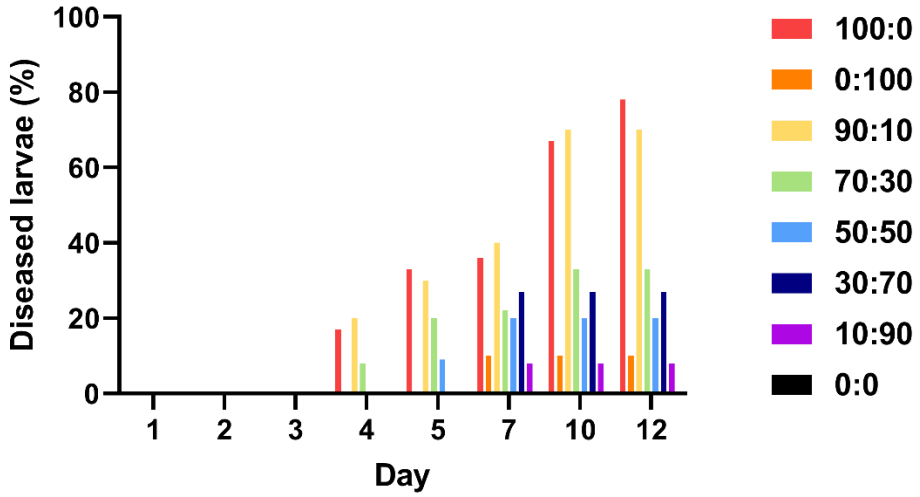


Figure 6.1. The changes in proportion of diseased larvae (n = 12) over 12 days following challenge with mixed ratio inocula of A1MO2 and 5.6 at a rate of 6.76 x 10^6 CFU/mL for A1MO2 and 6.31 x 10^6 CFU/mL for 5.6. Ratios given represented as A1MO2:5.6. A 0:0 ratio indicates no inoculant was used.

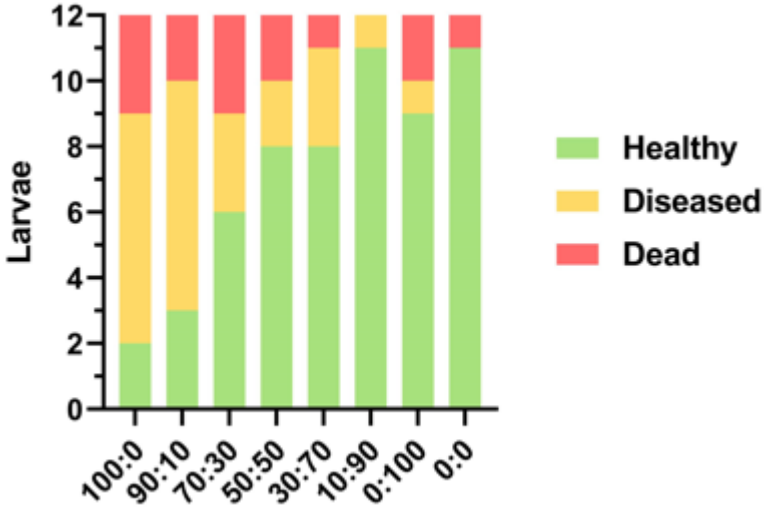


Figure 6.2. The conditions of larvae (n = 12) after 12 days following challenge with mixed ratio inocula of both A1MO2 and 5.6 at a rate of 6.76 x 10^6 CFU/mL for A1MO2 and 6.31 x 10^6 CFU/mL for 5.6. Ratios given represented as A1MO2:5.6. A 0:0 ratio indicates no inoculant was used. Disease was classified as amber discolouration and feeding cessation.

Table 6.1. Group-wise logistic regression model results comparing amber disease incidence at 14 days in grass grub larvae challenged with A1MO2 (100:0) against larvae challenged with a mixed ratio of A1MO2 and 5.6. Treatment ratios representative of A1MO2:5.6.

Treatment	Coefficient	Coefficient SE	95 % CI	Z-value	*P*-value
100:0	0.336	0.586	(-0.811, 1.484)	0.57	0.566
90:10	0.000	0.828	(-1.623, 1.623)	0.00	1.000
70:30	-1.435	0.887	(-3.174, 0.304)	-1.62	0.106
50:50	-1.946	0.971	(-3.849, -0.043)	-2.00	0.045
30:70	-1.435	0.887	(-3.174, 0.304)	-1.62	0.106
10:90	-2.73	1.20	(-5.08, -0.39)	-2.28	0.022
0:100	-2.73	1.20	(-5.08, -0.39)	-2.28	0.022

6.2.2 The impact of mixed ratio inoculation with A1MO2 and 477 on amber disease efficacy

The impact of a mixed inoculant consisting of A1MO2 and 477 was also assessed for how it might impact amber disease progression in larvae, and how the results compared to mixed inoculation of A1MO2 and 5.6. This allowed for further comparisons to be made between A1MO2 and 477, a non-pathogenic wild-type *S. entomophila* strain non-isogenic to A1MO2. Larvae were challenged with separate inocula of A1MO2 (100:0) and 477 (0:100), as well as the same array of mixed ratio inocula (90:10, 70:30, 50:50, 30:70, 10:90, A1MO2:477) used previously in Chapter 6.2.1. The 5 µL inocula were prepared from overnight cultures to produce a dose rate of 6.76 x 10^6 CFU/mL for A1MO2 and 5.62 x 10^6 CFU/mL for 477. An additional control group of uninoculated larvae (0:0) were also assessed.

The challenging of larvae (n = 12) with the 100:0 A1MO2 single strain inoculant first resulted the onset of amber disease in one larva (8 %) at three dpi, progressing to four larvae (33 %) four dpi (Fig. 6.3). The number of diseased larvae in this treatment continued to increase over the duration of the bioassay, resulting in seven larvae (58 %) becoming diseased at 12 dpi. A similar rate and progression of disease was also observed in the larvae challenged with the 90:10 and 50:50 inocula (A1MO2:477), with amber disease first occurring four dpi. Over the duration of the assay seven larvae (67 %) challenged with the 90:10 inoculant became diseased, and six larvae (64 %) challenged with the 50:50 inoculant became diseased.

A lower disease rate was seen between the larvae challenged with the 70:30, 30:70, and 10:90 inocula (A1MO2:477, respectively), where only two to three larvae had become diseased 12 dpi (18 – 27 %). In addition, increasing the proportion of 477 within the inoculant did not delay disease onset, as diseased larvae were first recorded four dpi following challenge with the 10:90 inoculant. Only one

diseased larva (9 %) was observed at ten dpi following challenge with the 0:100 477 single strain inoculant. All of the uninoculated control larvae remained healthy and continued to feed over the duration of the bioassay. Furthermore, a low mortality rate of 7 % was recorded amongst the challenged larvae, with most of the deaths occurring in the larvae challenged with the 30:70 inoculant (Fig. 6.4). A single larva challenged with the 0:100 477 single strain inoculant died, and none of the uninoculated larvae died during the bioassay.

When comparing the incidence of amber disease amongst the larvae at day 14 of the assay, a significant reduction in disease was only recorded in the 10:90 mixed ratio treatment group (A1MO2:477) ($p < 0.05$) (Table 6.2).

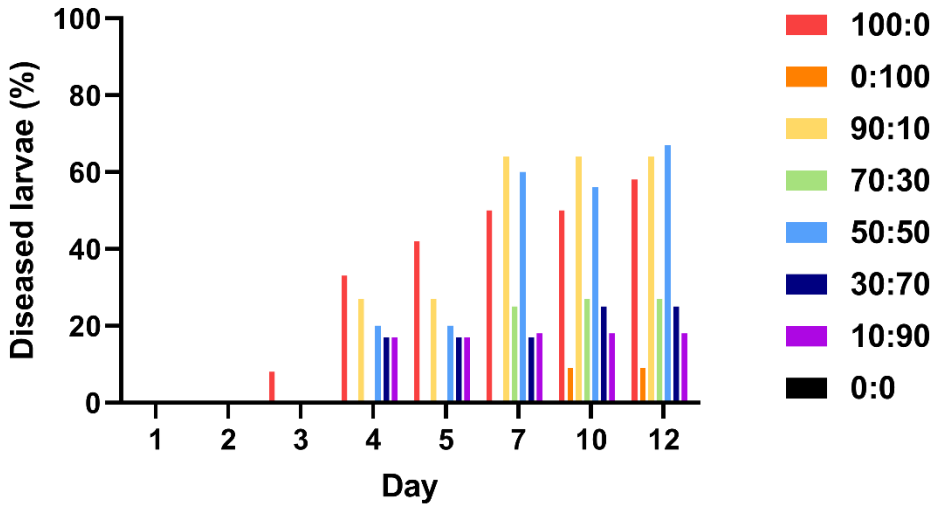


Figure 6.3. The changes in proportion of diseased larvae (n = 12) over 12 days following challenge with mixed ratio inocula of A1MO2 and 477 at a rate of 6.76 x 10^6 CFU/mL for A1MO2 and 5.62 x 10^6 CFU/mL for 477. Ratios given represented as A1MO2:477. A 0:0 ratio indicates no inoculant was used.

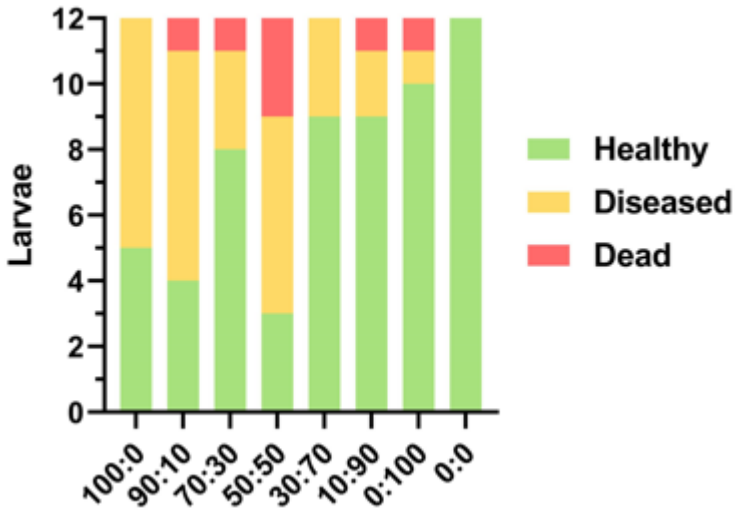


Figure 6.4. The conditions of larvae (n = 12) after 12 days following challenge with mixed ratio inocula of both A1MO2 and 477 at a rate of 6.76 x 10^6 CFU/mL for A1MO2 and 5.62 x 10^6 CFU/mL for 477. Ratios given represented as A1MO2:477. A 0:0 ratio indicates no inoculant was used. Disease was classified as amber discolouration and feeding cessation.

Table 6.2. Group-wise logistic regression model results comparing amber disease incidence at 14 days in grass grub larvae challenged with A1MO2 (100:0) against larvae challenged with a mixed ratio of A1MO2 and 477. Treatment ratios representative of A1MO2:477.

Treatment	Coefficient	Coefficient SE	95 % CI	Z-value	*P*-value
100:0	0.336	0.586	(-0.811, 1.484)	0.57	0.566
90:10	0.000	0.828	(-1.623, 1.623)	0.00	1.000
70:30	-1.435	0.887	(-3.174, 0.304)	-1.62	0.106
50:50	-0.336	0.822	(-1.948, 1.275)	-0.41	0.682
30:70	-1.435	0.887	(-3.174, 0.304)	-1.62	0.106
10:90	-1.946	0.971	(-3.849, -0.043)	-2.00	0.045
0:100	-2.73	1.20	(-5.08, -0.39)	-2.28	0.022

Comparing the results of mixed inoculation of larvae with A1MO2 and 477 against A1MO2 and 5.6 shows that whilst reductions in amber disease incidence were reported in both bioassays, often it was not significant when compared to the A1MO2-only groups. However, in both bioassays the 10:90 ratio of the respective treatments did cause significant reduction in amber disease incidence. This outcome may have been the combination of a lower dose rate for A1MO2 relative to 477, as mentioned above with regards to A1MO2 and 5.6, and competition between the strains.

6.2.3 Assessing A1MO2 pathogenicity in larvae pre-inoculated with non-pathogenic *Serratia* spp.

The presence of non-pathogenic *Serratia* strains 5.6 and 477 led to a reduction in disease rates, and delayed the onset of amber disease, when inoculated simultaneously at mixed ratios with A1MO2. Based on these results further investigation was conducted to assess whether the pre-inoculation of larvae with the non-pathogenic strains 5.6 and 477, followed by subsequent challenge with A1MO2 would produce a similar outcome. The rates of each strain used to challenge the larvae are listed in Table 6.3.

Table 6.3. The CFU/mL count of the 5 µL application rates of 5.6 and 477 used to pre-inoculate larvae at varying rates, and the CFU/mL count of the 5 µL rate of A1MO2 used to challenge the larvae after three days.

	Applied dose rate (CFU/mL)			
Strain	**Full strength**	**10^{-1}**	**10^{-2}**	**10^{-3}**
A1MO2	-	6.61×10^6	-	-
5.6	5.89×10^7	5.89×10^6	5.89×10^5	5.89×10^4
477	7.08×10^7	7.08×10^6	7.08×10^5	7.08×10^4

- Denotes no inoculant used

Prior to the pre-inoculation of larvae with non-pathogenic strains an initial check was made to ensure that the 10^{-1} dilution of A1MO2 (effective rate of 3.31×10^5 CFU/mL at 5 µL) was still able to cause amber disease within challenged larvae (n = 12) (Fig. 6.5). A general assessment over a 14-day period showed that at four dpi, when amber disease symptoms are typically exhibited, a total of six larvae (50 %) had become infected with amber disease, progressing to 11 larvae (100 %) at 14 dpi, with a single larval death occurring.

The pre-inoculation of larvae with varying doses of 5.6 (Table 6.3), followed by subsequent challenge with the 10^{-1} dilution of A1MO2 three days later resulted in a clear delay to the onset of amber disease in the challenged larvae (Fig. 6.5). Typically, challenge with A1MO2 causes disease within three to four dpi, however, following pre-inoculation with 5.6, amber disease first occurred five dpi in the larvae pre-inoculated with the 10^{-2} and 10^{-3} dilutions of 5.6. Furthermore, amber disease was first recorded ten dpi in the larvae pre-inoculated with the full strength and 10^{-1} dilutions of 5.6, indicating that the delay in amber disease onset was proportional to the 5.6 rate, with a higher rate delaying amber disease onset for longer. By the end of the assay at 14 dpi, a total of nine larvae pre-inoculated with the full strength 5.6 rate (82 %) and the 10^{-2} 5.6 rate (75 %) were displaying symptoms of amber disease with amber discolouration and a cessation of feeding behaviour. A total of six larvae (50 %) were

displaying disease symptoms following pre-inoculation with the 10^{-1} and 10^{-3} rates of 5.6. When compared to the disease rates for the larvae challenged separately with A1MO2 this shows that in addition to delaying disease onset, pre-inoculation of larvae with 5.6 also reduced the incidence of disease.

A significant reduction in amber disease incidence was recorded in the larvae pre-inoculated with 5.6 at rates of 10^{-1} and 10^{-3} and then challenged with A1MO2 at a rate of 10^{-1} after three days when compared to larvae only challenged with A1MO2 ($p < 0.05$) (Table 6.4). Whilst a reduction in disease incidence was recorded in the 5.6 full strength and 10^{-2} treatments, this reduction was not significant.

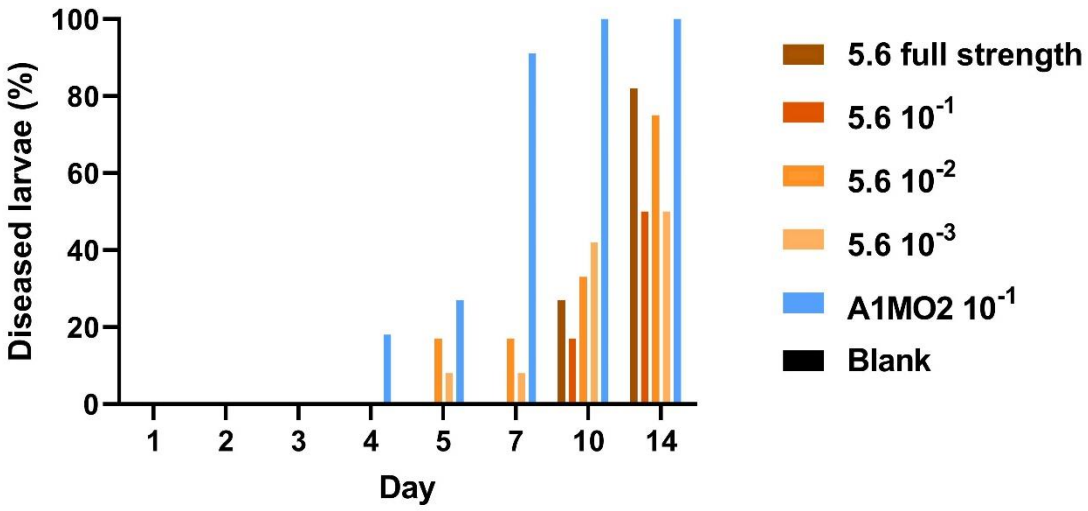


Figure 6.5. The changes in proportion for diseased larvae (n = 12) over 14 days following pre-inoculation with varying strength rates of 5.6, with a CFU/mL of 5.89 x 10^7 (5.6 full strength), 5.89 x 10^6 (5.6 10^{-1}), 5.89 x 10^5 (5.6 10^{-2}), and 5.89 x 10^4 (5.6 10^{-3}), prior to challenge with a 6.61 x 10^6 CFU/mL rate of A1MO2 after three days. An A1MO2-only control group (A1MO2 10^{-1}) was also challenged with a single 6.61 x 10^6 CFU/mL rate of A1MO2. Blank larvae were uninoculated. Disease was classified as amber discolouration and feeding cessation.

Table 6.4. Group-wise logistic regression model outputs when comparing amber disease incidence at 14 days post-inoculation in A1MO2 10^{-1} control group against larvae pre-inoculated with 5.6 prior to challenge with A1MO2.

Treatment	Coefficient	Coefficient SE	95 % CI	Z-value	*P*-value
A1MO2 (10^{-1})	2.40	1.04	(0.35, 4.45)	2.30	0.022
5.6 Full strength	-1.30	1.24	(-3.73, 1.13)	-1.05	0.294
5.6 (10^{-1})	-2.40	1.19	(-4.74, -0.06)	-2.01	0.045
5.6 (10^{-2})	-1.30	1.24	(-3.73, 1.13)	-1.05	0.294
5.6 (10^{3})	-2.40	1.19	(-4.74, -0.06)	-2.01	0.054

The pre-inoculation of healthy larvae with varying strength rates of 477 (Table 6.3) prior to challenge with a 10^{-1} dilution of A1MO2 after three days resulted in a similar dynamic (Fig. 6.6). A delay in amber disease onset was recorded following pre-inoculation with 477, however the delay was not as clear as when the larvae were pre-inoculated with 5.6, with disease first occurring at three dpi, and occurring in all treatments prior to seven dpi. In contrast, pre-inoculation of larvae with 5.6 delayed the onset of amber disease until ten dpi. Similarly, a higher incidence of disease occurred following pre-inoculation with 477 than with 5.6, with between 75 to 91 % of challenged larvae becoming diseased by 14 dpi, compared to 50 to 82 % for 5.6. The incidence of amber disease following pre-inoculation of larvae with 477 was still slightly lower than when larvae were challenged separately with A1MO2. However, a significant reduction in disease incidence was only recorded in the 477 full strength treatment group ($p < 0.05$).

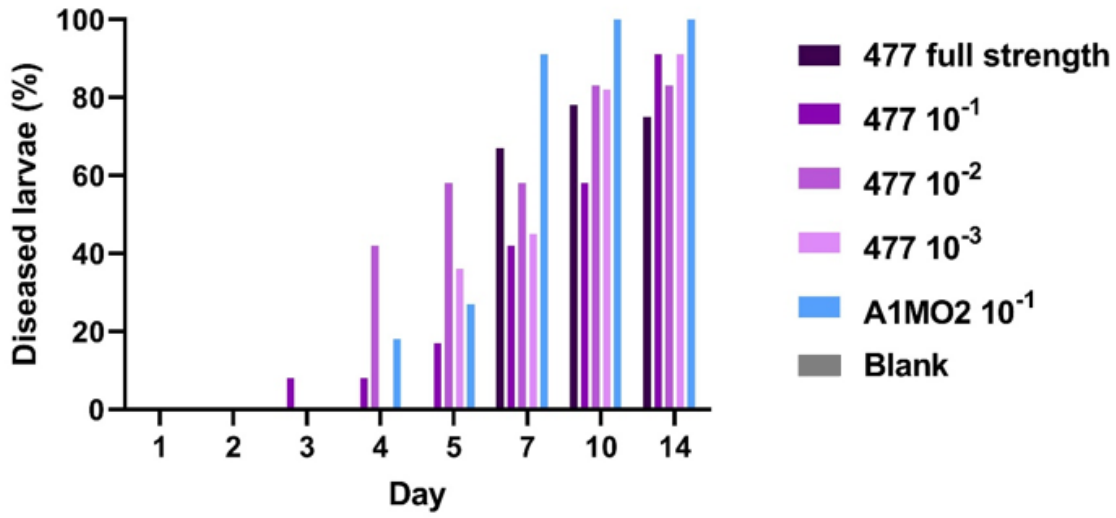


Figure 6.6. The changes in proportion for diseased larvae (n = 12) over 14 days following pre-inoculation with varying strength rates of 477 with a CFU/mL of 7.08 x 10^7 (477 full strength), 7.08 x 10^6 (477 10^{-1}), 7.08 x 10^5 (477 10^{-2}), and 7.08 x 10^4 (477 10^{-3}), prior to challenge with a 6.61 x 10^6 rate of A1MO2 after three days. An A1MO2-only control group (A1MO2 10^{-1}) was also challenged with a single 6.61 x 10^6 rate of A1MO2. Blank larvae were uninoculated. Disease was classified as amber discolouration and feeding cessation.

6.2.4 Persistence of tagged *Serratia* strains within larvae when inoculated at mixed ratios

Further exploration was made using mixed ratio inocula of A1MO2_tk and 5.6_s to determine how the persistence of the strains within the larval macerate samples compared to the persistence observed in both liquid culture and soil. The inocula were prepared using the same method as previously during *in vitro* culturing (method: Chapter 2, section 2.5.3). However, in addition to the preparation of separate

inocula for both A1MO2_tk (100:0) and 5.6_s (0:100), the mixed ratio inocula used were comprised either primarily of A1MO2_tk (90:10, 70:30, A1MO2_tk:5.6_s), or with an equal proportion of both strains (50:50, A1MO2_tk:5.6_s). The 5 µL inocula were prepared from diluted overnight cultures to produce a combined total dose rate of 5 x 10^5 CFU/mL for both A1MO2_tk and 5.6_s, with the inoculant being pipetted directly onto a carrot cube. The number of viable cells within the larvae were determined using whole-larval macerates of challenged larvae, with three larvae sequentially selected from each of the different groups and macerated individually on the appropriate days (method: Chapter 2, section 2.5.4).

Challenging the larvae with separate A1MO2_tk (100:0) and 5.6_s (0:100) inocula resulted in the generation of similar average CFU counts (n = 3) from the larvae macerated at one dpi, with counts of 2.27 x 10^5 CFU/mL for A1MO2_tk and 5.00 x 10^5 CFU/mL for 5.6_s (Fig 6.8. A - B). Over the duration of the 14-day bioassay the amount of A1MO2_tk isolated from the macerated larvae showed no significant changes. In contrast, a significant difference was recorded between the CFU counts for 5.6_s in the larvae macerated at one dpi and 14 dpi ($p < 0.05$). However, despite these changes, there was no statistical difference between the rates of CFU change in the larvae inoculated only with A1MO2 or with 5.6. These changes are also reflected in the multiple regression models, which show a similar non-significant decrease in CFU for both strains (Table 6.2).

Challenging the larvae with mixed ratio inocula often led to a comparable outcome, in which both A1MO2_tk and 5.6_s showed a similar persistence within the larvae macerated over the duration of the bioassay, except for the 90:10 treatment (A1MO2_tk:5.6_s) (Fig. 6.8. C). In the 90:10 treatment a significant difference was recorded in the rates of CFU change ($p < 0.05$), in which 5.6_s showed a positive CFU trend, and A1MO2_tk showed a negative trend. The fitted multiple regression models indicate that in each of the 90:10, 70:30, 50:50, and 30:70 treatments (A1MO2_tk:5.6_s) A1MO2_tk exhibited a significant CFU change per day ($p < 0.05$), whilst 5.6_s only exhibited significant change in the 90:10 and 30:70 treatments (Table 6.2). Furthermore, the AUC measurements for 5.6_s were significantly higher than A1MO2_tk in the 30:70 and 10:90 treatments (A1MO2_tk:5.6_s), which likely stemmed from the starting inocula ratios.

During the plating process 19 of 189 macerate replicates (~ 10 %) did not yield a countable number of cells. Several of these larvae displayed symptoms of amber disease, suggesting that the number of viable cells within the macerate samples may have been below detection limits of approximately 10^2 CFU/mL, or the applied dose was insufficient to cause disease within these larvae. This would have approximated to retrieving fewer than ten inoculated cells per larva when assessing 50 µL of macerate at a 1:10 dilution. These replicates were distributed across the treatments, and are highlighted in the CFU data, available in Appendix E.1.

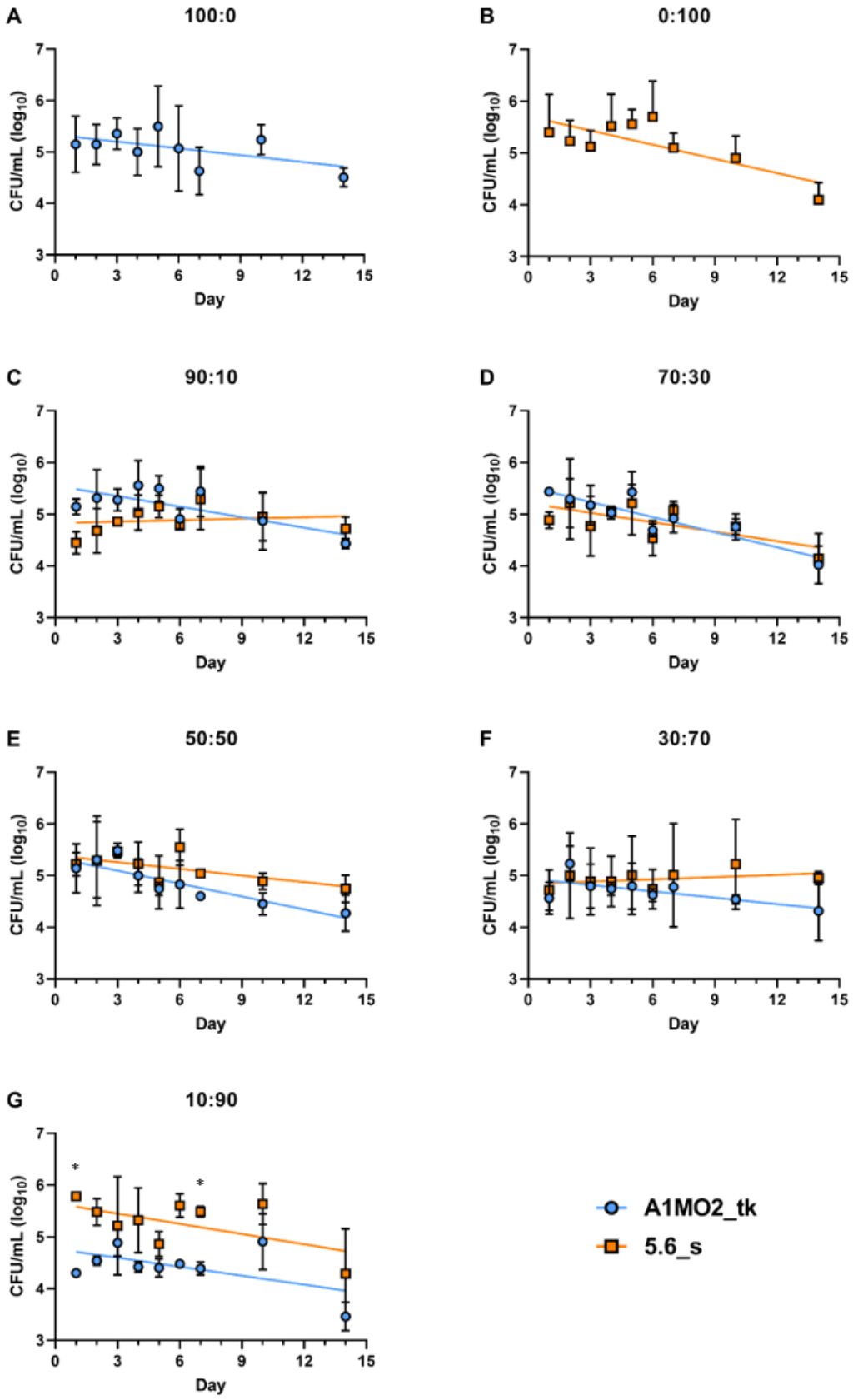


Figure 6.7. Changes in average log_{10} CFU/mL (n = 3) for A1MO2_tk and 5.6_s in whole-larval macerate samples after 14 days following challenge with mixed ratio inocula with a total 5 x 10^5 CFU/mL dose rate. The strains were inoculated separately (A) 100:0 and (B) 0:100, and together at mixed ratios (C) 90:10, (D) 70:30, (E) 50:50, (F) 30:70, and (G) 10:90 (A1MO2_tk:5.6_s). Log CFU/mL counts are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. The CFU data is available in Appendix E.1.

Table 6.5. The multiple regression models of log_{10} CFU/mL changes for A1MO2_tk and 5.6_s in larval macerate samples when inoculated separately (100:0 and 0:100) and together in a mixed ratio inoculant (90:10, 70:30, 50:50, 30:70, 10:90, A1MO2_tk:5.6_s) when the larvae were initially challenged at a rate of 5 x 10^5 CFU/mL. Regression model parameters include the adjusted R^2 value, the coefficient for each strain, the standard error and the *p* value of the coefficient (*P* value[1]). One-way ANOVA data includes the *F* statistic and corresponding p value (P value[2]). Models were calculated with six degrees of freedom.

Treatment	Strain	R^2	Coefficient	Std. err.	*P* value[1]	*F*	*P* value[2]
100:0	A1MO2_tk	0.47	-2.433	4.140	0.578	4.59	0.062
0:100	5.6_s		-5.566	2.766	0.091		
90:10	A1MO2_tk	0.93	-12.222	1.217	0.001	53.15	0.001
	5.6_s		11.805	1.722	0.001		
70:30	A1MO2_tk	0.78	-10.438	2.914	0.012	15.21	0.004
	5.6_s		3.248	3.763	0.421		
50:50	A1MO2_tk	0.74	-9.609	2.934	0.017	12.29	0.008
	5.6_s		0.684	4.245	0.877		
30:70	A1MO2_tk	0.65	-11.864	3.475	0.014	8.29	0.019
	5.6_s		14.667	5.721	0.043		
10:90	A1MO2_tk	0.16	-1.858	4.401	0.688	1.75	0.251
	5.6_s		-4.081	3.971	0.344		

Comparing the relative proportions of viable cells isolated from the larval macerate samples in the mixed ratio treatments showed that the proportion of 5.6_s continually increased over the duration of the assay, indicating a higher persistence in the macerated larvae (Fig. 6.8). This was most noticeable in the larvae challenged with the 90:10 inoculant, which began with a ratio of 83:17 (A1MO2_tk:5.6_s) at one dpi, progressing to 32:68 at 14 dpi. A similar outcome was observed in each of the larvae challenged with the 70:30, 50:50, and 30:70 inocula, where the proportion of 5.6_s increased following inoculation, after which a suppression of A1MO2_tk was maintained. In contrast, the proportions of each strain in the macerate of larvae challenged with the 10:90 inoculant showed a large degree of variation, beginning with a ratio of 3:97 (A1MO2_tk:5.6_s) at one dpi, progressing to 25:75 at five dpi, and then to 7:93 at 14 dpi. This indicated that whilst 5.6_s exhibited a higher fitness amongst the samples when inoculated as the minority proportion of the mixed inoculant, the same was not seen for A1MO2_tk when it was applied as the minority.

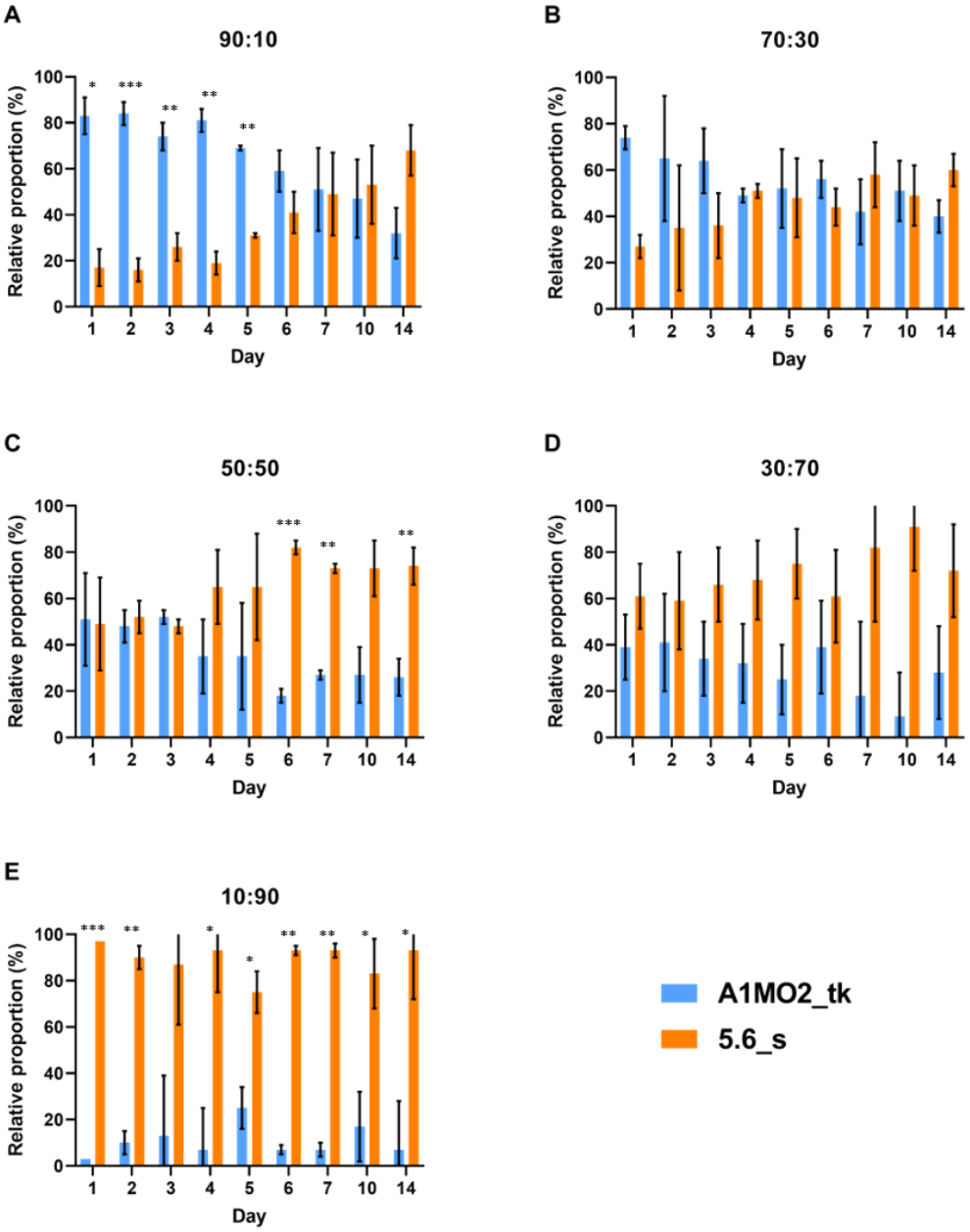


Figure 6.8. Changes in average relative proportion (n = 3) for viable A1MO2_tk and 5.6_s cells isolated from larval macerate samples over 14 days in larvae challenged with mixed ratio inocula of (A) 90:10, (B) 70:30, (C) 50:50, (D) 30:70, and (E) 10:90 ratios (A1MO2_tk:5.6_s). Inocula were prepared from cultures to provide a 5 x 10^5 CFU/mL total dose rate. Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

6.2.5 Assessing pADAP transfer between tagged *Serratia* strains within larvae

During the investigation of the persistence of *Serratia* strains within larvae (Section 6.2.4), further exploration was made regarding the occurrence of the horizontal transfer of pADAP between A1MO2_tk and 5.6_s in diseased larvae. During the plating of whole-larval macerates, a portion of the macerate was spread across agar plates containing kanamycin and spectinomycin to select for transconjugant colonies. During the investigation of persistence within the larvae, the strains were inoculated at a combined rate of 5 x 10^5 CFU/mL.

The first putative transconjugant cells were isolated from the larvae macerated at four dpi, where from the whole-macerate samples of 15 larvae challenged with the mixed ratio inocula (90:10, 70:30, 50:50, 30:70, and 90:10, A1MO2_tk:5.6_s) a total of seven putative HGT colonies were identified (Fig. 6.9). These putative cells were all isolated from a single larva challenged with the 50:50 inoculant, with the macerate being serially diluted 1:10 in PBS (equating a CFU count of 1.41 x 10^3 CFU/mL). In comparison, the total cells isolated for both A1MO2 and 5.6 from the same larva was 4.30 x 10^5 CFU/mL, resulting in the putative transconjugant cells comprising 0.3 % of the cells isolated from the larva. More transconjugant cells were isolated from larvae macerated on the subsequent days of the bioassay (Fig. 6.9), with between 17 and 52 putative HGT colonies being isolated at each of the time-points from the larvae macerated between five and 14 dpi, resulting in a total of 159 potential transconjugant cells being isolated from all macerate samples during the assay. Of these 159 total cells, 64 (40 %) were isolated from larvae macerated following challenge with the 50:50 inoculant. For the remaining putative transconjugants, 38 (24 %) were isolated from the larvae challenged with the 90:10 inoculant, 28 (18 %) were isolated from the larvae challenged with the 10:90 inoculant, 19 (12 %) were isolated from the larvae challenged with the 30:70 inoculant, and two (1 %) were isolated from the larvae challenged with the 70:30 inoculant. Furthermore, many of these cells were isolated from a single larva; for example, 50 of the 64 cells were isolated from a single larva challenged with the 50:50 inoculant macerated at 14 dpi, with all putative cells being isolated from the macerate of 14 separate larvae.

However, as mentioned previously, whilst HGT occurred during this assay, it did so at a low rate. The isolation of 159 potential transconjugant cells between days four and 14 (during six time-points assessing 90 larvae in total) from 50 µL of 1:10 whole-macerate dilutions spread across 90 antibiotic LB agar plates (Spec 100 and Kan 100). When compared against the total average number of cells isolated for both A1MO2_tk and 5.6_s (n = 3) from the larvae between these days the potential transconjugant cells constituted approximately 0.37 % of the cells isolated. Amongst the 135 larvae assessed, the occurrence of pADAP HGT was validated in nine of the larvae assessed.

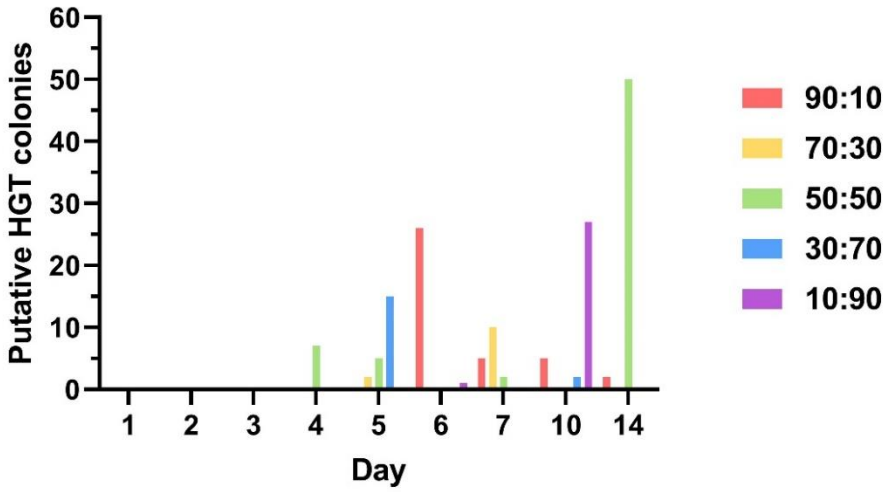


Figure 6.9. The total number of putative transconjugant cells isolated from whole-larval macerate samples produced over a 14-day period, with three larvae from each treatment being macerated individually on the designated days (135 larvae total). The cells were isolated from larvae challenged with mixed inocula of A1MO2_tk and 5.6_s at varying ratios: 90:10, 70:30, 50:50, 30:70, 10:90 (A1MO2_tk:5.6_s), with inocula being produced at a total rate of 5 x 10^5 CFU/mL.

The colonies were initially assessed by patching across LB agar containing either tetracycline, spectinomycin, or kanamycin (method: Chapter 2, section 2.5.5). The transconjugant cells were resistant to the 5.6 chromosomal spectinomycin-resistance cassette, and resistant to kanamycin due to the Kan cassette in pADAP derived from the kanamycin resistant donor cell (A1MO2_tk). All 159 colonies patched were resistant to both spectinomycin and kanamycin, and sensitive to tetracycline, denoting the successful conjugation of pADAP into 5.6_s for each of these cells.

Further validation of the colonies was conducted using PCR with primers designed to amplify the Spec cassette in 5.6_s, the Tet cassette in A1MO2_tk, and the Kan cassette in the tagged pADAP. A positive band for the Spec (~ 1.4 kb) and Kan (~ 4 kb) cassettes, and the lack of a band for the Tet (~ 1.6 kb) cassette would indicate successful HGT, as this would indicate the presence of pADAP in 5.6_s. All colonies assessed produced a positive band for the Spec cassette, and no band for the Tet cassette, indicating that all colonies assessed were 5.6_s. Some of the colonies assessed produced a positive band for the Kan cassette in pADAP, indicating that HGT of pADAP between A1MO2_tk and 5.6_s occurred within the larvae. The majority of the transconjugant cells which produced a band for the Kan cassette were isolated from the larvae macerated between four and five dpi, and fewer positive transconjugant cells were isolated between six and 14 dpi. The PCR gels showing banding for the Kanamycin primer set following an assessment of mini-prepped macerate putative transconjugant colonies is depicted in Figure 6.10.

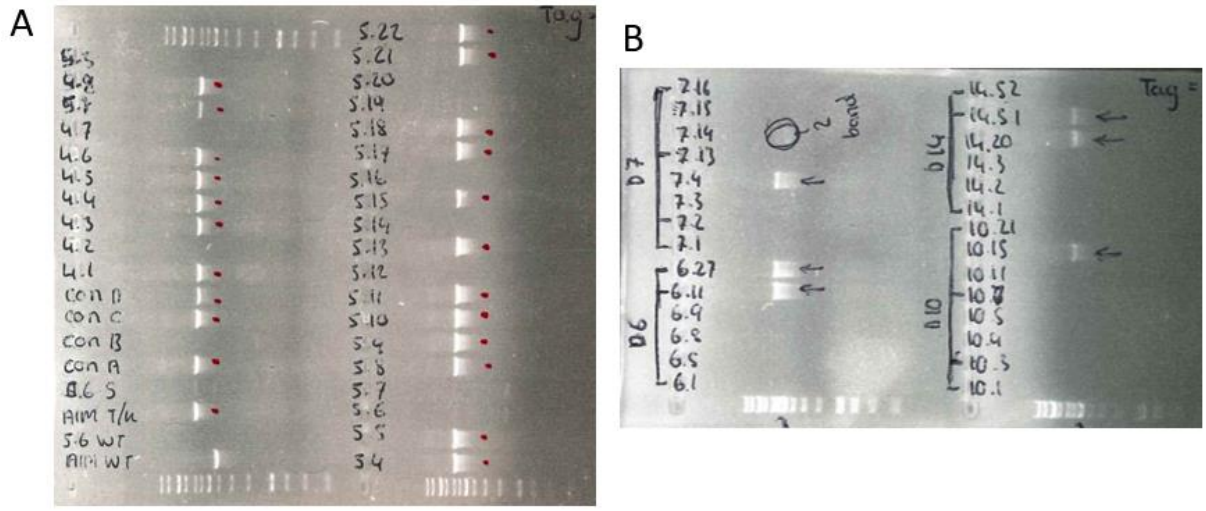


Figure 6.10. Agarose gels showing the PCR banding of putative transconjugant cells isolated from larval macerates. The amplicons were generated using a Kanamycin primer set (AFP_Kan_F/R) to validate the presence of the Kan cassette (~ 4kb) in transconjugants. All transconjugant cells depicted produced the correct banding for the Spec cassette (SpF/R), and no band for the donor-derived Tet chromosomal cassette (TcF/R). (A) Control samples (A1MO2 and 5.6 wild-type, A1MO2_tk and 5.6_s), day 4 macerate samples (4.1 - 4.7), day 5 samples (5.1 - 5.22), red dots denote a valid amplicon size. (B) Day 6 samples (6.1 - 6.27), day 7 samples (7.1 - 7.16), day 10 samples (10.1 - 10.21), day 14 samples (14.1 - 14.52), black arrows represent correct banding / validation.

Further assessments were made to ensure that the HGT observed between A1MO2_tk and 5.6_s within the larvae did not occur prior to the consumption of the carrot cube pieces used to inoculate the larvae. The inoculation of carrot cube pieces with an equal proportioned inoculant of A1MO2_tk and 5.6_s (50:50) from ~ 1 x 10^9 CFU/mL cultures of A1MO2_tk and 5.6_s resulted in a single transconjugant colony being isolated from carrot cube macerated samples (n = 3) at 24 hpi and validated with PCR. No transconjugant colonies were isolated from carrot cube pieces macerated after 48 hours using the same experimental procedure. This single colony was confirmed as a transconjugant cell via patching and PCR.

6.2.6 Assessing pADAP HGT between *S. entomophila* and *S. proteamaculans* within the larvae

Following the confirmation of pADAP transfer between A1MO2_tk and 5.6_s within the larvae, further HGT assessments were made to detect plasmid transfer to other naturally occurring non-pathogenic *S. entomophila* strains containing antibiotic tags (isolates 219_s, IDIA_s, 329_S, 477_s), and, a non-pathogenic *S. proteamaculans* isolate (Tukino_s). The bioassay was conducted using the same method as described above (Section 6.2.6) using a total of 162 larvae, with 18 per treatment, assessing three

whole-larval macerates at set points between four and 14 dpi following challenge with a dose rate of ~ 10^6 CFU/mL.

Many putative transconjugant cells (~ 250 +) were isolated from the larvae macerated over the duration of the assay following challenge with mixed inoculants of A1MO2_tk and either 219_s (*S. entomophila*), IDIA_s (*S. entomophila*), or Tukino_s (*S. proteamaculans*). Patching a proportion of these isolates across LB agar using the same process described in Chapter 6.2.6 resulted in all colonies showing resistance to both spectinomycin and kanamycin, and sensitivity to tetracycline. The resistance to spectinomycin indicated that none of the potential colonies were A1MO2_tk. Further validation of the colonies was performed via PCR, with all isolates tested producing a positive band for the Spec cassette (~ 1.4 kb), and no bands for the Tet cassette (~ 1.6 kb), and two of the 250 + isolates tested produced a positive band for the Kan cassette (~ 4 kb). These bands were produced by two isolates obtained from a single larva challenged with the A1MO2_tk and Tukino_s inoculant and macerated at four dpi. This indicated that HGT had occurred within the larvae, and also that pADAP had been transferred between A1MO2_tk and Tukino_s. None of the larvae macerated following challenge with A1MO2_tk and either 329_s, 477_s, or 5.6_s alone (all non-pathogenic *S. entomophila*) yielded any putative transconjugant cells from the whole-macerate samples. These results indicated that HGT within the larvae was a rare event, with only a single larva out of the 162 total larvae assessed during the experiment yielding two validated transconjugant colonies, constituting fewer than one percent of the total number of cells isolated from the larval macerates.

6.2.7 Assessing resistance of larvae to *S. proteamaculans* strain 143 after repeated challenge

An additional bioassay was conducted to explore the pathotype of the wild-type pathogenic *S. proteamaculans* isolate 143 and how the pathotype of the strain and variations in pADAP virulence regions may contribute to a reduction in efficacy. This strain has a variable infectivity causing disease in 60 to 70 % of infected larvae, which may stem from a truncated virulence region with differences in the *sepA* and *sepC* regions compared to A1MO2, and from a lack of an Afp orthologue (Hurst et al., 2011), causing reduced efficacy. A standard bioassay method (method: Chapter 2, section 2.5.2) was used to assess the larvae following challenge using a carrot cube rolled across a bacterial lawn, producing a dose rate exceeding 1 x 10^8 CFU/mL. The characteristics of the challenged larvae (n = 90) were compared to uninoculated control larvae (n = 12) over a nine-day period, after which larvae which remained healthy and exhibiting active feeding behaviour nine-days following the initial dosing were re-challenged and assessed for a further nine days.

Following the initial nine-day bioassay, 67 larvae (74 %) challenged with the 143 isolate showed symptoms of amber disease, such as discolouration and feeding cessation; this disease rate matched what was observed for the strains previously (Glare et al., 1993b) (Fig. 6.11. A). A total of 14 larvae (16 %) retained a healthy phenotype over the duration of the bioassay, where only nine of these larvae still displayed feeding behaviour. In addition, a mortality rate of 10 % was noted for the larvae challenged with 143. In contrast, 11 of the uninoculated larvae (92 %) remained healthy and feeding over the bioassay duration, with a single larva (8 %) becoming diseased prior to nine dpi.

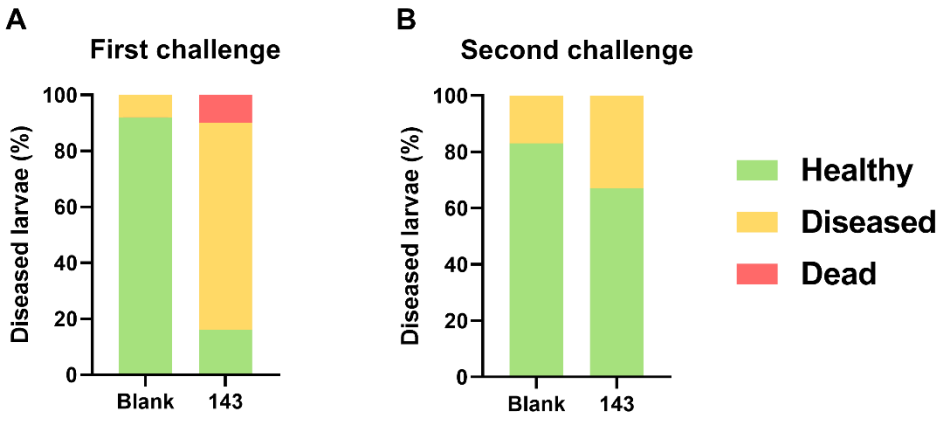


Figure 6.11. The condition of larvae following (A) challenge with *S. entomophila* isolate 143 (n = 90) at a rate exceeding 10^8 CFU/mL, and uninoculated larvae (n = 12) after nine days, and (B) condition of the remaining healthy larvae when re-challenged with the same dose of 143 (n = 9) and assessed for a further nine days, alongside the same uninoculated larvae (n = 12). Classification of disease was based on amber discolouration and lack of feeding behaviour.

The nine larvae which retained a healthy phenotype and feeding behaviour following the initial challenge with 143 were re-assessed using the same method and a similar dose rate exceeding 10^8 CFU/mL to determine whether any resistance to the strain would be identified. Alongside this, the 12 uninoculated control larvae were also assessed for a further nine days. A total of three larvae (33 %) re-challenged with 143 displayed amber disease symptoms at nine dpi, with six larvae (67 %) retaining a healthy phenotype and feeding behaviour, suggesting a potential resistance of the *C. giveni* larvae to strain 143 (Fig. 6.11. B). For comparison, ten larvae (83 %) in the control group remained healthy at nine dpi following the second challenge with 143, with one additional larva becoming diseased. No deaths were recorded amongst the challenged and uninoculated control larvae following the second treatment with 143.

Using group-wise logistic regression models, it was determined that there was a significant difference between disease incidence amongst the larvae challenged with *S. proteamaculans* 143 initially, and the larvae-re-challenged during the second 143 inoculation ($p < 0.005$). Furthermore, during the re-challenge of the larvae with 143 there was no significant difference in amber disease incidence between the larvae re-challenged with 143 and the uninoculated blank larvae.

6.3 Discussion

Assay results provided evidence for competition between pathogenic and non-pathogenic *Serratia* spp. within grass grub larvae, where the effects of this competition with 5.6 were found to reduce the efficacy of A1MO2. Interestingly, when larvae were challenged with each strain separately, A1MO2 persisted better within the larvae than 5.6, as it was likely able to establish itself within the larvae following the occurrence of amber disease, facilitating replication of the strain. However, during mixed inoculation of the strains, 5.6 displayed a better persistence than A1MO2, indicating an increased fitness for 5.6 in the presence of A1MO2. This conforms with previous findings using mixed strain inocula comprised of pathogenic and non-pathogenic *Bacillus* spp. by Raymond et al. (2007). These results showed that in mixed infections of diamondback moth larvae, the non-pathogenic *Bacillus* spp. (*Bacillus thuringiensis* subsp. *tenebrionis* or *Bacillus cereus*) displayed greater fitness and competitive abilities than the virulent *Bacillus* spp. (*Bacillus thuringiensis* subsp. *kurstaki*). Other studies focussing on *Bacillus* spp. have demonstrated a reduction in virulence during mixed inoculations as opposed to single-strain inoculation (Garbutt et al., 2011). However, a significant difference in the rate of CFU change was only recorded during one of the mixed ratio treatments with A1MO2 and 5.6 (90:10, A1MO2:5.6), in which the trends showed a decrease in CFU for A1MO2 and an increase for 5.6 over the assay duration. In each of the other mixed ratio treatments a similar level of persistence was reported for the strains.

Additionally, similarities can be seen when comparing the changes in proportion for A1MO2_tk and 5.6_s in mixed ratios during *in vitro* culturing in LB broth, and when isolated from the larval macerate samples (Fig. 6.12). This suggests that the larva provides a favourable environment for the growth of 5.6. This also showed that whilst the outcome of competition within the larvae might be comparable to competition *in vitro*, the onset of competition between A1MO2 and 5.6 took longer to occur within the larvae than in liquid cultures. This may have been due to fewer available nutrients in the larval gut, or the lower rate at which the strains were applied in the respective assays (10^5 CFU/mL during the bioassay and 10^{7-8} during the *in vitro* culturing), with competition occurring after two days in the liquid cultures, and by four dpi in the larvae.

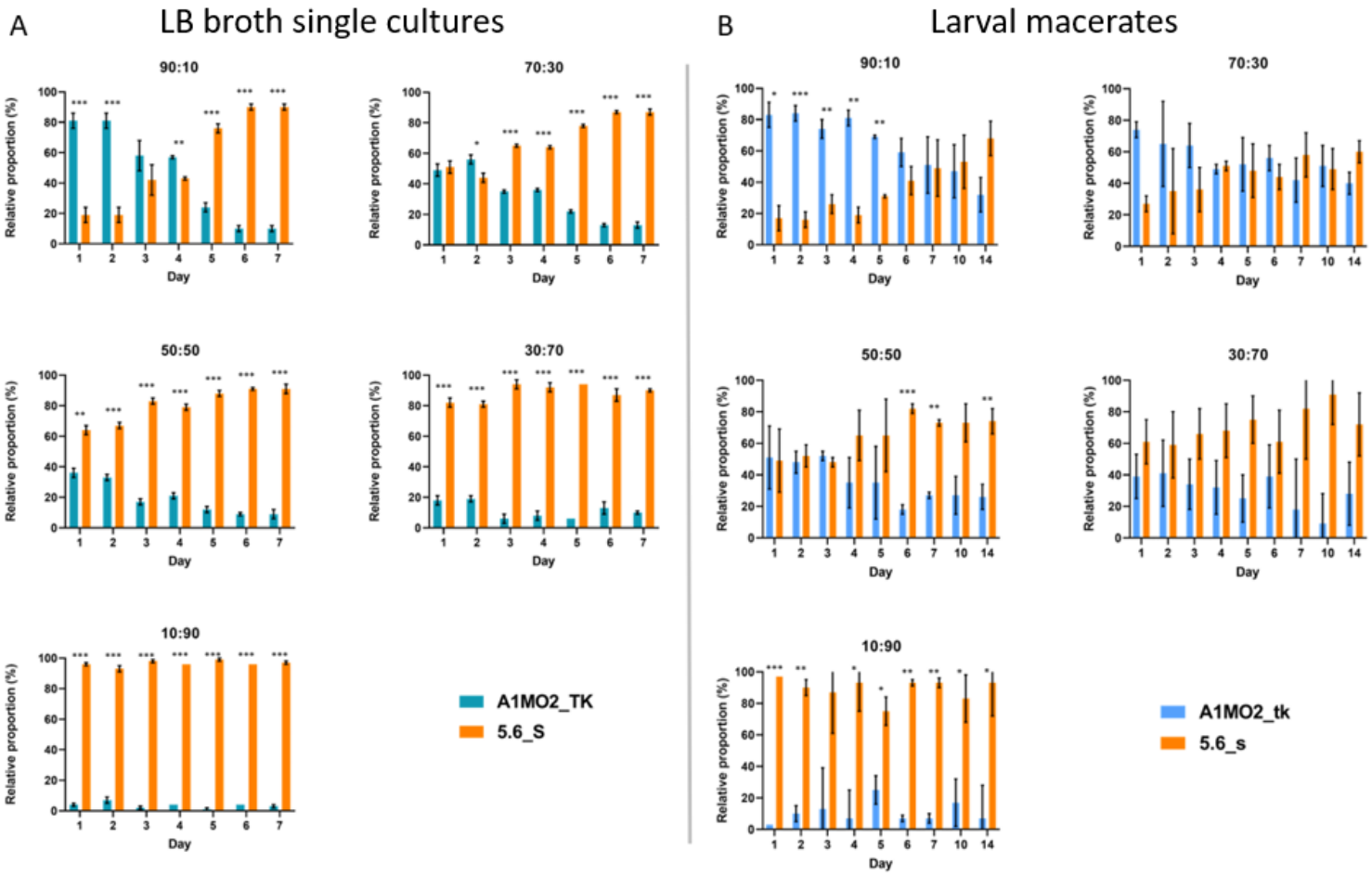


Figure 6.12. A comparison between the changes in the average relative proportion (n = 3) over a seven-day period of A1MO2_tk and 5.6_s isolated from either (**A**) LB broth cultures inoculated once and maintained over seven days, or (**B**) whole-macerate larval samples following challenge with A1MO2_tk and 5.6_s at mixed ratios. Ratios represent the proportion of A1MO2_tk and 5.6_s in the initial inocula (A1MO2_tk:5.6_s). Results are presented with standard deviation error bars. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Furthermore, the results obtained also show that the ratio of non-pathogenic strains, such as 5.6 or 477, in the initial inocula used to challenge the grass grub larvae can influence the efficacy of A1MO2, with a significant reduction in amber disease incidence occurring the lower the proportion of A1MO2 in the initial inoculant. Whilst this outcome may have been dependent on competition, a reduction in disease may have also resulted from lower doses of A1MO2 not being able to establish an amber disease infection, with a minimum of 10^4 CFU/mL being required to cause amber disease in 50 % of challenged larvae (Jackson et al., 2001), and a rate of 7 x 10^4 CFU/mL being used for A1MO2 in the bioassay. Whilst a reduction in disease was documented for both 5.6 and 477, the A1MO2 isogenic strain 5.6 reduced the efficacy of A1MO2 to a greater extent than 477. A similar outcome was observed when larvae were pre-inoculated with either 5.6 or 477 and then challenged with A1MO2 after three days. In this instance, pre-inoculating the larvae with the non-pathogenic strains resulted in delayed symptom development and a reduced incidence of disease. A similar effect has been reported in both viral and fungal systems. The pre-inoculation of pigs with non-pathogenic strains of African swine fever virus (ASFV) led to a delay in disease onset by 14 days following challenge with virulent ASFV strains, or full resistance, as seen in one pig (Boinas et al., 2004). Furthermore, the pre-inoculation of cucumber plant leaves with non-pathogenic isolates of fungal species (*Alternaria cucumarina* or *Cladosporium fulvum*) led to systemic resistance responses in the plants when later challenged with pathogenic species (*Sphaerotheca fuliginea*) (Reuveni and Reuveni, 2000). This caused a reduction in disease by up to 80 %.

Another outcome observed during the *in vivo* testing was the occurrence of pADAP HGT between A1MO2 to 5.6 within larvae during mixed strain inoculation assessments. Many of these transconjugant cells were isolated from the macerate of larvae challenged with an equal proportion of both strains (50:50, A1MO2:5.6), suggesting that even numbers of both strains was more conductive to plasmid transfer, which corresponds with previous studies of HGT in *Serratia* spp. (Dodd, 2003). Despite the isolation of an increasing number of putative transconjugant cells over the duration of the assay, the rate of HGT decreased, with the highest relative rate of HGT occurring between four and five dpi, at a rate of 0.35 % when comparing transconjugants to total cells isolated from the macerate. In this study HGT was defined as the transfer of the Kanamycin resistance cassette (located on pADAP in A1MO2_tk) to 5.6_s, allowing it to grow on LB agar plates with both spectinomycin and kanamycin, it was assessed using the number of transconjugant cells isolated from the whole macerate sampes, compared with the total number of inoculated cells isolated from the macerates. This rate is low when compared to other bacterial species, such as *Staphylococcus aureus*, which displays a higher rate of *in vivo* HGT for bacteriophages and three plasmids (McCarthy et al., 2014). Similarly, during an assessment of horizontal transfer between gut-associated members of the Acetobacteraceae family was found to be widespread and occurring at a rate described as ‘extensive’ in red carpenter ants

(Brown and Wernegreen, 2019), with up to 16 % of the genome for the strains being acquired from horizontal transfer. The rate of HGT in the Brown and Wernegreen (2019) study was determined through integrated microbial genome comparative analysis. The low occurrence of pADAP transfer between *Serratia* spp. has been reported previously in other studies (Dodd, 2003; Vaughan, 2021), with pADAP HGT not being a guaranteed event within the larvae. This was evident based on the results obtained in this study, as during the investigation of HGT between *Serratia* spp., only the macerate of a single larva out of 108 larvae assessed produced any transconjugant cells at a 1:10 dilution on LB agar plates. This suggested a similarly low rate of pADAP transfer between A1MO2 and 5.6, with the macerate of 14 larvae out of 324 assessed yielding any putative HGT cells (fewer than 50 transconjugant cells isolated from the macerate of 324 larvae) and between A1MO2 and Tukino (2 transconjugant cells being isolated from the 108 macerated larvae) under the conditions assessed. It can be estimated that fewer than 1 per cent of cells isolated from the macerated larvae would be transconjugant cells.

During the assessments of HGT it was also noted that plasmid transfer could occur on the carrot cube pieces used to inoculate the larvae, as a single confirmed transconjugant cell was isolated following maceration of carrot cube pieces after 24 hours following inoculation. However, no other transconjugant cells were isolated from carrot cube pieces after 48 hours, indicating that whilst HGT can occur on the carrot, it is a rare event that often falls below detection limits of 10^2 CFU/mL. In addition, in the bioassays conducted it was commonly found that larvae would consume the entire carrot cube within a 24-hour period, further reducing the likelihood for HGT to occur prior to consumption.

Another point of consideration was the failure for some of the macerated larvae (~ 10 %) to yield any viable cells when a portion was spread on agar plates despite presenting symptoms associated with amber disease, such as amber discolouration and a lack of feeding. This may have stemmed from bacteria detection limits, where a macerate sample might have contained too few cells to produce a countable number on agar, despite being plated at low serial dilutions on LB and CTA media (10^{-1} and 10^{-2}), as all other macerated larvae yielded countable cell numbers. This would indicate that the larvae received a sufficient dose to initiate disease, but not enough to produce a countable number of cells. A similar degree of variance was seen in the CFU results for the cells isolated from the macerate samples, indicating that whilst the data shows an increased persistence for 5.6, the dataset would be strengthened with the inclusion of additional replicate larvae to reduce the observed variance. This setback might be alleviated by the utilisation of a more sensitive selection method for the cells, namely quantitative PCR, with multiple test first screening for *Serratia* via a section of the chromosome, and then differentiating between the strains based on plasmid using a Kado plasmid visualisation method.

Whilst these results show that the presence of non-pathogenic *Serratia* strains can reduce the efficacy of pathogenic *Serratia* strains, further work is needed to determine if a similar outcome is likely to occur under natural conditions in the field. Field validation of these results is challenging as many environmental and biological factors interact to cause high levels of natural variability within the results, such as temperature and precipitation, or other bacterial species in the rhizosphere. As a first step towards simulating field conditions, competition between pathogenic and non-pathogenic *Serratia* spp. was explored further in a shade-house pot trial (Chapter 7).

Chapter 7

Competition between pathogenic and non-pathogenic *Serratia* spp. during a pot trial

7.1 Introduction

One of the overarching goals in this project was the characterisation of interactions between pathogenic and non-pathogenic *Serratia* strains, in particular, how competition between the strains might influence the occurrence of amber disease within grass grub populations. So far, this concept has been approached through *in vitro* (in liquid culture and soil microcosms) and *in vivo* (within larvae) mixed inoculation experiments, presented in Chapters 3, 4, 5, and 6, respectively. The results from these experiments showed an increased replication rate and persistence for the non-pathogenic *S. entomophila* strain 5.6, relative to the isogenic pathogenic *S. entomophila* strain A1MO2, when both strains were inoculated together during competition assays. This was most evident during bioassays when larvae were pre-inoculated with the non-pathogenic *S. entomophila* strain 5.6 (and also the non-pathogenic *S. entomophila* strain 477), which led to a reduction of amber disease rates following challenge with A1MO2 (Chapter 6, section 6.2.3). Moreover, the pre-inoculation of larvae also delayed amber disease onset by up to four days (Chapter 6, section 6.2.1). These experiments were conducted under laboratory conditions in the absence of soil, thus further experimentation was needed to validate these results under conditions more representative of the field environment. Pot trials using natural soil samples have often been used as a surrogate for field trials to analyse interactions between microbial species (Ogunkunle and Beckett, 1988; Kumar et al., 2018; Samayoa et al., 2020). Using pots allows for control over other changeable factors such as the soil moisture content and temperature, and also provides an ease of management, greatly reducing any potential experimental errors and maintaining a more realistic soil environment than laboratory-based assays.

Alongside competition between A1MO2 and 5.6, the interactions between A1MO2 and 477, a naturally occurring non-pathogenic *S. entomophila* isolate, were assessed. This allowed for a comparison between the potential impacts on A1MO2 efficacy between its isogenic plasmid-cured variant (5.6) and a natural non-pathogenic isolate such as 477, which is more representative of non-pathogenic strains which may be more readily encountered in a pasture than a laboratory-derived heat-cured strain plasmid-free. Furthermore, using the pot trial competition between 5.6 and 477, and the hyperpathogenic *S. proteamaculans* strain AGR96X, were assessed. This experiment would determine if the exposure to the non-pathogenic strains would lead to a reduction in AGR96X-induced mortality rates.

7.2 Results

7.2.1 Assessment of larval feeding behaviour in pots using ryegrass germination

The pots and soil were prepared as described previously in Chapter 2, section 2.4.4, where each pot contained three perennial ryegrass seeds (*Lolium perenne*; cultivar Sampson nil endophyte) used as an indicator of larval health; each pot also contained a single healthy grass grub larva, with the grub being added three days following the inoculation of the soil and sowing of the seeds. If the larvae remained healthy, they actively fed on the grass roots, impacting plant growth. However, larvae which become infected with amber disease cease feeding, resulting in the germination and growth of the plants. Table 7.1 summarises the differing experimental treatments used. The number of germinated seeds was recorded following the conclusion of the pot trial after four weeks, when the health of the larvae remaining in the soil was also recorded.

Table 7.1. A summary of the various treatments used in the pot trial. Each treatment consisted of ten replicate pots, with each pot containing three ryegrass seeds and a single actively feeding and healthy grass grub larva.

Treatment	Treatment name	Pre-inoculant non-pathogen	Rate (CFU/g)	Challenge pathogen	Rate (CFU/g)
1	Blank	-	-	-	-
2	A1MO2 ctrl	-	-	A1MO2	5×10^5
3	5.6 ctrl	5.6	5×10^5	-	-
4	477 ctrl	477	5×10^5	-	-
5	AGR96X ctrl		5×10^5		
6	5.6 (5×10^4)	5.6	5×10^4	A1MO2	5×10^5
7	5.6 (5×10^5)		5×10^5		
8	5.6 (5×10^6)		5×10^6		
9	477 (5×10^4)	477	5×10^4		
10	477 (5×10^5)		5×10^5		
11	477 (5×10^6)		5×10^6		
12	* 5.6 (5×10^5)	5.6	5×10^5	AGR96X	5×10^5
13	* 477 (5×10^5)	477	5×10^5		

- denotes no strain added, * denotes larvae were challenged with AGR96X instead of A1MO2.

In the pots containing uninoculated soil and a single healthy larva a total of 18 of the 30 seeds planted had germinated by the end of the pot trial after four weeks (60 %), with the seedlings being distributed between seven of the ten pot replicates (Fig. 7.1). The high rate of germination in this treatment indicated a lack of feeding by the larvae. However, as noted later in Section 7.2.3, many of these plants grew in pots from which larvae were not retrieved following the conclusion of the pot trial. In the pots

containing a single healthy larva and soil inoculated with a single dose of A1MO2 at a rate of 5 x 10^5 CFU/g, 14 of the 30 ryegrass seeds planted (47 %) between the ten replicate pots (three seeds per replicate) germinated over the four-week assay. The germinated seeds were distributed between seven of the ten replicates, with no growth occurring in the three remaining pots.

In the pots containing soil inoculated with a single dose of 5.6 at a rate of 5 x 10^5 CFU/g nine of the 30 seeds planted germinated (30 %), with growth occurring in five of the ten replicate pots (Fig. 7.1). The lower rate of germination suggested that many of the larvae held in the pots inoculated with 5.6 remained healthy and were still able to feed on the plants. The pre-inoculation of the soil with varying dose rates of 5.6 (5 x 10^4, 10^5, and 10^6 CFU/g), followed by the addition of a single dose of A1MO2 at a rate of 5 x 10^5 CFU/g after three days resulted in the germination of 23 to 40 % of the seeds in the treatments after four weeks. This growth occurred in four to five of the ten replicate pots, which, akin to the single dose of 5.6, suggested that many of the larvae were healthy and still feeding.

In the pots containing soil inoculated with a single dose of 477 at a rate of 5 x 10^5 CFU/g 12 of the 30 seeds planted between the ten replicate pots (40 %) had germinated after four weeks (Fig. 7.1). This growth was recorded between four of the pots, with none of the seeds germinating amongst the other six pots of this treatment. Further analysis of this data, presented in Section 7.2.3, shows that no larvae were recovered from the four pots in which plant growth was recorded. This suggested that all larvae held in pots which were retrieved from the soil were still feeding, preventing plant growth. The pre-inoculation of soil with varying rates of 477 (5 x 10^4, 10^5, and 10^6 CFU/g) followed by a single dose of A1MO2 at a rate of 5 x 10^5 CFU/g after three days led to 23 to 37 % of seeds geminating after four weeks. This plant growth was recorded in four to five of the ten replicate pots in each of the treatments, indicating that many of the larvae contained in these pots remained healthy and able to feed.

The inoculation of soil with a single dose of AGR96X at a rate of 5 x 10^5 CFU/g resulted in 14 of the 30 seeds germinating (47 %), with a germination rate similar to the pots containing soil inoculated with a single dose of A1MO2. The higher germination rate suggested that many of the larvae in these pots were unable to feed following ingestion of the pathogen from the soil due to disease or mortality. The pre-inoculation of soil with either 5.6 or 477 at a rate of 5 x 10^5 CFU/g followed by the addition of a single dose of AGR96X at the same rate after three days led to ten of the 30 seeds germinating (33 %) in soil pre-inoculated with 5.6, and 11 of 30 seeds germinating (37 %) in soil pre-inoculated with 477. The lower germination rate suggested that many of the larvae held in the pre-inoculated soil were healthy and able to feed. An example of the plant growth recorded at the end of the assay is presented in Figure 7.2, and a summary of the initial experimental data is presented in Table 7.2.

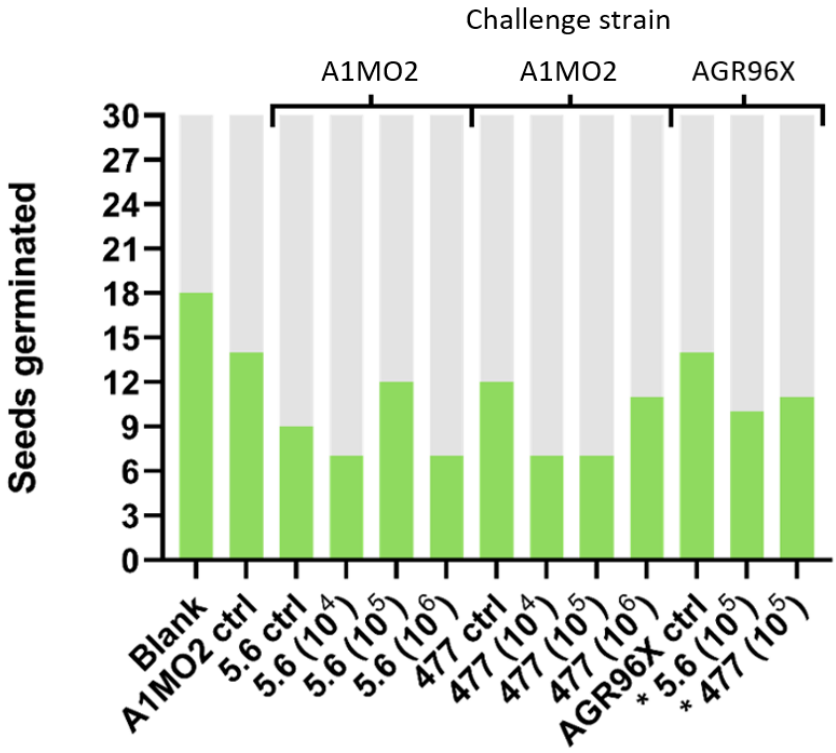


Figure 7.1. The total number of ryegrass seeds (n = 30) which germinated over a four-week period in soil inoculated with *Serratia* spp. Each treatment comprised of ten pots, with each pot containing a single healthy grass grub larva and three ryegrass seeds. The soil was inoculated with either a single dose of A1MO2, 5.6, 477, or AGR96X (ctrl) at a rate of 5 x 10^5 CFU/g or was pre-inoculated with either 5.6 or 477 at varying rates (5 x 10^4, 10^5, and 10^6 CFU/g), followed by the addition of a single dose of A1MO2 or AGR96X at a rate of 5 x 10^5 CFU/g after three days. The blank treatment contained uninoculated soil and a single healthy grass grub larva. * Denotes the addition of AGR96X after three days instead of A1MO2.

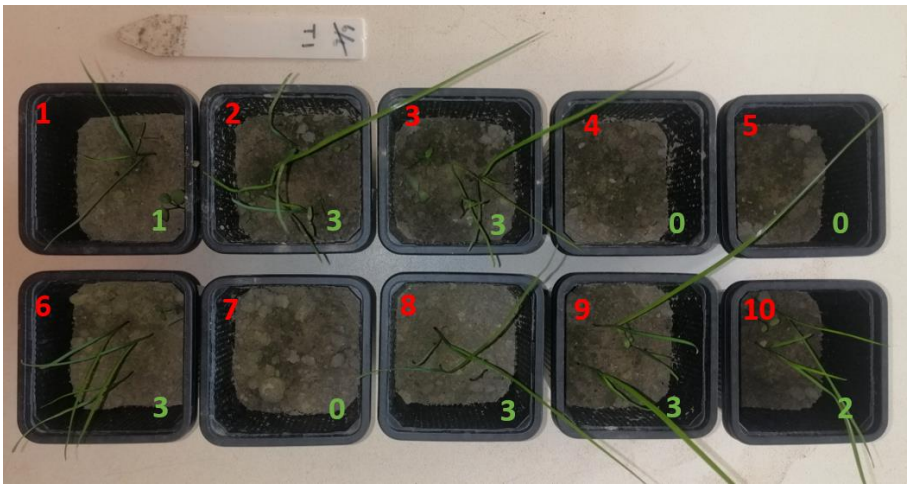


Figure 7.2. The growth of ryegrass recorded in the ten blank treatment replicate pots containing uninoculated soil, three ryegrass seeds, and a single healthy grass grub larva after four weeks. Red denotes the replicate number, and green denotes the number of observed ryegrass plants at four weeks. Non-ryegrass plants were not counted.

Table 7.2. The rates of bacteria used in each of the treatment (CFU/g), the number of ryegrass seeds (n = 30) which germinated amongst the replicates of each treatment (n = 10) after four weeks, the percentage of seeds which germinated, and the number of pot replicates (out of 10) in which ryegrass germination was recorded.

Treatment	Non-pathogen (CFU/g rate)	Pathogen (CFU/g rate)	Seeds germinated	% Germinated	Replicates with growth
1	-	-	18	60	7
2	-	A1MO2 (10^5)	14	47	7
3	5.6 (10^5)	-	9	30	5
4	477 (10^5)	-	12	40	4
5	-	AGR96X (10^5)	14	47	7
6	5.6 (10^4)	A1MO2 (10^5)	7	23	4
7	5.6 (10^5)		12	40	5
8	5.6 (10^6)		8	27	4
9	477 (10^4)	A1MO2 (10^5)	7	23	5
10	477 (10^5)		7	23	5
11	477 (10^6)		11	37	6
12	5.6 (10^5)	AGR96X (10^5)	10	33	5
13	477 (10^5)		11	37	7

- Denotes no bacteria added

7.2.2 Assessing larval health with a bioassay following retrieval from inoculated soil

At the conclusion of the pot trial after four-weeks, the soil in each pot was independently searched for the added larva, and each larva assessed for disease status. The main assessment criterion of the larvae following retrieval from the soil was amber colouration of the gut, which would indicate the clearance of material from the gut and the occurrence of amber disease These larvae were then monitored for a further two weeks and provided with carrot cube pieces to determine feeding behaviour, with the results presented below in Section 7.2.4. In addition, three 20 g soil samples were collected randomly from each of the treatments to determine the presence of any of the inoculated strains; these results are presented later in Section 7.2.5.

In some cases, the larvae added to the soil were not found in the pots during an assessment of the soil; this includes the absence of any parts of a grass grub cadaver. Furthermore, the liner material contained in each of the pots to prevent the escape of the larvae through the holes at the bottom of the pots remained intact. These larvae, classified as missing, were presumed to be dead, however it was unclear as to whether they were diseased prior to becoming unaccounted for.

Four larvae were retrieved from the pots containing uninoculated soil, with each of these larvae presenting a healthy phenotype with a dark-coloured gut (Fig. 7.3). The remaining six larvae in this treatment were not found and were classified as missing. A total of eight of the ten larvae were retrieved from soil inoculated with a single dose of A1MO2 at a rate of 5 x 10^5 CFU/g; of these eight larvae, seven larvae retained a healthy phenotype with a darker-coloured gut. This may suggest that the larvae did not initially ingest a sufficient dose to cause immediate disease. However, as detailed later in Section 7.2.4, whilst these larvae were healthy following retrieval from the soil, they would begin to exhibit amber disease symptoms later during the subsequent incubation, indicating that they were infected with amber disease, but not yet showing symptoms. The other larva retrieved from this treatment was diseased, with an amber-coloured gut, indicative of gut clearance.

Eight larvae were also retrieved from the pots containing soil inoculated with 5.6 at a rate of 5 x 10^5 CFU/g, with all eight of these larvae retaining a healthy phenotype (Fig. 7.3). All ten larvae were retrieved from the soil pre-inoculated with 5.6 at a rate of 5 x 10^4 CFU/g, with nine larvae retaining a healthy phenotype and a single larva displaying amber disease symptoms. Similarly, seven larvae were retrieved from soil pre-inoculated with 5.6 at a rate of 5 x 10^5 CFU/g, with six larvae remaining healthy and a single larva displaying an amber discolouration. Eight larvae were retrieved from soil inoculated with 5.6 at a rate of 5 x 10^6 CFU/g, with all eight larvae displaying a healthy phenotype.

A similar number of larvae were retrieved from soil inoculated with 477 at a rate of 5 x 10^5 CFU/g, with seven larvae being retrieved from the pots (Fig. 7.3). Of these seven larvae six displayed a healthy phenotype, with a desiccated cadaver being retrieved from one pot. Nine larvae were retrieved from soil pre-inoculated with 477 at a rate of 5 x 10^4 CFU/g, with all nine larvae displaying a healthy phenotype. Nine larvae were also retrieved from the soil pre-inoculated with 477 at a rate of 5 x 10^5 CFU/g, of which eight retained a healthy phenotype and one larva exhibited amber discolouration indicative of amber disease or pre-pupation. Eight larvae were retrieved from soil pre-inoculated with 477 at a rate of 5 x 10^6 CFU/g, with all eight larvae exhibiting a healthy phenotype.

A higher mortality rate was recorded amongst the larvae contained in soil inoculated with AGR96X at a rate of 5 x 10^5 CFU/g, with eight larvae being retrieved of the ten larvae initially distributed between the ten pot replicates (Fig. 7.3). Of these eight larvae five retained a healthy phenotype whilst the cadavers of three other larvae were retrieved. The cadavers retrieved from this treatment were blackened with a soft morphology, which typifies AGR96X-induced mortality. Six larvae were retrieved from the soil pre-inoculated with 5.6 and challenged with AGR96X, with all six larvae retaining a healthy phenotype. Similarly, eight larvae were retrieved from soil pre-inoculated with 477 and challenged with AGR96X, of which all eight retained a healthy phenotype (Fig. 7.3).

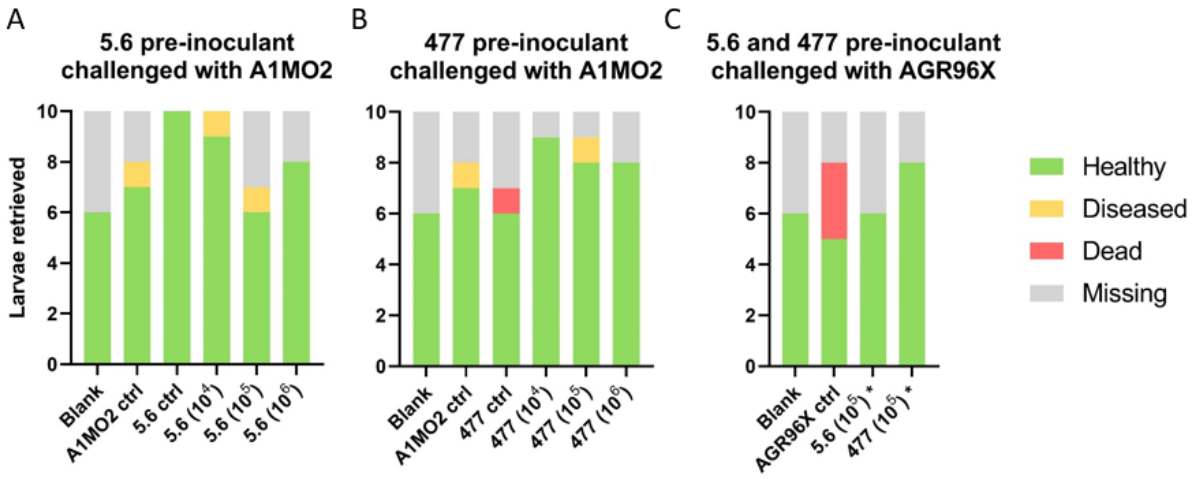


Figure 7.3. The phenotype of each larva retrieved from the ten pots containing soil inoculated with *Serratia* spp. after four weeks. Larvae were classified based on gut colouration, with a dark gut indicative of a healthy larva, and an amber coloured gut indicative of amber disease. Missing larvae were not retrieved following a thorough search of the soil. The soil was pre-inoculated with either (A) 5.6 at varying rates following challenged with A1MO2 at a rate of 5 x 10^5 CFU/g after three days, pre-inoculated with (B) 477 followed by the same challenge rate of A1MO2, or (C) both 5.6 and 477, followed by challenge with AGR96X. The blank treatment pots contained uninoculated soil. * Denotes the use of AGR96X instead of A1MO2.

7.2.3 Further incubation of larvae following retrieval from pots to assess feeding behaviour

Following their retrieval from the soil the larvae were assessed over a further two weeks using a standard ice-cube tray bioassay method (method: Chapter 2, section 2.5.2) to identify any latent amber disease infections. The feeding behaviour and phenotypes of the larvae were assessed to determine whether the pre-inoculation of soil with the non-pathogenic *S. entomophila* spp. 5.6 or 477 had any impact on the efficacy of pathogenic *Serratia* spp. A1MO2 or AGR96X.

Of the four larvae retrieved from the uninoculated soil one larva began to show symptoms of amber disease during the first day of the assay and then died prior to the second day. The remaining three larvae from this treatment remained healthy and feeding over the duration of the 14-day assay (Fig. 7.4). The highest rate of amber disease was identified in the larvae retrieved from the soil inoculated with a single dose of A1MO2 at a rate of 5 x 10^5 CFU/g. By the end of the 14-day assay six of the eight larvae retrieved from the pots were exhibiting amber disease symptoms, primarily a lack of feeding and amber discolouration, indicative of the voiding of gut material. These results suggest that whilst seven of the eight larvae retrieved from the soil in this treatment group initially appeared healthy, as mentioned previously in Section 7.2.2, they may have been carrying a latent amber disease infection which manifested during the subsequent incubation period. Over the duration of the assay the larvae in this treatment which became diseased showed little-to-no feeding behaviour, either not eating the

provided carrot cube pieces, or masticating very small pieces, indicating they were not actively feeding during the assay.

Eight larvae were assessed following retrieval from the soil inoculated with a single dose of 5.6 at a rate of 5 x 10^5 CFU/g. All larvae within this group continued to exhibit feeding behaviour and a dark-coloured gut over the duration of the assay, however one larva died prior to day five of the assay but did not display amber disease symptoms prior to death (Fig. 7.4). All ten larvae retrieved from the soil pre-inoculated with 5.6 at a rate of 5 x 10^4 CFU/g prior to challenge with A1MO2 at a rate of 5 x 10^5 CFU/g were assessed during the observation assay. Of these ten larvae two larvae displayed amber disease symptoms prior to the second day of the assay, progressing to seven larvae by day 14. This rate of amber disease was similar to the rate of disease recorded in larvae exposed to a single dose of A1MO2. Additionally, whilst the three remaining larvae in this treatment continued to exhibit a dark-gut colour only one was still actively feeding by day 14 of the assay.

Increasing the rate at which 5.6 was used to pre-inoculate the soil to 5 x 10^5 CFU/g, prior to challenge with A1MO2 at the same rate, resulted in a reduction in the occurrence of amber disease within the larvae (Fig. 7.4). Of the seven larvae in this treatment, one began to exhibit amber disease symptoms after the first day of the assay, progressing to a total of three diseased larvae by day seven, with no changes by the end of the assay at day 14. Increasing the rate of 5.6 used to pre-inoculate the soil further to 5 x 10^6 CFU/g prior to challenge with A1MO2 at a comparatively lower rate of 5 x 10^5 CFU/g resulted in the lowest occurrence of amber disease amongst the soil treatments pre-inoculated with 5.6. Of the eight larvae assessed in this treatment one exhibited amber disease symptoms within the first day of the assay, with another becoming diseased by day seven. The remaining six larvae in this treatment remained actively feeding over the duration of the assay. No changes were recorded for these larvae by the end of the assay at day 14.

These results suggest that the pre-inoculation of soil with 5.6 at a rate equal to, or in excess of the rate of A1MO2 used to challenge led to a reduction in A1MO2 efficacy. The reduction in amber disease occurrence was found to be significant in larvae retrieved from soil pre-inoculated with 5.6 at a rate of 10^6 CFU/g ($p < 0.05$), but non-significant in both of the other treatments when compared to the larvae only challenged with A1MO2 (Table 7.3).

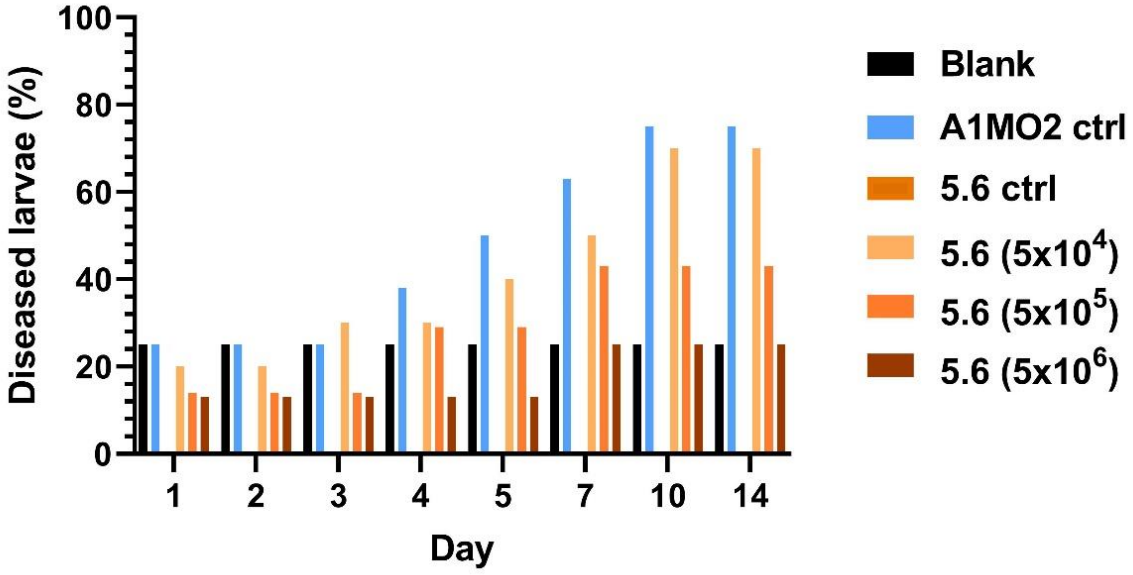


Figure 7.4. The changes in proportion (%) of larvae which exhibited amber disease symptoms during a 14-day observation assay conducted following retrieval of larvae from inoculated soil during a four-week pot trial. The soil was inoculated with a single dose of either A1MO2 or 5.6 at a rate of 5 x 10^5 CFU/g (ctrl) or pre-inoculated with 5.6 at varying rates (5 x 10^4, 10^5, and 10^6 CFU/g) prior to challenge with A1MO2 at a rate of 5 x 10^5 CFU/g after three days. Larvae from the 'Blank' treatment were retrieved from uninoculated soil. Proportions are adjusted to account for missing larvae which were not retrieved from the pots.

Table 7.3. Group-wise logistic regression results comparing disease occurrence in the larvae exposed only to A1MO2 (A1MO2 ctrl) against larvae retrieved from soil pre-inoculated with 5.6. - Denotes no data.

Treatment	Coefficient	Coefficient SE	95 % CI	Z-value	*P*-value
A1MO2 ctrl	1.099	0.816	(-0.502, 2.669)	1.35	0.178
5.6 ctrl	-	-	-	4.90	0.007[1]
5.6 (5x10^4)	-0.25	1.07	(-2.35, 1.84)	-0.24	0.814
5.6 (5x10^5)	-1.39	1.12	(-3.58, 0.81)	-1.24	0.215
5.6 (5x10^6)	-3.04	1.35	(-5.68, 0.41)	-2.26	0.024

[1] Fisher's exact P-value used to compare treatments with 0 % disease rates (5.6 ctrl) to non-0 % disease rates (A1MO2 ctrl).

Six larvae were retrieved from the pots containing soil inoculated with a single dose of 477 at a rate of 5 x 10^5 CFU/g and assessed during the 14-day assay (Fig. 7.5). One larva amongst these six had died by day ten of the assay, however this larva exhibited a dark-red colouration which may have be the result of a fungal infection. The other five larvae in this treatment continued to exhibit feeding behaviour over the bioassay duration. In comparison to the pre-inoculation of soil with 5.6, the pre-inoculation of soil with the naturally occurring non-pathogenic *S. entomophila* isolate 477 resulted in consistent amber disease suppression, regardless of the rate at which it was initially applied to the soil. This

resulted in a total of two larvae from each of the treatments with soil pre-inoculated with 477 at a rate of 5 x 10^4 or 5 x 10^6 CFU/g, prior to challenge with A1MO2 at a rate of 5 x 10^5 CFU/g after three days displaying amber disease symptoms by day 14 of the assay from a total of nine and eight larvae assessed in the respective treatments. Of the nine larvae assessed from the soil pre-inoculated with 477 at a rate of 5 x 10^5 CFU/g prior to challenge with A1MO2 at the same rate after three days only three larvae exhibited amber disease symptoms by day 14 of the assay.

These results highlighted a key finding of how the pre-inoculation of soil with the non-pathogenic *S. entomophila* isolate 477 was effective at reducing the efficacy of A1MO2 when applied at a field rate of 5 x 10^5 CFU/g, reducing the occurrence of amber disease amongst the challenged larvae. This reduction was found to be significant when comparing larvae challenged with A1MO2 against larvae retrieved from soil pre-inoculated with only 477 ($p < 0.05$), and in larvae contained in soil pre-inoculated with 477 at a rate of 10^4 CFU/g ($p < 0.05$) (Table 7.4). Whilst a clear reduction in amber disease incidence amongst the 10^5 and 10^6 CFU/g treatment groups was noted the difference in disease occurrence between these groups and the A1MO2 control group was not significant. However, this reduction in disease may influence down-stream effects such as pathogen recycling in the field.

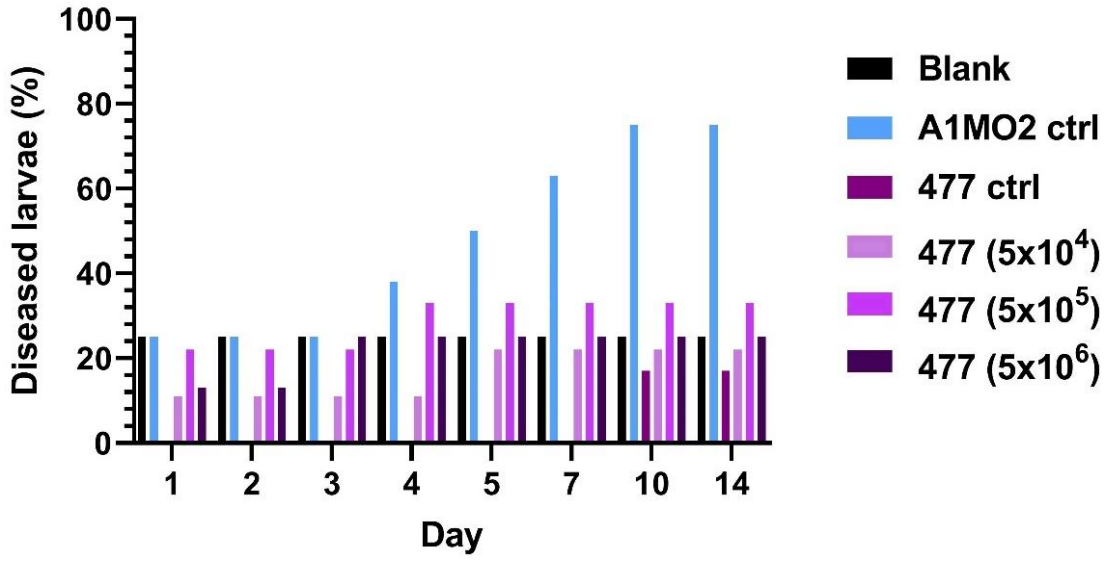


Figure 7.5. The changes in proportion (%) of larvae which exhibited amber disease symptoms during a 14-day observation assay conducted following retrieval of larvae from inoculated soil during a four-week pot trial. The soil was inoculated with a single dose of either A1MO2 or 477 at a rate of 5 x 10^5 CFU/g (ctrl) or pre-inoculated with 477 at varying rates (5 x 10^4, 10^5, and 10^6 CFU/g) prior to challenge with A1MO2 at a rate of 5 x 10^5 CFU/g after three days. Larvae from the 'Blank' treatment were retrieved from uninoculated soil. Proportions are adjusted to account for missing larvae which were not retrieved from the pots.

Table 7.4. Group-wise logistic regression results comparing disease occurrence in the larvae exposed only to A1MO2 (A1MO2 ctrl) against larvae retrieved from soil pre-inoculated with 477.

Treatment	Coefficient	Coefficient SE	95 % CI	Z-value	*P*-value
A1MO2 ctrl	1.099	0.816	(-0.502, 2.669)	1.35	0.178
477 ctrl	-2.71	1.37	(-5.39, - 0.03)	-1.98	0.047
477 ($5x10^4$)	-2.35	1.14	(-4.59, 0.11)	-2.05	0.040
477 ($5x10^5$)	-1.79	1.08	(-3.91, 0.33)	-1.66	0.097
477 ($5x10^6$)	-2.20	1.15	(-4.46, 0.07)	-1.90	0.057

The final group of larvae assessed were those retrieved from the soil challenged with the hyperpathogenic *S. proteamaculans* strain AGR96X. In contrast to the larvae challenged with A1MO2, a lower rate of amber disease occurrence was anticipated in larvae challenged with AGR96X, as infections with AGR96X typically transition from cream, to brown, to black, and then die within 12 days of infection, leaving a soft and blackened cadaver. A total of four larvae were assessed following initial retrieval from soil inoculated with a single dose of AGR96X at a rate of 5 x 10^5 CFU/g (Fig. 7.6). Of these four larvae one died prior to the second day of the observation assay, with another beginning to transition to an amber/brown colour by day five of the assay, indicative of an AGR96X-induced infection. A reduction in disease and mortality rates and a delay in disease onset was observed in the larvae retrieved from soil pre-inoculated with either 5.6 or 477, and, akin to the previous results, a greater reduction in AGR96X efficacy was recorded in larvae retrieved from soil pre-inoculated with 477. One larva amongst the six retrieved from soil pre-inoculated with 5.6 at a rate of 5 x 10^5 CFU/g and challenged with the same rate of AGR96X after three days died prior to the second day of the assay. The remaining five larvae in this treatment continued to show active feeding behaviour until day seven of the assay, after which one larva died prior to day ten and day 14, resulting in three deaths amongst the six larvae. All three of the dead larvae exhibited a blackened colour associated with AGR96X infections. Seven larvae were assessed following retrieval from pots containing soil pre-inoculated with 477 at a rate of 5 x 10^5 CFU/g and challenged with AGR96X at the same rate after three days. All seven of these larvae showed active feeding behaviour until day four of the assay, at which point one larva began to show amber discolouration and died by day five with a blackened discolouration. Another larva began to exhibit amber disease symptoms by day five of the assay and exhibited a cessation of feeding over the end of the 14-day assay.

Whilst these results indicated that the pre-inoculation of soil with either 5.6 or 477 was able to cause a reduction in AGR96X efficacy when it was applied at the same rate, this reduction was not significant when compared to larvae challenged only with AGR96X.

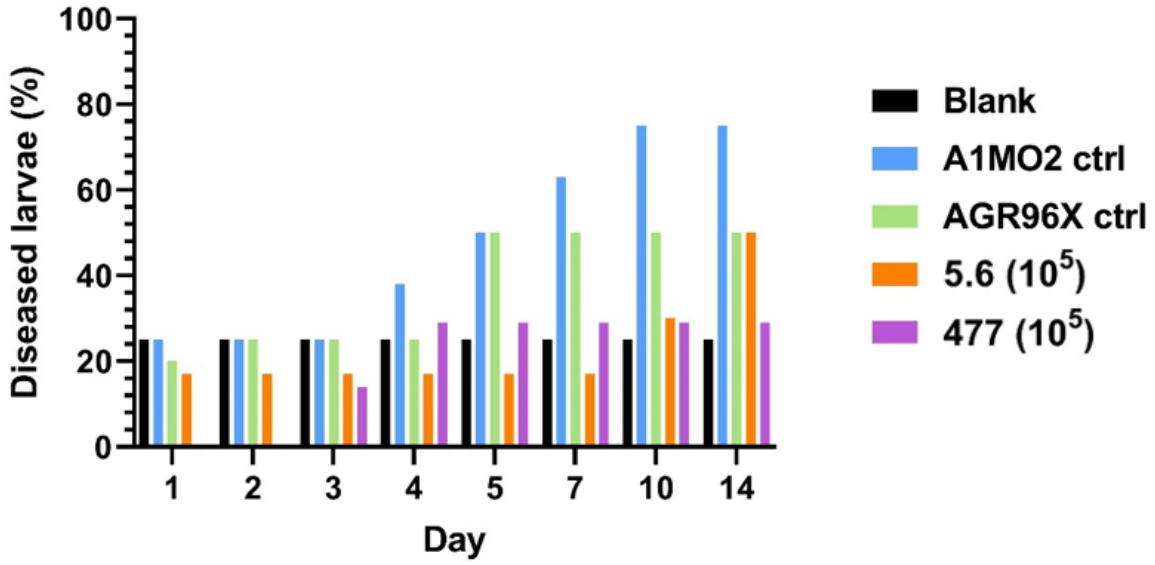


Figure 7.6. The changes in proportion (%) of larvae which exhibited amber disease symptoms and mortality during a 14-day observation assay conducted following retrieval of larvae from inoculated soil during a four-week pot trial. The soil was inoculated with a single dose of either A1MO2 or AGR96X at a rate of 5 x 10^5 CFU/g (ctrl) or pre-inoculated with 5.6 or 477 at the same rate (5 x 10^5 CFU/g) prior to challenge with AGR96X at a rate of 5 x 10^5 CFU/g after three days. Larvae from the 'Blank' treatment were retrieved from uninoculated soil. Proportions are adjusted to account for missing larvae which were not retrieved from the pots.

The results collected during the 14-day observation assay indicate that the pre-inoculation of soil with the non-pathogenic *S. entomophila* strains 5.6 or 477 led to a reduction in amber disease rates following challenge with either A1MO2 or AGR96X after three days (Fig. 7.7). The reduction in amber disease exhibited by 5.6 was dependent upon dose rate, where an increase in the dose rate of 5.6 corresponded with a reduction in amber disease rates. In contrast, the pre-inoculation of soil with 477 resulted in consistent disease suppression, regardless of the rate of 477 dose. In the larvae challenged with AGR96X the pre-inoculation of soil with 5.6 or 477 led to a reduction in disease and mortality rates, with the data showing 477 to be more effective at reducing AGR96X efficacy. This can have significant implications on field trial efficacy for the pathogens, where plasmid-free variants such as 477 are more likely to be prevalent.

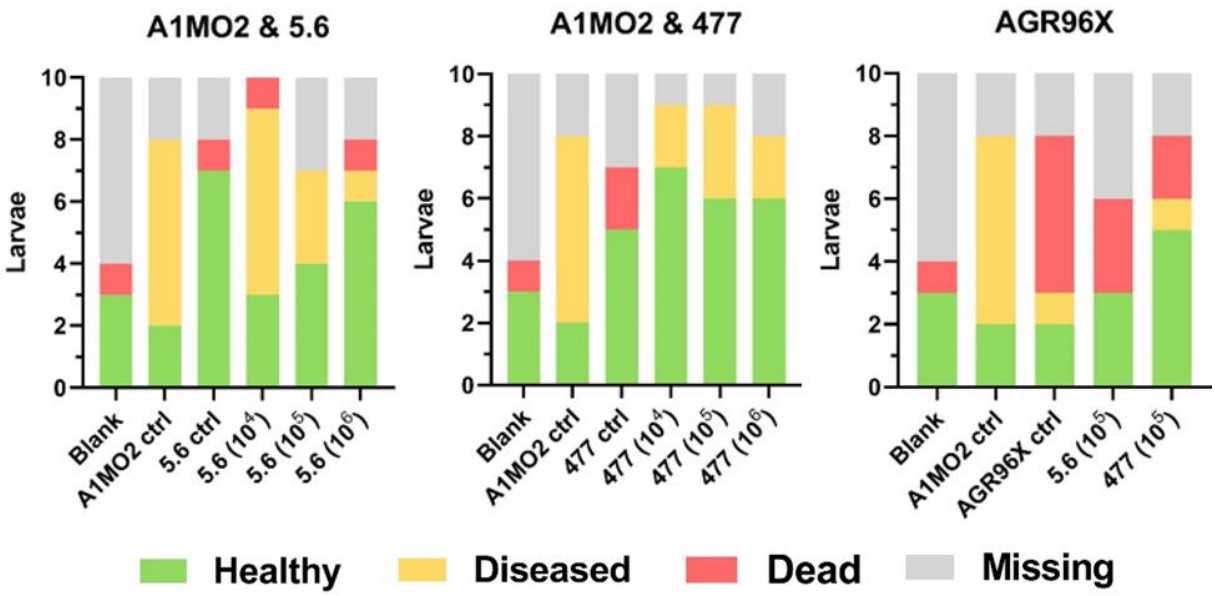


Figure 7.7. The phenotypes of larvae following the conclusion of the 14-day observation assay conducted following the retrieval of larvae from soil inoculated with (A) a single dose of A1MO2 or 5.6 at a rate of 5 x 10^5 CFU/g, or pre-inoculated with 5.6 at rates of 5 x 10^4, 10^5, and 10^6 CFU/g, followed by a dose of A1MO2 at a rate of 5 x 10^5 CFU/g after three days. (B) From soil inoculated with a single dose of 477 at a rate of 5 x 10^5 CFU/g or pre-inoculated with 477 at rates of 5 x 10^4, 10^5, and 10^6 CFU/g, followed by a dose of A1MO2 at a rate of 5 x 10^5 CFU/g after three days. (C) From soil inoculated with a single dose of AGR96X at a rate of 5 x 10^5 CFU/g, or pre-inoculated with 5.6 or 477 at rates of 5 x 10^5 CFU/g, followed by a dose of AGR96X at the same rate after three days. Larvae from the 'Blank' treatment were retrieved from uninoculated soil. Larvae were classified as diseased based on amber discolouration and feeding behaviour. Missing larvae were not found in the soil following the initial pot trial.

7.2.4 Linking ryegrass germination to larvae missing from pots

Following the removal of the larvae from the soil the replicates from which larvae were not retrieved and were classified as missing was cross-referenced against the replicates in which the ryegrass seeds germinated. This would determine whether there was a link between the absence of the larva in the soil and the germination of the seeds.

A comparison between the number of ryegrass seeds which germinated in each of the pots after four weeks and the conditions of the larvae retrieved from the soil established a link between the absence of larvae from the pots and the germination of the seeds (Fig. 7.8). Due to the lack of larvae recovered from these pot replicates this also provides an explanation for the high rate of ryegrass germination in the blank treatment with a larva in uninoculated soil, in which little-to-no plant growth was expected. In these instances the use of the non-pathogenic strains, such as 5.6 and 477 would not have caused amber disease and inhibited larval feeding, preventing plant growth. As plant growth was recorded, this might suggest that the larvae died quickly following their addition to the soil, allowing plant growth in these replicates. Furthermore, in some cases one or two of the three planted ryegrass seeds

germinated in pots from which the larva was retrieved with a healthy phenotype. In these instances, the larva may have consumed the seedlings within the first one to three days following their addition to the pots alongside the challenge dose of A1MO2 or AGR96X and consumed the seedlings prior to becoming infected with amber disease, with amber disease onset preventing further feeding. Conducting a Mantel correlation test between the two matrices indicated that there was a correlation between the distribution of the larvae and the consumption of the plants in the pots ($p < 0.0001$).

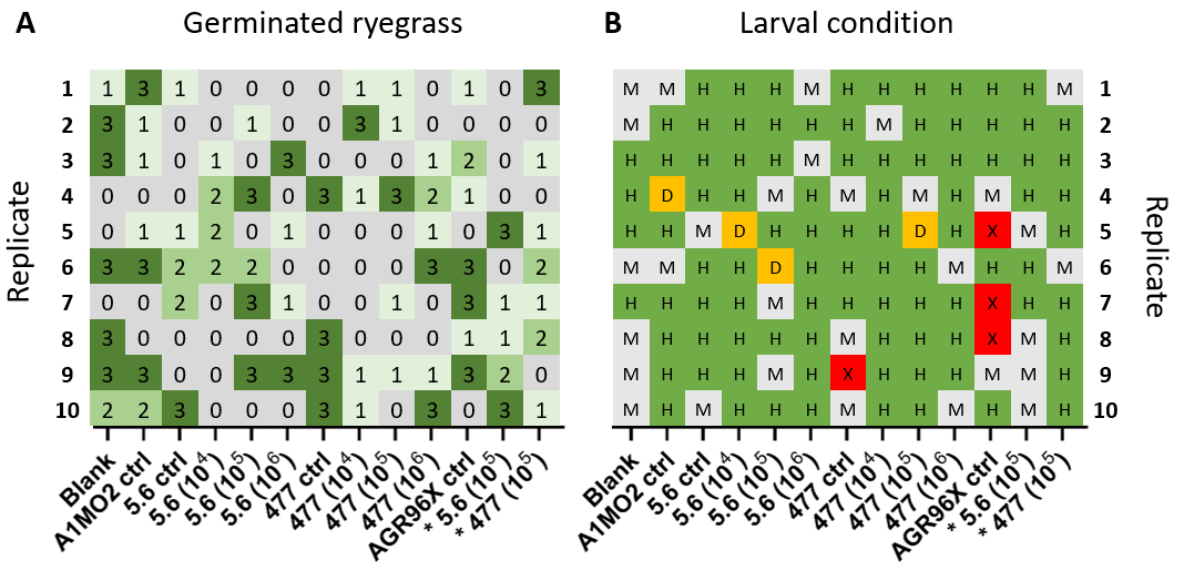


Figure 7.8. A comparison between: (A) the number of ryegrass seeds which germinated in each pot over a four-week period, with each pot containing three seeds and a single healthy grass grub larva, with the number representing how many of the three planted seeds germinated. (B) the condition of the larva retrieved from the corresponding pot, where H/green denotes a healthy phenotype, D/yellow denotes amber disease, X/red denotes the retrieval of a cadaver, and M/grey denotes no trace of the larvae was found in the soil for the corresponding pot. Missing larvae were considered dead.

7.2.5 Isolating viable *Serratia* cells from inoculated soil samples

During the retrieval of the larvae from the soil following the conclusion of the pot trial, presented previously in Section 7.2.2, a 20 g soil sample was collected randomly from three of the ten replicate pots. These soil samples were assessed using an enumeration method (method: Chapter 2, section 2.4.5) to determine the number of viable *Serratia* cells remaining in the soil.

The enumeration of the soil samples provided total CFU counts for inoculated *Serratia* cells ranging between 10^3 and 10^5 CFU/g (Fig. 7.9). Amongst the treatments inoculated with a single dose of either A1MO2, 5.6, 477, or AGR96X at rates of 5 x 10^5 CFU/g the highest CFU counts were recorded in the soil inoculated with a single dose of 477, with a CFU count significantly higher after four weeks than each

of the soil samples inoculated with A1MO2 ($p < 0.005$), 5.6 ($p < 0.05$), or AGR96X ($p < 0.05$). There were no significant differences between the CFU counts of A1MO2, 5.6, or AGR96X in the soil following the conclusion of the pot trial, indicating a similar persistence of the strains. Further assessments were conducted on the cells isolated from the soil pre-inoculated with either 5.6 or 477 and later challenged with AGR96X to determine the ratio of *S. entomophila* to *S. proteamaculans* in the samples, with a detection limit of 10^2 to 10^3 CFU/g. Using DNAse and adonitol typing agars it was confirmed that all colonies isolated from the soil inoculated with a single dose of AGR96X were *S. proteamaculans*. An assessment of the soil pre-inoculated with *S. entomophila* 5.6 at a rate of 5 x 10^5 CFU/g, and then challenged with AGR96X at the same rate revealed that all patched colonies were classified as *S. proteamaculans*, with no *S. entomophila* colonies identified in the assessed samples. In the soil pre-inoculated with 477 a total of 55 % of assessed colonies were classified as *S. entomophila* and presumed to be 477. The increased persistence of 477 in these assessed samples in comparison to 5.6 might also reflect the natural origins of the strain, in comparison to 5.6 which is a laboratory derived heat-cured variant of A1MO2, which may indicate that the strains is more able to compete against A1MO2 under natural circumstances, in contrast to 5.6, which is able to readily compete against A1MO2 in a laboratory setting. No *Serratia* cells were isolated from the uninoculated soil samples.

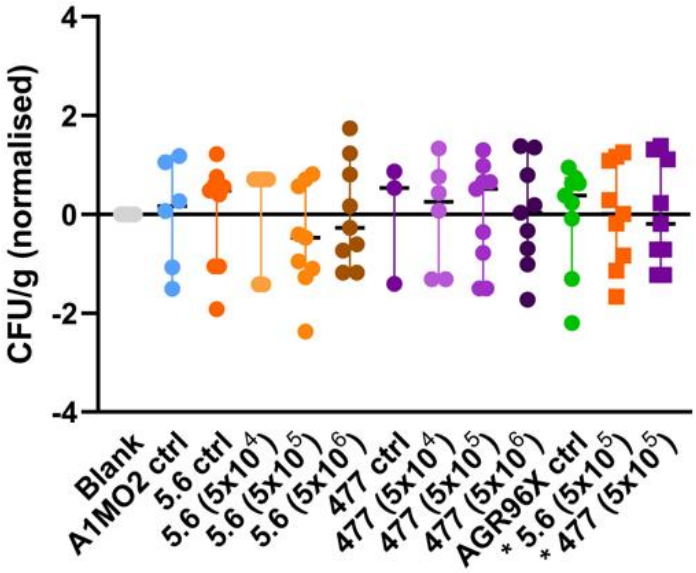


Figure 7.9. Normalised $\log_{10}$ CFU/mL counts of *Serratia* cells enumerated from three 20 g soil samples randomly selected from each treatment following the initial four-week pot trial. Results are presented with standard deviation error bars. Blank soil was uninoculated. * Denotes the addition of AGR96X to the soil instead of A1MO2.

7.2.6 Isolating viable *Serratia* cells from larvae macerated following the bioassay

Following the conclusion of the 14-day feeding observation assay, three larvae from each treatment were selected and macerated to determine the presence of viable *Serratia* cells remaining within the larvae (method: Chapter 2, section 2.5.4). Where possible, the larvae selected for maceration from each treatment exhibited amber disease symptoms, or, in the case of larvae inoculated with AGR96X, the blackened cadavers were selected. The whole-larval macerate samples were plated on a CTA medium with a detection limit of 10^2 CFU/mL, and resultant colonies were patched onto DNAse and adonitol typing agar to also differentiate between *S. entomophila* and *S. proteamaculans* cells.

The plating of the whole-larval macerate samples yielded total CFU counts between 10^2 and 10^5 CFU/g (Fig. 7.10), with the highest CFU counts being recorded in the larvae inoculated with AGR96X, which is typical for the strain. Furthermore, plating of the macerates of the larvae retrieved from soil inoculated with a single dose of 5.6 at a rate of 5 x 10^5 CFU/g resulted in colonies forming on one plate out of nine when serially diluted 1:10 in PBS, indicating either a low persistence or clearance of the strain in the assessed larvae. Additionally, whilst there were no instances of significant difference between the CFU counts of A1MO2, 477, and AGR96X from the macerated larvae, the CFU count for 5.6 was significantly lower than both 477 ($p < 0.05$) and AGR96X ($p < 0.005$).

Patching the cells isolated from the macerates of larvae retrieved from soil inoculated with a single dose of AGR96X to DNAse and adonitol agar resulted in all colonies being classified as *S. proteamaculans* and presumed to be AGR96X. When patching the cells isolated from the macerates of larvae retrieved from soil pre-inoculated with 5.6 and later challenged with AGR96X a total of 8 % of the assessed cells were classified as *S. entomophila* and, based on the absence of *Serratia* from the soil used in the production of the pot trial, are presumed to be the inoculated 5.6 cells. The remainder of the cells were classified as *S. proteamaculans*. Similarly, in the macerates of larvae retrieved from soil pre-inoculated with 477 and challenged with AGR96X, a total of 13 % of the isolated cells were classified as *S. entomophila* following patching, with the remaining cells being classified as *S. proteamaculans*. No *Serratia* cells were isolated from the larvae retrieved from the uninoculated soil samples.

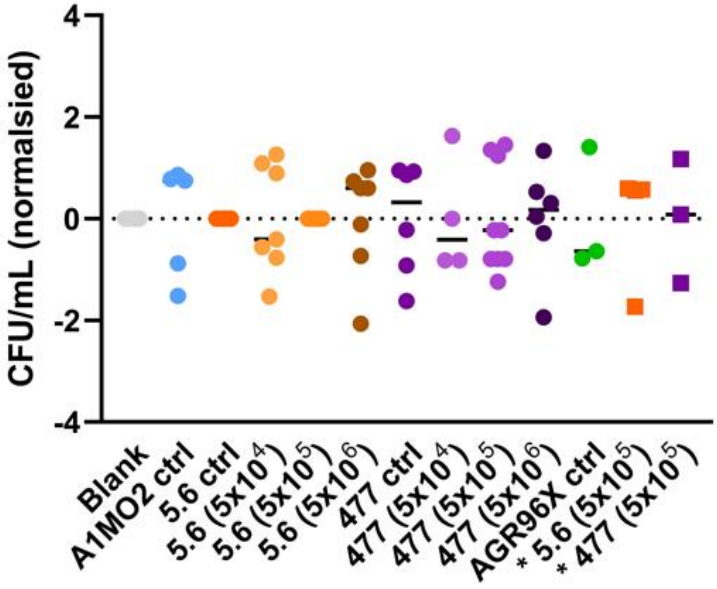


Figure 7.10. The normalised log_{10} CFU/mL counts of *Serratia* cells isolated from the whole-macerate of larvae macerated following the conclusion of the 14-day feeding observation assay. Results are presented with standard deviation error bars. Larvae from the 'Blank' treatment were retrieved from uninoculated soil. * Denotes the addition of AGR96X to the soil instead of A1MO2.

7.3 Discussion

The results obtained during the pot trial and follow-up feeding observation assay suggested that the pre-inoculation of soil with the non-pathogenic *S. entomophila* strains 5.6 and 477 could cause a significant reduction in the efficacy of the pathogenic *S. entomophila* A1MO2 when A1MO2 was subsequently applied to the soil at an expected field rate (5 x 10^5 CFU/g) after three days. The reduction in A1MO2 efficacy caused by the pre-inoculation of soil with 5.6 was found to be dependent on the rate at which 5.6 was applied, with a higher rate of 5.6 (10^6 CFU/g) in the soil resulting in a significant reduction in A1MO2 efficacy. This correlated with results from the *in vivo* pre-inoculation bioassays with grass grub larvae (Chapter 6, section 6.2.3), where the pre-inoculation of larvae with 5.6 led to a reduction and delay in amber disease onset following subsequent challenge with A1MO2 after three days. Furthermore, the results obtained during the pot trial follow-up feeding assay suggested that the pre-inoculation of soil with 5.6 at a rate one log lower (5 x 10^4 CFU/g) than the A1MO2 challenge rate (5 x 10^5 CFU/g) had little-to-no effect on A1MO2 efficacy. In contrast, when the soil was pre-inoculated with 5.6 at a rate one log higher (5 x 10^6 CFU/g) than A1MO2 a significant reduction in A1MO2 efficacy was observed.

This indicated that in order to maintain the expected levels of A1MO2 efficacy in the presence of 5.6, A1MO2 would need to be present at the field at a rate at least one to two log CFU/g higher in the soil. A reduction in pathogen efficacy following pre-inoculation with a non-pathogenic species in the field has also been demonstrated with *Streptomyces scabies* (Wanner et al., 2013). In this study the pre-inoculation of potatoes with non-pathogenic *Streptomyces* spp. reduced the incidence and severity of potato common scab following challenge with *S. scabies*. This method, termed competitive exclusion (Hardin, 1960), has also been used as a method for the biological control. It focuses on the establishment of a favourable competitor species with a high relative fitness preventing a cheat species from gaining a foothold within a specific niche. This method has been explored with *Bacillus cereus* to control the fish pathogen *Aeromonas hydrophila*, where, due to a faster growth rate, *B. cereus* is able to outcompete *A. hydrophila*, thereby depriving *A. hydrophila* of resources required for growth and preventing disease of fish in aquaculture (Laloo et al., 2010). Competitive exclusion has also been tested with pathogenic and non-pathogenic *Fusarium* endophytes species, in which the pre-inoculation of banana roots with the non-pathogenic species was able to cause a significant reduction in the occurrence of Fusarium wilt (Panama disease) following challenge with the pathogenic species (Belgrove et al., 2011; Bubici et al., 2019), similar to the results of the pot trial in the present study.

Another finding from the post-pot trial feeding bioassay was that the pre-inoculation of soil with 477, a naturally occurring non-pathogenic *S. entomophila* isolated from the Canterbury region, was also able to reduce A1MO2 efficacy by a significant amount. Moreover, unlike 5.6, the reduction in efficacy

caused by 477 was not found to be dependent on the rate at which 477 was initially applied to the soil. In this instance the reduction in A1MO2 efficacy was consistent between all of the pre-inoculation dose rates, however, only the pre-inoculation of soil with 477 at a rate of 10^4 CFU/g was found to cause a significant reduction. This difference between 5.6 and 477 may stem from the natural origin of 477, leading to an increased fitness within the soil. Recent studies by Sitter (2020) and Vaughan (2021) have shown that the naturally occurring plasmid-free *Serratia* variants have likely always been plasmid-free, and thus may be more acclimated to competition with other microbial species in the soil. The bioassay results for 477 also demonstrate a departure from *in vitro* mixed inoculation of A1MO2 and 477 in LB broth (Chapter 3, section 3.2.4.b) where in the liquid medium A1MO2 was shown to have a similar or higher fitness that 477. Interestingly, a similar dynamic was demonstrated with *Bacillus* spp. by Raymond et al. (2007). In their study the pathogenic *B. thuringiensis* subsp. *kurstaki* (Btk) outcompeted the non-pathogenic *B. thuringiensis* subsp. *tenebrionis* (Btt) in LB broth, but during *in vivo* assessments with diamondback moth larvae the non-pathogenic Btt was shown to outcompete Btk. However, this competition *in vivo* did not result in a reduction of Btk efficacy. This may reflect a difference between the insecticidal actions of the pathogens, where Btk kills insects through rapid-acting extracellular toxins.

A reduction in efficacy caused by 5.6 and 477 was also noted for the hyperpathogenic *S. proteamaculans* strain AGR96X. Infections of grass grub larvae with AGR96X have been shown to cause mortality in up to 87 % of challenged larvae (Hurst et al., 2018). However, during the pot trial bioassay the pre-inoculation of soil with 5.6 and 477 led to a reduction in mortality from ~ 63 % in the larvae challenged with a single dose of AGR96X to 50 % in larvae pre-inoculated with 5.6, and 25 % in larvae pre-inoculated with 477. The acclimation of 477 in the soil was also evident from the proportions of *S. entomophila* and *S. proteamaculans* in the assessed soil samples. In the samples of soil inoculated with both 477 and AGR96X 55 % of the colonies produced during enumeration were *S. entomophila*, whereas none of the cells in the soil inoculated with 5.6 and AGR96X were *S. entomophila*. Additionally, *S. proteamaculans* cells presumed to be AGR96X constituted a higher proportion of the cells isolated from the whole-larval macerate samples or larvae retrieved from the soil samples. This indicates an increased fitness of AGR96X in the larvae, wherein it has been shown to attain a high bacterial load (Hurst et al., 2018). This finding is also of importance as AGR96X has become a potential candidate for the development of new biopesticides for the management of both grass grub and manuka beetle larvae. Therefore, further testing using a wider array of dose rates is required to see if the reduction in AGR96X is dependent upon the dose rate of the non-pathogenic strain in the soil, as was seen with A1MO2. This would also allow for the testing of pathogenic *Serratia* strains alongside non-pathogenic *Serratia* strains at the rates they are often found in the soil (10^3 to 10^5 bacteria/g of pasture soil: O'Callaghan et al., 1999).

The pot trial and follow-up bioassay results obtained also correlate with a model based on the interactions between *S. entomophila* and *C. giveni* larvae described by Godfray et al. (1999). This model describes how saprophytic reproduction of *S. entomophila* in the soil may lead to a reduction in the number of grass grub larvae required to sustain an infection amongst a grass grub population. This may link to the reduction in disease observed during the pot trial, where the presence of the non-pathogenic strain prevents infections from occurring. The model also predicts that a reduction in the host threshold is diminished during competition for saprophytic substrates with non-pathogenic *Serratia* spp. free-living in the soil, as the non-pathogenic strains were speculated to be better competitors. As such, competition between the pathogenic and non-pathogenic *Serratia* spp. would lead to a reduction in efficacy, limiting the use of pathogenic strains to control grass grub populations in situations of high loadings of non-pathogenic strains in the soil. Whilst this shows promise, increasing the number of replicates used, for both pots and larvae, would help to strengthen the results obtained. Using an increased number of replicates would also help to alleviate the number of losses that were observed during the present trial, allowing more opportunities to gather data from any remaining larvae.

These results show that under the assessed conditions the pre-inoculation, or presence, of non-pathogenic *Serratia* spp. in natural soil samples appears to have the potential to reduce the efficacy of pathogenic *Serratia* spp. such as A1MO2 and AGR96X. This correlates with the results obtained from *in vivo* bioassays with grass grub larvae in which the pre-inoculation of larvae with non-pathogenic *Serratia* spp. also led to a reduction in pathogen efficacy. Further testing with additional naturally occurring plasmid-free *Serratia* isolates is required to determine whether a similar outcome would be observed when tested during large-scale field trials using an increased number of grass grub larvae.

Chapter 8

General discussion

8.1 An investigation of *Serratia* dynamics

The purpose of this study was to explore the dynamics between pathogenic and non-pathogenic *Serratia* strains, and to determine whether these interactions have any impact on the efficacy of pathogenic *Serratia* spp. when used as biopesticides to manage grass grubs. These dynamics are of importance because it is estimated that between 40 to 60 % of background *Serratia* spp. within the soil of pastures are non-pathogenic to grass grub larvae (Jackson et al., 2001). Therefore, if a link between the presence of non-pathogenic *Serratia* strains and a reduction in the efficacy of pathogenic *Serratia* strains is established, this will help to shape both the development of future biopesticides, whilst also guiding the optimal method for their application in the field. This premise was approached using constructed *Serratia* strains with antibiotic markers inserted into an intergenic region of the chromosome and pADAP backbone to differentiate between pathogenic and non-pathogenic strains during mixed inoculation assays. Of chief interest during this study were the interactions between A1MO2, a chronic *S. entomophila* strain used as a biopesticide for grass grub larvae, and 5.6, an isogenic plasmid-cured non-pathogenic variant of A1MO2. In addition to A1MO2 and 5.6, the non-pathogenic *S. entomophila* strain 477, and the hyperpathogenic *S. proteamaculans* strain AGR96X were also assessed during mixed inoculation assays. This provided a reference for the dynamics of both a naturally occurring plasmid-free *S. entomophila* strain (477), and also for a pathogenic *Serratia* isolate associated with increased virulence and mortality towards both grass grub larvae and manuka beetles (AGR96X) with potential to be used in biopesticide development (Hurst et al., 2018).

8.2 The links between the *Bacillus* and *Serratia* biopesticide systems

At many points during this study comparisons were made to similar work conducted using *Bacillus* spp., particularly *B. thuringiensis*, which are also used as biopesticides for the management of a wide variety of pest species (Argôlo-Filho and Loguercio, 2013). Many of these studies document the occurrence of competition between *Bacillus* spp. which produce extracellular insecticidal toxins, and strains which do not. In these instances, the non-pathogenic *Bacillus* spp. take advantage of the toxin effects whilst providing nothing in return and are termed as ‘cheats’. This interaction can sometimes cause an overall reduction in the density of pathogenic *Bacillus* strains (Raymond et al., 2007; Garbutt et al., 2011), as it is more efficient for the non-pathogenic strains to cheat, rather than producing these metabolically costly toxins, a phenomenon often referred to as the ‘tragedy of the commons’. It has

been speculated that a similar dynamic might occur between pathogenic and non-pathogenic *Serratia* spp., where an increased competitiveness of non-pathogenic *Serratia* spp. leads to a lower occurrence of amber disease in grass grub larvae. A previous model of competition between pathogenic and non-pathogenic *Serratia* spp. predicted this outcome, as the non-pathogenic strains were assumed to be better competitors due to the lack of plasmid carriage (Godfray et al., 1999). The results obtained during the present study also support this model, as the mixed inoculation of pathogenic and non-pathogenic *Serratia* spp. led to a reduction in the virulence of A1MO2 and AGR96X during grass grub bioassays and the pot trial. In this instance, competitive exclusion by the non-pathogenic strains may be preventing the pathogenic *Serratia* species from exerting insecticidal actions and causing disease within the larvae. In contrast to the *Serratia* system, mixed inoculation *in vivo* experiments with pathogenic and non-pathogenic *Bacillus* spp. still resulted in similar mortality rates amongst challenged diamondback moth (96 % mortality) (Raymond et al., 2007), with mixed inoculation not having a significant impact on mortality. This might suggest a distinct difference between the systems, where the presence of non-pathogenic *Bacillus* cheat strains does not limit the insecticidal effects of the extracellular crystal toxins, whereas the presence of non-pathogenic *Serratia* strains can limit the efficacy of the pathogenic strains through competitive exclusion.

8.3 Growth dynamics in a simplified liquid-broth system

The use of *in vitro* assays with liquid media served as the starting point for an assessment of competition between the *Serratia* strains, using a controlled nutrient-rich environment (LB broth) to allow for optimal growth. When using an LB broth medium 5.6 was shown to consistently outcompete A1MO2 during mixed inoculation in two, distinct ways. Firstly, during the exponential growth period 5.6 exhibited a faster doubling time than A1MO2, and secondly, when cultured over a period of seven days 5.6 demonstrated a higher persistence in the medium, relative to A1MO2. It was also noted that this outcome was not dependent upon the initial cell density of 5.6 within the mixed inocula, as 5.6 was able to outcompete A1MO2 even when it comprised a small proportion of the initial inoculant. This outcome links to previous work with cheats in other bacterial species, which suggests that the reduced frequency of a cheat strain allows for the greater exploitation of communal resources (Patel et al., 2019), such as the nutrients in the broth. It also aligns with the hypothesis for this aspect of the project, in which 5.6 was predicted to outcompete A1MO2 in the liquid broth.

Interestingly, the same dynamic was not observed during the mixed inoculation of A1MO2 and 477 in LB broth. In this instance A1MO2 was able to show either a similar, or slightly higher persistence within the cultures than 477, suggesting strain variability whereby other non-pathogenic strains might not carry the same traits leading to increased competitive behaviour as 5.6, or may be lamadapted to the

liquid medium. Further testing using additional naturally occurring non-pathogenic *Serratia* strains is needed to validate the competitive interactions between A1MO2 and 5.6. As these interactions occurred under optimal conditions in a nutrient-rich environment, it might not translate to the outcome of competition between pathogenic and non-pathogenic strains in the field environment. The mixed inoculation of A1MO2 and 5.6 into a restricted-nutrient M9 medium using either glucose or casamino acids as the supplemental carbon source led to a different outcome. Whilst 5.6 exhibited a faster doubling time than A1MO2 in the M9 medium in the mid to late exponential growth phase, and thus obtained higher cell counts, both strains demonstrated a similar level of persistence within the culture over a seven-day period. This outcome correlates with previous findings which detail how stressors such as nutrient scarcity within the medium can lead to the onset of co-existence between two competing species (Kümmerli et al., 2010). Another key component of the *in vitro* assessments was the use of competition coefficients to describe how competitive exclusion may occur within the flasks over a longer period of time. As 5.6 typically exhibited a higher competition coefficient than A1MO2, and a lower degree of self-limitation, as derived from the Lotka/Volterra-style competition models in LB broth, it can be assumed that 5.6 is more able to outcompete A1MO2 in the liquid cultures. In a closed culture, with no nutrients being added, this would lead to the earlier death of A1MO2. Interestingly, the opposite was seen with A1MO2 and 477, where the competition coefficients suggested that 477 was the poorest competitor. This provides further evidence that there may be intrinsic differences between the competitive capabilities of the non-pathogenic strains in a liquid medium, as was speculated earlier. Though, as mentioned, increasing the level of replication would help to increase the model accuracy, resulting in more reliable coefficient values.

8.4 Chromosomal differences between the strains impacting competition

The results obtained during the *in vitro* culturing of A1MO2 and 5.6 prompted further investigation into any chromosomal genetic differences between the strains, as the only difference between the strains was the carriage of pADAP. This allowed for an assessment of how plasmid carriage might impact the expression of chromosomal genes, and how this might link to the trends exhibited by 5.6 during mixed inoculation with A1MO2 thus far. Through RNA sequencing of 5.6 and 5.6+pADAP (an A1MO2 proxy strain) it was discovered that there were many chromosomal genes with differing levels of expression between the strains. The most notable was the significantly increased expression of genes for maltose and trehalose transport and metabolism operons in 5.6 during the mid-exponential growth phase. These findings indicated that there were differences in chromosomal gene expression between the pathogenic and non-pathogenic strains, as initially hypothesised, with the chromosomal genes identified during this study able to confer an increased competitive ability to 5.6. During follow-

up *in vitro* assessments using an M9 medium with these carbon sources 5.6 exhibited a faster doubling time than A1MO2 during exponential growth, coinciding with the increased expression of these operons. However, when grown in cultures over seven days 5.6 displayed a much greater competitive behaviour within the maltose medium, outcompeting A1MO2 within one day, and then maintaining significantly higher CFU counts over the assay duration. Subsequent investigation with the re-inoculation of a spent maltose medium led to an initial co-existence of the strains within the spent medium over three days, likely due to nutrient scarcity, after which 5.6 showed an increase in growth relative to A1MO2. This growth may have also stemmed from the utilisation of resources from dead and lysed cells, as in each of the 48-hour growth curve experiments in both LB broth and M9 media no additional growth was recorded for the strains after 24 hpi.

The increased expression of the maltose operon in 5.6 may confer increased competitive capabilities of the strain within the field, as maltose can accumulate at plant roots (Lu et al., 2006). The consumption of plant roots by grass grub larvae, and the release of maltose into the soil may provide a favourable environment for the growth of 5.6, enabling it to outcompete A1MO2. Additionally, maltose and maltodextrins have been utilised as a preferential sugar by *E. coli* when switching from a sequential to simultaneous substrate utilisation, ahead of other sugars such as galactose and glycerol (Baev et al., 2006). A similar preferential utilisation of the maltose may have occurred in the restricted M9 medium, where the increased expression of the maltose-related genes allowed 5.6 to outcompete A1MO2 when cultured over seven days.

Trehalose is found within the insect haemolymph, so the increased expression of this operon is unlikely to confer an immediate fitness advantage for 5.6, as trehalose typically would not be encountered until the final stages of an amber disease infection. Therefore, whilst the presence of trehalose would not present an immediate benefit to 5.6, the increased expression of the trehalose-related genes in the RNAseq data might provide a competitive advantage during the latter stages of an infection once the haemocoel has been breached. This could facilitate the increased growth of 5.6 within the larval cadaver, relative to A1MO2. A study on the trehalose content of the grass grub larvae, in relation to other sugars such as glucose would help to provide a reference for how beneficial the increased expression of the trehalose genes may be. Further RNA sequencing would help to determine the differences in chromosomal gene expression for other *Serratia* strains, and what differences there may be between a naturally occurring non-pathogenic strain such as 477 and A1MO2.

8.5 Competition between pathogenic and non-pathogenic *Serratia* spp. in soil

Less competition was seen between A1MO2 and 5.6 when inoculated together in soil microcosms and assessed over a two-month period, likely reflecting limited and slower growth in a soil environment. When inoculated into soil-only microcosms both strains showed a similar level of persistence, with the number of viable cells decreasing at a steady rate over the assay duration. This is consistent with previous findings for *Serratia* spp. when inoculated into soil (O'Callaghan and Gerard, 2005), and may suggest a co-existence of the cells in the soil. However, following the addition of a single healthy grass grub larvae to the microcosms a higher proportion of 5.6 cells were isolated from the soil, relative to A1MO2, over the duration of the assay. This resulted in a significant difference in the CFU counts in some of the mixed ratio treatments. This outcome might suggest that the presence of larva within the microcosms provided a change to the soil conditions, which were favourable to the growth of 5.6, however it is unclear what this change might have been. This increase in 5.6 proportion was primarily observed after a period of 28 days. Due to this delay, the increase in proportion observed for 5.6 may have resulted from the release of sugars such as glucose and fructose, and starches, which can be metabolised into maltose, from carrot cube pieces added to the microcosms to feed the larvae. This may have been due to the carrot cube pieces not being retrieved during a cursory search of the microcosms, allowing for their continued breakdown in the soil. These findings partly align with the initial hypothesis made for the soil-based assessments, which predicted 5.6 outcompeting A1MO2 in the soil, as it did during the initial *in vitro* experimentation. Whilst 5.6 did not outcompete A1MO2 in the soil-only microcosms 5.6 did outcompete A1MO2 in the microcosms which contained healthy grass grub larvae. Therefore, further experimentation with other non-pathogenic *Serratia* strains, such as *S. entomophila* 477, would help to determine if a similar persistence between pathogen and non-pathogenic is consistent and repeatable. Additionally, further experimentation with larvae might help to elucidate the impact of their presence in the microcosms, and how this altered the outcome of competition. The increased persistence of 5.6 in the soil might also link to the speculation that plasmid-free *Serratia* spp. carry adaptations that make them more able to survive within the soil than plasmid-bearing strains (Vaughan, 2021).

Despite the occurrence of amber disease in the larvae contained in the soil microcosms, no viable *Serratia* cells were isolated from the plating of whole-larval macerates. The cells may have been present, but at levels below the limit of detection for the assay (~ 10^3 CFU/mL), or it may have been due to the larvae being in a pre-pupa state, in which they would have taken on an amber colouration due to fat build-up and would have exhibited less feeding than regular 3rd instar grass grub larvae. For comparison, the larvae macerated after the conclusion of the pot trial observation assay (six weeks total) contained ~ 10^{2-3} CFU/mL from 50 µL of a 1:10 serial dilution of whole-larval macerate.

8.6 Competition within the grass grub larvae

During laboratory-based bioassays the effects of competition between A1MO2 and 5.6 were most noticeable during *in vivo* assessments of mixed inoculation with grass grub larvae. The initial strain density in the mixed inoculant impacted the onset of amber disease, where the inoculation of 5.6 at a higher density reduced disease occurrence and delayed the onset of disease in larvae challenged with A1MO2, with significant reductions in disease being noted in some mixed ratio treatments. Whilst this might indicate a potential rate effect, where a lower dose of the pathogen correlates with a lower incidence of disease, the minimum rate of a pathogenic *Serratia* spp. required to cause disease in 50 % of challenged larvae between 10^4 and 10^5 CFU/mL (Jackson et al., 2001; Vaughan, 2021). During a preliminary version of the pre-inoculation bioassay, a dose of A1MO2 at a rate of ~ 5 x 10^4 CFU/mL was able to cause amber disease in 75 % of challenged larvae. This may indicate that the non-pathogenic *Serratia* strains were acting as cheats within the larvae, where their presence caused a reduction in efficacy by limiting the growth of the pathogenic strains. This would allow the non-pathogenic strains access to nutrients in the diseased larvae without being excreted but could potentially prevent disease from occurring. This could lead to a scenario where the increased presence of cheating non-pathogenic *Serratia* strains prevents disease from occurring in a grass grub pasture population. This would lead to fewer diseased larvae, and would prevent, or limit, access of the strains to the larvae. A similar outcome was observed during the pre-inoculation of larvae with 5.6 and subsequent challenge with A1MO2, where an increased rate of 5.6 correlated with a reduction in amber disease occurrence, and a delay in disease onset, with significant disease reductions also being recorded. This is one of the key findings of the project, supporting the theory that the prior presence of non-pathogenic *Serratia* strains can reduce the efficacy of pathogenic *Serratia* strains. This outcome also supports the initial hypothesis, indicating that 5.6 was able to reduce A1MO2 efficacy in the larvae during both mixed inoculation and pre-inoculation experiments. A reduction in disease onset was also observed during mixed inoculation and pre-inoculation bioassays using A1MO2 and 477, which also led to significant reductions in amber disease incidence in some of the mixed and pre-inoculant treatments when larvae were challenged with A1MO2. This further supports the hypothesis, providing evidence for the competitiveness of other non-pathogenic *Serratia* strains during mixed inoculation experiments. To ensure that this finding is consistent, further work with additional non-pathogenic strains is needed.

Evidence for a low rate of pADAP horizontal gene transfer (HGT) occurrence between A1MO2 and 5.6 was also found during *in vivo* assessments, with HGT first detected four days after inoculation during a 14-day assessment. These transconjugant cells were more often isolated from the whole-macerate of larvae inoculated with equal proportions of A1MO2 and 5.6, suggesting that a balance in cell density favoured HGT under the conditions assessed. During this experiment HGT was validated in nine larvae out of the 135 larvae challenged with a mixed ratio inoculant (6 % of challenged larvae). In addition,

HGT was also recorded between A1MO2 and Tukino, a non-pathogenic *S. proteamaculans* isolate, indicating that plasmid transfer can occur between *Serratia* species. However, the low rate of HGT under the conditions assessed suggested that it is unlikely to have a large impact on the densities of pathogenic and non-pathogenic *Serratia* spp. within the field. HGT was not observed in the soil microcosms under the conditions assessed, or in liquid cultures, as the continuous mixing of medium within the liquid culture flasks likely impeded cell-to-cell contact. Previous work on HGT between pathogenic and non-pathogenic *Serratia* spp. by Dodd (2003) found evidence for HGT in both soil microcosms and within the larvae occurring at a higher rate. However, under these conditions the strains were inoculated at rates two log CFU/g of soil higher than the present study, thus providing a higher chance for HGT to occur, though at a rate less realistic under field conditions, in which pathogenic *Serratia* strains are usually applied at a rate between 10^5 and 10^6 CFU/g of soil.

8.7 Competition during a pot trial

The assessment of competition between pathogenic and non-pathogenic *Serratia* spp. using a pot trial came closest to assessing the potential interactions between the strains under natural field conditions. The pre-inoculation of soil with either 5.6 or 477 prior to challenge with A1MO2 at a field rate of 5 x 10^5 CFU/g of soil led to a reduction in efficacy for A1MO2 when applied as a liquid drench. This reduction in amber disease incidence caused by the presence of 5.6 and 477 was found to be significant in some of the treatments (5 x 10^6 CFU/g for 5.6, and 5 x 10^4 CFU/g for 477). Amber disease incidence was reduced from 75 % in the A1MO2-only control group to lower than 22 % of larvae where the reduction in amber disease caused by 5.6 was found to be dependent upon the strength of the initial 5.6 dose. A strong reduction in amber disease incidence was recorded in all 477 pre-inoculated treatment groups. This presented a contrast to previous bioassay experiments conducted during this study, in which the direct pre-inoculation of larvae with 477 only caused a slight reduction in amber disease occurrence, highlighting how 477 may be more competitive in a soil environment than 5.6 or A1MO2. In addition, the pre-inoculation of soil with 5.6 and 477 led to a reduction in efficacy for AGR96X, though the reduction was not statistically significant. Previous *in vivo* experimentation with this strain resulted in a mortality rate of approximately 87 % (Hurst et al., 2018), with an estimated morality rate of 50 % during the pot trial in the present study. However, pre-inoculating soil with 5.6 resulted in a reduced morality rate of 30 % in retrieved larvae, whilst the pre-inoculation of soil with 477 reduced mortality rates to 20 % in retrieved larvae. Whilst this shows that AGR96X efficacy can be impacted by non-pathogenic strains, potentially limiting its utility, further work is required to determine how altering the dose-strength of the strains may change mortality rates.

Like the *in vivo* experiments, the findings obtained during the pot trial support the initial hypothesis that the pre-inoculation of soil with non-pathogenic *Serratia* spp. would cause a reduction in pathogen efficacy when subsequently applied after three days. The reduction in disease noted during the experiment may be due to the concept of competitive exclusion, in which, due to the presence of the non-pathogenic strains (more-so in the treatments with soil pre-inoculated with the non-pathogens at a higher rate), the larvae did not ingest a sufficient number of pathogenic *Serratia* cells to initiate disease. This concept has been explored previously with both fungal species, and pathogenic and non-pathogenic bacterial species in pot and field trials, and often shows how the pre-inoculation or exposure to non-pathogenic species prior to challenge with a pathogen can result in a significant reduction in disease incidence amongst the challenged species (Laloo et al., 2010; Raymond et al., 2010; Belgrove et al., 2011; Wanner et al., 2013; Bubici et al., 2019).

This finding also links back to the concept of competitive exclusion, in which the actions of the non-pathogenic strains in the soil (causing a reduction in efficacy for A1MO2) leads to fewer grass grub larvae becoming diseased, which might detrimentally impact the populations of *Serratia* in the soil. Whilst the non-pathogenic strains can acquire nutrients in larvae with established amber disease infections during mixed infections, a reduction in the efficacy of the pathogen will lead to fewer opportunities for mixed infections, as the presence of the non-pathogenic strain would prevent disease from occurring. This links back to the predictions of Godfray et al. (1999), where a reduction in pathogen efficacy correlates with a reduction in the host threshold density for the larvae, leading to fewer infections because there are not enough pathogenic bacteria to sustain an infection amongst the larvae population in the pasture.

8.8 Steps to improve the efficacy of *Serratia* biopesticides

Whilst the effects of 5.6 and 477 regarding reduction of efficacy for A1MO2 and AGR96X are clear in this data, further work using a full-scale field trial is required to validate these findings, to determine if a similar outcome would be observed for larvae in pastures. As non-pathogenic *Serratia* spp. comprise between 40 to 60 % of *Serratia* spp. within the soil of New Zealand's pastures it can be speculated that these background populations are limiting the efficacy of strains such as A1MO2 when applied as a biopesticide or contributing to field variability of disease occurrence. Based on the pot trial data, a practical approach for overcoming this limitation would be to survey the density of non-pathogenic *Serratia* strains within a given area of soil, followed by the application of A1MO2 at a rate two log CFU/g of soil higher than the non-pathogen population, with a minimum applied field rate between 10^6 and 10^7 CFU/g soil. However, further testing with 477 is required to determine the rate at which it no longer causes a reduction in efficacy for A1MO2, as in the pot trial 477 was effective at

reducing A1MO2 efficacy at all pre-inoculated rates, which can have further impacts on pathogen recycling in the field. A similar solution might be expected with AGR96X, where increasing the rate of application of AGR96X to exceed the background level of non-pathogenic *Serratia* spp. within the soil allows for optimal efficacy of the pathogen, as AGR96X was shown to be more competitive than A1MO2 during the pot trial based on the reductions caused by the non-pathogenic strains. This would also allow for continued development of AGR96X as a biopesticide of grass grub larvae, providing a fast-acting alternative to A1MO2. In addition, the results from the *in vitro* experiments showed that when inoculated together at equal proportions in an LB broth medium, both A1MO2 and AGR96X were able to co-exist, with neither strain outcompeting the other, indicating a potential for a combination treatment. However, additional pot trials using both pathogenic strains in tandem would be required to determine their efficacy when compared to each strain when used separately.

In summation, the results obtained over the course of this study demonstrate the competitive capabilities of non-pathogenic *Serratia* strains, particularly 5.6 and 477, and how mixed inoculation can lead to a reduction in efficacy for pathogenic *Serratia* strains, limiting their use as biopesticides. Further work with larger pot trials and full-scale field trials and other non-pathogenic *Serratia* strains is required to fully validate these results, as this will allow for the inclusion of other factors such as the bacterial and fungal species present in the rhizosphere which may also influence competition via competition for resources and competitive exclusion. In addition to larger-scale field trials, the continued assessment of pasture soil samples would allow for the isolation of variant *Serratia* spp. which may exhibit more competitive behaviours. These findings would also be strengthened with additional assessments covering a variety of other naturally occurring non-pathogenic *Serratia* spp. to determine whether any competitive differences exist between the different strains.

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Appendix A

Chapter 2

Below follows the recipes used in various media and reagents, described previously in Chapter 2 (Methods and Materials).

A.1 Medias and agar

Caprylate thallous agar solution A:
0.3 g $MgSO_4.7H_2O$
1.36 g KH_2PO_4
5.22 g K_2HPO_4
2 mL 0.1M $CaCl_2$ solution
2 mL Trace element solution (recipe below)
2.2 mL $CH_3(CH_2)_6COOH$
0.5 g Tl_2SO_4
0.2 g Yeast extract
Adjusted to 1000 mL with Milli-Q water, adjusted to pH 7.20, and autoclaved

Caprylate thallous agar Solution B:
1 L Milli-Q water
14 g NaCl
2 g $(NH_4)_2SO_4$
30 g Bacto Agar (Difco)
Adjusted to pH 7.20 and autoclaved

Trace element solution:
19.6 g H_3PO_4
0.556 g $FeSO_4.7H_2O$
0.287 g $ZnSO_4.7H_2O$
0.223 g $MnSO_4.H_2O$
0.025 g $CuSO_4.5H_2O$
0.030 g $Co(NO_3)_2.6H_2O$
0.062 g H_3BO_3

DNAse agar:
42 g DNAse test agar (Remel) + 900 mL Milli-Q water
0.05 g Toluidine blue + 100 mL Milli-Q water
Autoclaved separately and mixed

Adonitol agar:
8.33 g Bacteriological peptone
4.17 g NaCl
Dissolved in 500 mL Milli-Q water and adjusted to pH 7.40
10 mL Bromothymol blue
5 g Adonitol
12.5 g Standard granulated agar
Made to 1 L with Milli-Q water and autoclaved

Itaconate agar:
6.0 g Na_2HPO_4
3.0 g KH_2PO_4
0.5 g NaCl
1.0 g NH_4Cl
Dissolved in 1 L Milli-Q water and adjusted to pH 7.0
15 g Agar and autoclaved
1 mL 0.1 M $CaCl_2$
1 mL 1.0 M $MgSO_4.7H_2O$
10 mL 20 % itaconate solution

A.2 Miniprep solutions

Solution I:
5 mL 1M Tris-HCL (pH 8)
4 mL 0.5M EDTA (pH 8)
182 mL Milli-Q water and autoclaved
9 mL 20 % Glucose
2.5 µL RNAse per 50 mL of solution I

Solution II:
1 mL 2 M NaOH
1 mL 10 % SDS
8 mL autoclaved Milli-Q water

A.3 PCR parameters

PlatniumTaq

PCR step	Temperature (°C)	Duration (minutes)
1	95	3
2	95	0.25
3	X	0.25
4	72	Y
5	Repeat steps 2-4 five times	
6	95	0.25
7	X	0.25
8	72	Y
9	Repeat steps 6-8 30 times	
10	4	∞

2X ReddyMix Master Mix

PCR step	Temperature (°C)	Duration (minutes)
1	95	7
2	94	1
3	X	1
4	65	Y
5	Repeat steps 2-4 30 times	
6	65	15
7	4	∞

2X DreamTaq

PCR step	Temperature (°C)	Duration
1	98	2
2	92	0.15
3	X	0.15
4	72	Y
5	Repeat steps 2-4 four times	
6	92	0.15
7	X	0.15
8	72	Y
9	Repeat steps 6-8 34 times	
10	68	15
11	4	∞

BOX PCR – 2X ReddyMix Master Mix

PCR step	Temperature (°C)	Duration (minutes)
1	95	7
2	94	1
3	53	1
4	65	8
5	Repeat steps 2-4 thirty times	
6	65	15

A.4 RegionA antibiotic insertion site sequence

The Apa1 cut site used to insert antibiotic cassettes is highlighted.

ATCGGCCAACTATCGCCGGTCGGCATGATGTTTGCCAGCGTGCTGATTTTAAAAGAGCGCATGCGCATTACCCAGGTGATT
GGCGCGCTGATGCTGATCTGCGGGTTGCTGTTGTTCTTCAACGTCAGCCTGATTGAAATTTTCACCCGCCTGACGGATTAC
ACCCTTGGGGTGTTGTTGGGTGTGTGTGCGGCAATGGTCTGGGTTACTTATGGCGTTGCGCAGAAAGTGTTGCTGCGTCG
ATTGGCCTCGCCGCAAATCCTGGTAATGTTGTACACTTTATGTGCAGTGGCGTTATTTCCTCTGGCTAGGCCGGAGGTGAT
TTTCCAGCTCAGCGGTTGGCAATTAGCCTGTCTGCTGTTCTGTGGTGCCAACACGCTGATTGGTTATGGTGCGCTGGCTGA
GGCCATGGCGCGCTGGCAGGCGGCACAGGTGAGCGCGTTAGTGACGCTGACACCGCTGTTTACCCTGCTTTTTTCAGATTT
ATTGGCGCTGGCCTGGCCACACGTATTCGCCGCCCCGACATTAAATGTCGTCGGCTATGTCGGTGCTTTCGTGGTGGTAGC
GGGCGCCATGTTTTCCGCAATTGGTCACCGTTGGTGGCCGCGACGGGCAGAACCCCGCCTGGTTGCTCCTTTGAAGCAGC
CTGGTGAATGATTTACGGAGACGGTAAATGAAGTTTGTAGATGAAGCAGCGATTTTGGTCGTTGCAGGTGACGGTGGTAA
TGGTTGCGTCAGCTTCCGTCGCGAAAAGTATATCCCGAACGGCGGGCCTGATGGTGGTGACGGCGGCGATGGCGGCGAC
GTCTATCTGTTGGCGGACGAAAACCTCAACACGCTGATCGACTACCGCTTTGAGAAATCTTTCCGCGCCGAACGTGGCCAG
AACGGTCAGAGCCGTGACTGTACCGGCAAGCGTGGCAAAGATATTACCATCAAGGTACCGGTAGGTACCCGCGTGCAGG
ATCAGGGCACCGGCGAGATCCTCGGTGACATGACCCGTCATGAGCAGCGTCTGATGGTGGCGAAGGGCGGTTGGCACGG
CTTGGGCAACACCCGTTTTAAATCATCGGTCAACCGTGCTCCGCGTCAGAAAACGTTGGGTACCGCCGGTGAAGCTCGTGA
CATCCTGCTGGAGCTGTTACTGCTGGCCGATATGGGTATGTTAGGCCTGCCGAACGCCGGCAAGTCGACCTTTATCCGCGC
CGTGTCTGCTGCCAAGCCGAAAGTGGCTGACTATCCGTTTACCACTCTGGTACCAAGCCTGGGTGTGGTGCGTATGGACCA
CGAGCAAAGCTTCGTGGTTGCCGATATCCCAGGGCTGATCGAAGGTGCCGCTGACGGCGCTGGTCTGGGTATTCGCTTCC
TGAAGCATCTGGAGCGTTGCCGCGTGCTGTTGCACCTGGTGGATATCGCCCCAATCGATGAGTCTGACCCGGTAGAAAAT
GCCAAAATCATCATCAATGAGTTGAATCAGTATAGCGAGAATCTGTCGCAGAAGCCTCGCTGGCTGGTTTTCAACAAGATT
GATGTGATAGGCGAAGAAGAAGCTGCCGAACGTGCCAAGGTGATTGCAGAGGGCATGGGTTGGGAAGGTAAATACTAC
ATGATCTCCGCCGTCAATCGCGAAGGTGTCAATGCGCTGTGCTGGGATGTGATGAAGTTTATCAATACCCAACCGAAAGCC
ATGGCCATCGCAGAAAGCGCGCCGGAGAAAGTCGAGTTCATGTGGGATGATTACCATCGTGAACAGATCGCTGAAGTGG
AAGCTGAAGCGGATGACGACTGGGATGACGACTGGGATGAAGAAGACGACGAAGGCGTCGAAATCGTTTACCAGAAGT
AATCTTGCGAAGAGAGGGGCTCAGTTAATACT**GGGCCC**TTTTTTTATTTCAGTACGTTTCCGGCAACCAGCATTGAGCATTG
AGCCCGTGGGTATCCTGACGGTTTTCCAACGTTAAGCGGCCCTGATGCAGCTGTACAATACGGGTCACGATGTTCAGCCCC
AGCCCACTGCCGCCATAGCGCTGATCCATACGGCGGAAGGCCTGGGTGAGTTCGCCGACCATTTTCTCTTTAACGCCCGGT
CCTTCATCGATCACCTGTAGCCGATGCCCGCGATCCTGCCGAGTCAGTTGCACCTGGATCCTGCTGTCCTCGGGGCTATAAC
GATGGGCATTCTCCACCAGGTTACGCAGCAGCAGACGTAACAGCACCGGGTCGCCATGTACCCGCGCGGTGGCCGGAAG
CTGCCATTGCAGACTTTGTCCGCGTTGCGCAGCCAACTCCTCGAGTTCTTCCCGTAGCGGTTGAATAACGTCGCTCACCCAA
TCGAAGCGTTGATAATGGCCACTGGCAAAATCTTGCCCGGCGCGCGCAAGCATCAGCAATTGCTCGACGGTGTGCATCAG
TTGGTCAATGCGGGTAATCAGCGGTCCGCTTTGCGGGATCCCCTGCTTTTCCATTAACTCCAGATGCAGACGGATCCCCGC
CAGAGGTGTACGCAGCTCATGGGCGGCATCGGCGGTAAACAACCGTTCCTGTTGGATGGTGTTATCCAGTCGCGAGAACA
ATTGGTTGAGTGTGGTGGTAACCGCCATCATTTCCAGACTGTCACTGTTGAGCGGCAGTGGCGTCAGGTTATCGGCGGAG
CGCTTTTCCAGCCGTTGCTGCAGTTGATTAAGCGGTCGGATAATCCAGCTGATAGCCCAAAATGAGGCCAGCAGGGTGAC
GATCATCATGATCAGTGAAGGGGCGAGCAACGAGGCTATCGCTTCGGCGATTTCCGTATCGACACGCTCGGTACGAACCT
TGGCTGACAGGGTTTCATCCACCAGGAAGCTGATTTGCTCCTGGCTTTCATGCCACAGCCAAAAGGCACTGATAAGCTGGG
TGACCAAAAGGATCAGCGCCAGCATCAACAACAGACGACGGCGCATGCTGATCATGATGAGGATTCCAGGCGATAGCCG
ATACCGCGAACGGTACGGATGCGATCCTTACCCAATTTGCGGCGCAGGTTATGGATATGCACTTCCAGCGTGTTAGAGCCC
AAATCATCCTGCCAGGTATAAAGATCCTGCTGCAGCAGTTCACGGTTGACCGTTTGTCCGGCGCGCATCATCAGACGTGAC
AGGATGGCGAACTCTTTTGGAGTGACTTCCACCGGCTGTTGTTGAACATAAACCTGCTGACTGGAGAGATTGAGCTGGAG
ATCGTCCAACTGCATGAGATTGTCACTGTGGCCCTGGTAGCGGCGGATCAGCGCTCGCACGCGCGCCTGCAGCTCAACCA
AAGCAAAGGGCTTCACCAGGTAATCGTCGGCGCCGGCATCCAGCCCGTCGACACGATCTTCCAGTGCGTCACGTGCCGTC
AGAATCAGTACCGGCAATTCAACTTGCTGGCGGCGCCACTGCCGAAGCAGGCTGGCACCGTCCATGTCGGGCAGGCCCAG
ATCGAGGATAATCATACTGTACTGACTGGTGAGGAGCAGAGCACTGGCCTCCGCCGCCGTGGCGGCGCAGTCGCAGGCA
TAGCCTTCCCCGGCTAATGCCAGTGCCAGGCCTTGTTGCAGCAGCTCATCGTCTTCAACAATCAGCAGTTTCATCATAACCC
TCAGTTATTCTGATAAATATCCCGGTACAGGCGGCTTTCGAAACGCACCAAGGGAGCACGGCGCTGTTTCTGATCTTCCGG
CGGCACGGCATAGCCTGACAGATACTGTACGAAGGCAAAACGCTGTCCGCTGGCGGTGGTGATAAACCCGGCCAGGTTA
TACACGCCTTGCAGCGCACCGGTTTTGGCAGAAACCTTGCCGTCGACACCGGCTTCATGCAGACCGCCACGGTAACGCAG
GGTTCCGTCGTAGCCGGATAACGGCAGCATCGAAATAAAGTTCAGCTCGCTGTCATGCTGCGCAATGTACTGCAATGCCTG
CATCATGGTTGCCGGGGCCAGCAGGTTATGGCGTGACAGGCCTGAGCCGTCTACCACGATGCTGTTACCCAAATCGACAC
CGGCTTTCTGACGAAGGACCTGACGCACCGCGTCGGCACCGGCACGCCAGGTGCCCGGAACGCCAAAACGCTCATGGCC
GATAGTGCGGAATACGGTATCGGCGATCATGTTGTCCGATTTCTTCAGCATGATCTTCAACAACTCGTGCAGCGGTGCGGA
TTGAGCTTGGGCGATCACCGTTCCTTTCAGGCCTGGCTGGGTCTGACGCTTCAGGTGGCCGTCGATCTGAATGCCGGCCTG
TTTCAGCTCATCTTTTAAAATAGCACCGGCATAGCTGGCGCCATCCTGGATAGCAAAAGCCAGCGGCAGTGGGTCGCTGC
GTTGGGTC

A.5 Kado and Liu (1981) megaplasmid visualisation solutions

2 X E-buffer:
0.968 g Tris base
3.3 mL 3 M Na acetate
4 mL 0.5M EDTA
Made to 100 mL with Milli-Q water and adjusted to pH 7.9 with glacial acetic acid
Working solution was diluted 1:10 with Milli-Q water to achieve 2 X

Lysis solution:
0.060 g Tris base
3 mL 10 % SDS
0.41 mL 2 M NaOH
Made to 10 mL with Milli-Q water

A.6 Nutrient profile of Wakanui silt loam soil

The Wakanui silt loam soil profile.

Wakanui_Soil_Profile.pdf

Appendix B

Chapter 3

Below follows the CFU data from the various competition experiments conducted during Chapter 3 (Competition between *Serratia* spp. in liquid culture).

B.1 A1MO2_tk and 5.6_s LB broth competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into an LB broth medium both separately and at mixed ratios.

A1M&5.6_Surv.xlsx

B.2 A1MO2_tk and 477_s LB broth competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 477_s when inoculated into an LB broth medium both separately and at mixed ratios.

A1M&477_Surv.xlsx

B.3 A1MO2_tk and AGR96X_s LB broth competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and AGR96X_s when inoculated into an LB broth medium both separately and at mixed ratios.

A1M&96X_Surv.xlsx

B.4 A1MO2_tk and 5.6_s Re-seeded LB broth competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into an LB broth medium and re-seeded daily into fresh LB broth daily both separately and at mixed ratios.

A1M&5.6_Seed.xlsx

B.5 A1MO2_tk and 5.6_s M9 glucose medium competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into an M9 medium containing a 20 % glucose supplemental carbohydrate source both separately and at mixed ratios.

A1M&5.6_Glu.xlsx

B.6 A1MO2_tk and 5.6_s M9 casamino acids medium competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into an M9 medium containing a 20 % casamino acids supplemental carbohydrate source both separately and at mixed ratios.

A1M&5.6_Cas.xlsx

B.7 Competition coefficients from Lotka-Volterra-style models

An xlsx spreadsheeting containing the primary and optimised competition coefficients from seven-day in vitro experiments conducted between A1MO2, 5.6, 477, and AGR96X in both LB broth and M9 medium. An example of the code used to calculate the parameters is also available.

LV_Comp_Data.xlsx

LV_Code

Appendix C

Chapter 4

Below follows the RNAseq spreadsheet and CFU data from competition experiments conducted during Chapter 4 (Transcriptomics of pathogenic and non-pathogenic *Serratia* spp.).

C.1 RNAseq gene expression data for 5.6+pADAP and 5.6 replicates

A collated spreadsheet of RNAseq data for the gene expression profiles of +pADAP and 5.6 replicates sampled during mid-exponential growth. The spreadsheet is colour-coded, where blue indicates increased gene expression in +pADAP replicates, and green indicates increased expression in 5.6 replicates. The spreadsheet also contains information relating to the expression scores of each replicate, the matched gene name, and the predicted product.

RNAseq_Data.xlsx

C.2 A1MO2_tk and 5.6_s M9 maltose medium competition CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into an M9 medium containing a 20 % maltose supplemental carbohydrate source both separately and at mixed ratios.

A1M&5.6_Malt.xlsx

Appendix D

Chapter 5

Below follows the CFU data from the soil microcosm competition experiments conducted during Chapter 5 (Competition between *Serratia* spp. in natural soil microcosms).

D.1 A1MO2_tk and 5.6_s competition in soil-only microcosms CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into 20 g natural soil microcosms both separately and at mixed ratios.

Soil_Microcosm.xlsx

D.2 A1MO2_tk and 5.6_s competition in soil microcosms containing a single grass grub larva CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when inoculated into 20 g natural soil microcosms containing a single healthy grass grub larva both separately and at mixed ratios. Cells highlighted in red represent the soil samples from which no *Serratia* cells were isolated.

Larvae_Microcosm.x
lsx

Appendix E

Chapter 6

Below follows the CFU data from the soil microcosm competition experiments conducted during Chapter 5 (Competition between *Serratia* spp. in natural soil microcosms).

E.1 A1MO2_tk and 5.6_s larval macerate CFU data

An xlsx spreadsheet containing the CFU and colony count data for A1MO2_tk and 5.6_s when isolated from grass grub larval macerate over a 14-day period. Cells highlighted in red represent the macerate samples from which no *Serratia* cells were isolated. Cells highlighted in yellow represent the days on which putative transconjugant cells were recorded growing on LB agar plates with Spectinomycin and Kanamycin antibiotics.

Larvae_Macerates.xlsx