Using Experimental Evolution to Understand the Relationship between the Motile Strategies and Biosurfactant Production on a Nutrient Gradient, in *Pseudomonas fluorescens*

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MSc (by research)

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Biology

December 2016

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Abstract

The role of flagellar based motility is well established amongst strains of *Pseudomonas fluorescens*. The benefits and overall necessity of motility give a large selection pressure for bacteria to be motile, especially in the colonisation of the plant root rhizosphere. As flagellar based motility is so important to *P. fluorescens,* non-motile *P. fluorescens* have a great benefit in evolving towards motility. Here I investigate the strength of reviving flagellar regulation in previously non motile strains of *P. fluorescens.* I show that the evolved flagella mechanisms are far weaker than the wild-type system and thus the mutant bacteria rely on other motile secondary metabolites, particularly the biosurfactant viscosin. I also investigate the different swarming phenotypes *P. fluorescens* express when introduced to a varied nutrient environment. I find that when bacteria are in a stress free nutrient rich environment, a smooth swarming phenotype is favoured. When the bacteria are under stress and starved of nutrients, the spidery phenotype is preferred. I also introduce a new novel swarming phenotype I have nicknamed 'sun-like', which appears to be a combination of both smooth and spidery motility. I theorise these distinct phenotypes can be directly influenced by the amount of biosurfacant present, where smooth spreading require increased biosurfacants and spidery spreading require the least. Moreover, I believe the phenotypes are a response to colonisation versus exploration, where biosurfacant can aid colonisation but is a hindrance to exploration.

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

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Acronyms

***fleQ* –** Transcriptional regulator – Bacterial Flagellar

ntrB – Two-component sensor - Nitrogen

***ntrC* –** Two-component response regulator – Nitrogen

viscC – Non-Ribosomal peptide synthase gene involved in making the lipopeptide surfactant visocsin

SBW25 – A Wild-type *Pseudomonas fluorescens* Strain

AR2 – Non-motile *Pseudomonas fluorescens* mutant. Deleted *fleQ* and Kanamycin resistant transposon interested into *ViscC*

1.0 Introduction

Bacteria play a vital role in the mutualistic symbiosis of many natural ecosystems (Lugtenberg and Dekkers 1999, Barea *et al.,* 2005), in other words they offer a 'give and take' relationship with an overall benefit for the community and habitat.

Research into plant – microbe bioremediation has provided us with an enhanced understanding of how we can take advantage of this bacterial symbiosis for our own gain in various industries (Anderson *et al.,* 1993 and Lu and Zhang 2006), of which agriculture remains at the forefront of research (Ciancio *et al.,* 2016, Singh *et al.,* 2016) and Dubey *et al.,* 2016). In fact, there is a strong focus on the use of bacteria in agriculture as a means to enhance plant growth and food production (Ciancio *et al.,* 2016 and Saharan 2011). Bacteria enable plants to better utilise their surrounding nutrients, and also fend off diseases (Vessey 2003 and Ciancio *et al.,* 2016). These bacteria reside in thriving populations at the plant root i.e. rhizosphere. Thus in theory, by increasing a bacterium's capacity to aid plants, we can use bacteria as a catalyst for plant nutrient metabolism. However, in order to do this, it is essential that we fully understand the role and process by which bacteria deliver and transport nutrients.

Studies have shown that crop yield and plant health can be increased through symbiotic bacteria by ensuring bacterial dominance and increasing the beneficial community where is it needed (van Loon *et al.,* 1999, Tautges *et al.,* 2016 and Kundu and Guar 1980). For attachment and colonisation of bacteria, motility is essential and the components of motility (i.e. flagella and biosurfacants) play a vital role in the bacterium's success in aiding plant growth (Martínez-Granero *et al.,* 2006). A prevalent bacterium within the soil microbe relationship is *Pseudomonas fluorescens* (Lugtenberg and Dekkers, 1999). *P. fluorescens* is a plant growth promoting bacteria which is part of the beneficial microbial symbiosis with in the plant-root rhizosphere (Hayat *et al.,* 2010). As *P. fluorescens* has such a positive effect on plants, it has the potential to be exploited for agricultural purposes to increase crop growth and defend against plant pathogens (Rainey 1999). Bacterial motility has a key function in the plant rhizosphere as it is fundamental in colonising plants roots, therefore by further understanding and evolving species of *P. fluorescens* we could aid the ecological performance of the bacteria (Martínez-Granero

et al., 2006). I aim to focus on the different motile phenotypes of *P. fluorescens* particularly the smooth and spidery phenotypes (Giddens *et al.,* 2007 and Alsohim *et al.,* 2014)**.** By understanding the mechanisms by which these motile phenotypes occur, and the specific benefits for the community when shifting between each phenotype, I hope to gain an insight into whether these can be put to use by inducing a motile phenotype to aid a healthy rhizospheric community.

1.1 *Pseudomonas fluorescens*

Pseudomonas fluorescens is a diverse species of opportunistic bacteria commonly found in soil, plants and water surfaces (Silby *et al.,* 2009) and is a beneficial microbe in the symbiosis between plants at the rhizosphere (Handelsman and Stabb 1996 and Naseby *et al.,* 2001). The bacteria are gram-negative rod shaped with an optimum growth temperature between 25-30^{o}C. *P. fluorescens* is a model bacterium for the study of motility due to its strong phenotypes and diversity (Speirs *et al.,* 2000, Bantinaki *et al.,* 2007 and Rainey and Travisano 1998). These same benefits mean *P. fluorescens* also holds many benefits for the study of bacterial evolution, especially with microbe-plant interactions.

1.2 *Pseudomonas* Motility

Motility is recognised as a fundamental necessity for bacteria, as motility aids colonisation, invasion and protection within a community (Chaban *et al.,* 2015). *P. fluorescens* is able to perform a number of motile phenotypes, including the flagella-dependent motilities swarming and swimming (Jarrell and McBride 2008). As a result there is a huge selective advantage for motility, particularly the methods involving flagella. The swarming and swimming motility expressions allow the bacteria to efficiently and quickly move through an environment and mutations in the bacterium's flagellar regulation affect it's chemotaxis towards attractants and their biofilm formation (Mastropaolo *et al.,* 2012). To aid swarming motility, the bacteria can produce biosurfactants, which allow the bacteria to glide with their flagella across a surface (Andersen *et al***.,** 2003).

Biosurfactants also have other beneficial roles for *P. fluorescens*, as they act as an antifungal and antibacterial compound to antagonise pathogens and competitors (Andersen *et al.,* 2003).

P. fluorescens is also able to utilise flagella -independent motilities. One example of this is twitching which requires pili and is controlled by the Poc complex (Cowles *et al*., 2013). The twitching motility allows the bacterium to travel across moist surfaces by retraction of the polar type IV pili, which operates as a grappling hook (Mattick 2002).

Twitching is also referred to as 'social gliding motility' and it gives a bacterial population an advantage in rapid colonisation in nutrient rich environments (Mattick 2002). Although each adaption of motilities provides a unique advantage for *P. fluorescens*, the focus of this project is on the flagella-based motiles and how the flagella evolve in response to environmental conditions.

1.3 Flagella and *fleQ*

The flagellum is a threadlike locomotor appendage that protrudes from the plasma membrane and cell wall of bacteria (Willey *et al.,* 2011). The expressions of flagella are mediated by the *fleQ* gene (Arora 1997). Gene expression for flagellar mediated motility is highly coordinated. The gene *fleQ* regulates the expression of two modes of movement (swimming and swarming) independently. Repression of *fleQ* can stop the flagellar movement as it is located at the top of the hierarchy which enables the expression of all the other flagellar genes (Giddens *et al.,* 2007). Therefore, the expression and regulation of *fleQ* is essential for the motility of bacteria.

Motility is recognised as an essential factor in the adhesion and colonisation of roots in plants (Piette and Idziak 1992) and thus there is a large selection pressure for bacteria to be motile. The development of non-motile strains of *P. fluorescens* can severely impair their ability to occupy plant roots (De Weger *et al.,* 1987). However, flagellar synthesis is costly (Zhao *et al.,* 2007), so the development of flagella in non-motile bacteria must provide a strong advantage to the population.

The flagella producing gene *fleQ* has a striking homology with the nitrogen regulating gene *ntrC* (Arora *et al.,* 1997). As a result it is possible that this homology may allow *ntrC* to express the role of *fleQ* in non-motile mutants with *fleQ* knocked out. Previous research at the University of Reading, UK, carried out by Alsohim (2010) found that if non-motile *Pseudomonas fluorescens* mutants were left to incubate at 27^{o}C for over 72 hours on 0.25% agar, they can begin to spread away from the site of inoculation, showing that non-motile *P. fluorescens* can acquire motility. The acquisition of motility was found to be the result of a mutation in *ntrC* where the homology resulted in *ntrC* replacing the function of the *fleQ gene*, in regulating flagellar synthesis.

1.4 Hypermotility

Hypermotility is the expression of multiple flagella through single point mutations in *fleN* (Dasqupta *et al.,* 2000 and van Ditmarsch *et al.,* 2013). Hypermotility increases the speed of flagella **-** dependent motilities and provides a benefit in competitive environments (Ditmarsch *et al*., 2013) and abundant healthy flagella are able to explore a diverse range of niches giving the bacteria an ecological advantage (Roth *et al.,* 2013). However, hypermotility comes at a cost to bacteria; the increased flagella numbers impair the bacterium's ability to form the strong biofilms that Pseudomonas spp. is known for (Ditmarsch *et al.,* 2013). The rhizosphere selects hypermotile mutants suggesting the in the soil environment motility is more important than biofilm formation (Barahona *et al.,* 2010 and Martínez-Granero *et al*., 2006).

1.5 *P. fluorescens* biofilm formation

The Pseudomonas genus is notorious for its ability to form strong biofilms (O'Toole *et al.,* 2000). Through cooperation and adhesion, biofilms are able to supply a bacterial community with essential oxygen and nutrients (Rainey and Rainey 2003). *P. fluorescens* is known to have many biofilm forming phenotypes each providing the community with a unique advantage, for example, the fuzzy spreader phenotype offers a resistance to some bacteriophage (Ferguson *et al*., 2013). The flagellum is essential for early biofilm development (O'Toole *et al*., 2000).

1.6 Biosurfactant

Biosurfactants are secondary metabolites produced by bacteria and are very useful in reducing surface tension, thus aiding motility (Alsohim *et al*., 2014). Along with being useful motile aids, biosurfactants are also known to restore and enhance swarming motilities under environmental stress, by creating a non-stressful micro-environment for the bacterium (Singh *et al.,* 2013).

Viscosin is a fundamental and well-studied polypeptide within the *Pseudomonas* genus; the properties of viscosin are numerous including antibiotic and biosurfactant roles. It is a known biosurfactant which aids *P. fluorescens* spreading motility and plant growth promotion (Alsohim *et al.,* 2014). The extent of the use of viscosin in *P. fluorescens* reflects the phenotypic motile response. This is due the negative correlation between biosurfactant production and flagella use, by allowing the bacteria to more efficiently slide across a surface (Nogales *et al.,* 2015).

1.7 The current study

To start I aimed to better understand whether the resurrection of motility of a *fleQ* mutant through nitrogen selection would prove to be as diverse and strong as the wild-type *P. fluorescens*. This research was based on the work of Taylor *et al*., (2015) who discovered that due to the homology of *fleQ* and *ntrC,* in the case of a catastrophic deletion of *fleQ,* flagellar regulation can be regulated by excessive NtrC-P, produced by a mutated *ntrB.* To achieve this mutants and wild-type strains of *P. fluorescens* (SBW25) were grown on a variety of nitrogen based mediums, as an active nitrogen regulation mechanism is required to stimulate the adaption of the *ntrB* gene for motile revival. However, through experimentation I noticed that the motile strategy employed by the wild-type SBW25 appeared to be media dependent and each method had a noticeable variation on the production of biosurfactants which are visible as a clear viscous liquid on the surrounding the bacteria.

I then aimed to explore these motile methods of *P. fluorescens* and to better understand the role of the biosurfactant viscosin, between the motile strategies. I aimed to investigate whether the different motile phenotypes exhibit a drastic change in the use of biosurfacants. As well, I pursue the effect of starvation stress on *P. fluorescens* and how this plays a role in different motile methods and the effect a limited nutrient environment has on the production of viscosin.

Currently there is little research into how environment can shape motile fitness and the phenotypic response. Therefore, this study aims to fill this research gap. I aim to investigate the role off the different motile phenotypes of *P. fluorescens* to see how motile fitness is affected under changes in nutrient environments. I also aim to understand what mechanics are behind each phenotype and the cost and benefits of employing different motility methods.

To summarize the aims are as follows:

- To investigate whether the evolved motility non-motile strains of *P. fluorescens* display as diverse motile strategies as the wild-type.
- To understand whether the non-motile strains will be as successful as the wild-type strain through area spread.
- To investigate the stimulus for the different motile methods of *P. fluorescens.*
- To understand the role of biosurfacants in the diverse motile phenotypes.

2.0 Methodology

2.1 Bacteria

The two strains of *P. fluorescens* I used in the selection were the wild-type SBW25 and a non-motile mutant (AR2) provided by The University of Reading, UK. The AR2 mutants have the *fleQ* gene knocked out and a kanamycin resistant transposon inserted into the *viscC* gene, resulting in strain with no primary flagella producing gene (*fleQ*) or viscosin gene (*viscC*). The kanamycin resistant transposon allows the identification of contamination in the AR2 strains, which gives confidence that any extreme motility observed in the AR2 strains is exhibited by the AR2 mutants and not the effect of cross contamination with an SBW25 strain or another contaminant.

2.2 Effect of nitrogen on motility

2.2.1 Media

Four treatments were used to create mutants within the SBW25 and AR2 strains. These selection treatments put the bacteria in a limited environment with regards to nitrogen. The environments supplied were LB, M9 minimal media, ammonia media and a media with no nitrogen (referred to as N-). These four mediums provide the bacteria with sufficient nitrogen for growth (LB), exclusive nitrogen environment (NH_4), and limited nitrogen environment (M9) and no nitrogen at all. Using these mediums I can assess; whether varied nitrogen conditions have any effect on motility and where in the nitrogen regulating cycle does the evolution of motility have the greatest impact. With the exception of the LB media, the mediums were created in a similar way except for their respective nitrogen requirement (Table 1). As we are assessing the swarming ability of these bacteria, 0.25% agar was used to allow the bacteria to swarm over the media (Rashid and Kornberg 2000).

Each stock media was created in a 1L Duran bottle. As all the supplements in the LB are able to be autoclaved these were simply mixed together and autoclaved with 1L dH_2O.

The other nitrogen limiting mediums required the addition of compounds which could not be autoclaved (such as glucose), therefore I filled a 1L Duran bottle with less than 500ml dH2O and added 2.5g of agar, which will create 0.25% agar after adding the further necessary solutions. I made a large batch of two different M9 salts, M9x10 for the minimal M9 salts media and M9x5 for the nitrogen exclusive or absent media which were both autoclaved (Table 1 and 2). These M9 salts (Table 2) provide the minimal nutrients for the bacteria to grow, so any change observed will be the effect of the added nitrogen source. The other solutions, 1M $CaCl_2$, 1M $MgSO_4$, 1M NH_4 and 20% glucose were sterilised by using 0.25µM syringe filter.

	M9	**NH3**	**No Nitrogen**
M9x5	n/a	500	500
M9x10	500	n/a	n/a
1M $MgSO_4$	2	2	2
20% Glucose	50	50	50
1M $CaCl_2$	0.250	0.25	0.25
1MNH_3	n/a	10	n/a

Table 1: The contents of each of the 3 nitrogen specific media made for 1L. Each was made in ~400ml water agar mixed with 2.5g agar. After autoclaving I added the relevant treatments to each bottle of water agar and topped the bottles up to 1l with dH_2O, giving a final agar concentrations of 0.25%.

	M9x5 Salts (g)	**M9x10 Salts (g)**
Na_2HPO_4	33.9	33.9
KH_2PO_4	15	15
NaCl	2.5	2.5
NH_4Cl	n/a	5.0

Table 2: The solution of M9 salts made to add to the nitrogen specific media. M9x5 salts contain no nitrogen and were specific to limited nitrogen mediums. M9x10 salts have added NH_4Cl and is for the M9 media. The salts were made in 1L

2.2.2 Selection

Within the different nitrogen selection, further artificial selection was added to assess how the different strains mutated within the media. This was achieved by using a positive, negative and random selection. The positive selection only transferred the bacteria that swarmed the furthest from the inoculation site; these bacteria will be the most motile therefore gives a positive selection for motility. The negative selection selected bacteria from the inoculation site, i.e. the bacteria that have swarmed the least or not at all, thus giving a negative selection for motility. The random selection used a square grid with a circle of a radius of 4.5cm in the centre, within this circle the square grid had numbers 1-60 written on. A random number generator from Microsoft Excel was used and the selection was taken from the number square that corresponded to the random numbers generated. This gives an effective neutral selection, neither selecting for the most motile or non-motile strains.

2.2.3 Storing mutants

The strains were grown over night at 28^{o}C and a sample was taken and added to 100µl LB in wells of a 96 well plate. These strains were then grown overnight and shaken at 28^{o}C. After growth, 100µl 50% glycine and frozen at -80^{o}C. The glycine was added to prevent the cells from bursting (Cleland *et al*., 2004).

2.3 LB Gradient Experiment

The results of the nitrogen selection experiment gave rise to an interesting discovery of the variation of motile methods between and a LB solution and a minimal M9 solution. Thus, this experiment aimed to understand the threshold of the motile methods used within these two mediums and how the bacteria would interact in a gradient combination of both. Therefore, the two foundations for the gradient experiment are an LB broth and M9 salts. This gradient creates an environment from the optimal bacterial growth, to the minimal requirements for growth. To achieve this I decreased the amount of LB in the media and substituted with minimal M9 salts. The gradient of LB

concentrations used were 50%, 10% 1% and 0% LB, all with 2.5g agar to create a 1L solution at 0.25% agar to allow for bacterial motility (Table 3).

A litre of non-sterilised LB was made with adding 20g in 1L dH_2O. Then the LB was pipetted to the different media concentrations as per table 1. The solutions were topped up to their relevant levels with dH_2O, with the addition of 2.5g agar and mixed until dissolved. The M9 salts (table 2) were made and sterilised separately and added after autoclaving. This is because it was found that the addition of M9 salts and agar in autoclaving compromised the media, making it turn a dark brown colour and affects the solidification of the agar after pouring. Once sterile, the plates were poured adding ~25ml of the media to each petri dish. These were left to dry under a laminar flow hood for 1 hour.

	LB stock (ml)	**M9 salts (ml)**	**20% Glucose (ml)**	**$MgSO_4$ (ml)**	**$CaCl_2$ (ml)**	**dH_2O (ml)**
50% media	500	250	25	1	0.125	250
10% Media	100	450	45	1.8	0.225	450
1% Media	10	495	49	1.9	0.247	495
0% Media	0	500	50	2	0.250	500

Table 3: The concentrations of LB and M9 salts used for 1L for the four separate mediums to measure the effect of bacteria movement on an LB gradient.

The ancestral of wild-type *Pseudomonas fluorescens* was point inoculated at the centre of each plate and grown at 28^{o}C for 48 hours. Due to varied growth rates of the bacteria on the mediums photos were taken at 24 hours and 48 hours, giving readings of initial and long term colonisation. The higher LB concentrations showed substantial motility very early on, where it took the lower concentrations longer to establish great motility. However, transfers were taken after 24 hours onto fresh media to allow for motility evolutions. I ran the experiment for 8 transfers or until I found significant differences in motile phenotype. I also used 10 replicates of each selection to ensure reliability.

This experiment also included the positive and negative selection to see whether selecting for motility affects the motile morphology of the bacteria. These two selections were taken the same as in the nitrogen experiment.

The data was analysed using ImageJ (Abramoff *et al.*, 2004). For the experiments here on out only the final transfer, mutants were used (transfer 8).

2.4 Cross Media Experiment

To assess whether the observed phenotypes were media specific or general mutations of the bacteria, I carried out a cross media experiments on the final transfer strains. For this, each media was inoculated with every mutant evolved at the final transfer (transfer 8) in the 50%, 10% 1% and 0% mediums, as well as the ancestral SBW25 strain and the non-motile AR2 strain as controls. Photos were taken at 24 hours and at 48 hours, using the same protocol as the gradient experiment.

2.5 Biosurfacant Experiment

The focus of this research was to understand the role of biosurfactants under nutrient stress and their effect on motile method. To ensure reliable results two experiments were used to measure biosurfacant production. Firstly, to positively detect for biosurfacant I used a novel paraffin oil experiment designed by Burch *et al*., (2010). A light mist of paraffin oil is sprayed on the visible bacteria and left to disperse for 15 minutes. After this time the paraffin behaves hydrophobicly towards the biosurfacant, so detection is seen by droplets of oil spread over the petri dish (Figure 1).

This method was useful to achieve biosurfacant detection; however, it did not give a clear measurement of biosurfacant which was required. To measure biosurfacant I designed a experiment using crystal violet. By pipetting 70% crystal violet (diluted with glycerol to keep viscosity) at the sight of inoculation, I found the crystal violet would spread across the plate with surface tension keeping the dye within the area of biosurfactant production (Figure 1). This method not only gave the option to measure the area spread of viscosin through ImageJ, but also provided and a distinct observation of the various patterns in which viscosin spread across the petri dish. This information could then be used to understand whether the different motile phenotypes employ different methods of viscosin secretion, or whether this is indeed of any significance.

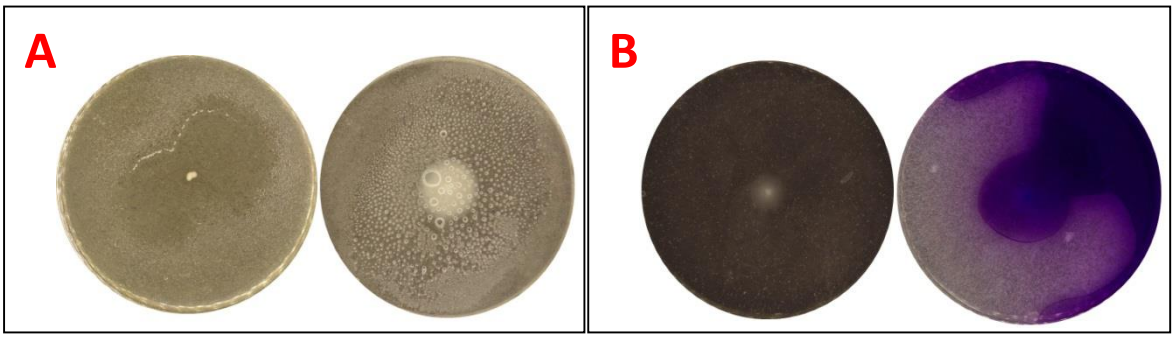

Figure 1: Investigating methods of identifying biosurfactant production using paraffin oil (**A**) and crystals violet (**B**).

The paraffin oil experiment (**A**) sufficed only as a detection method. Biosurfacants are detected by oil droplets onto of the surfactant where it is behaving hydrophoibcally towards the surfactant. Left shows a non-motile (AR2) mutant of *P. fluorescens* where now biosurfacant could not be detected. Right shows a wild-type motile *P. fluorescens* where biosurfacant is detected.

The crystal violet method (**B**) was an effective Biosurfacant measurement technique. Left shows the bacterial growth after 16 hours at room temperature before the addition of crystal violet. Right shows the addition of crystal violet applied immediately. The purple spread of crystal violet signifies the area of biosurfacant produce by the bacteria.

2.6 Data Analysis

Statistical differences ($P<0.05$) between experiments were analysed by ANOVA. Data analysis was done with the software SPSS 23. Graphs were constructed using GraphPad pro.

3.0 Results

3.1 Diversity of Motility on Nitrogen

This experiment aimed to follow the previously mentioned work of Taylor *et al* (2015) to understand whether a flagellar evolved in *fleQ* knockouts (the primary flagellar regulating gene) will be as strong as a wild-type flagellar in carrying out the diverse motile phenotypes wild-type *P. fluorescens* expresses.

I found that in the *fleQ* knockout strain (AR2) the speed of motility acquisition is significantly reflected by nutrient environment and that motile strength increases through time ($F_{5,43}$=20.065, $p<0.05$) (Figure 2). However, there is a fitness cap compared to the wild-type. Motile diversity is also restricted in the AR2 mutants. Two common strategies of *Pseudomonas* motility are smooth spreading and spidery spreading. In the non-motile AR2 mutants, there was no evidence of spidery spreading and these strains relied solely on smooth spreading (Figure 3).

In the wild-type SBW25 strain, the two most distinct motile phenotypes were found on the LB and M9 mediums. LB showed a large diversity in motility, however, favoured the smooth spreading and a new novel form of *Pseudomonas* motility named **'**sun-like' (Figure 4). The wild-type SBW25 strains were consistently motile throughout (Figure 5). The strains evolved on minimal M9 media favoured the spidery motility associated with hypermotility (Figure 6). These motile methods seem to be media specific; however the fitness benefits and mechanisms of the particular methods are unclear at this stage. This is why I pursued an investigation into the comparison between *Pseudomonas fluorescens* motility on an LB gradient mixed with M9 salts.

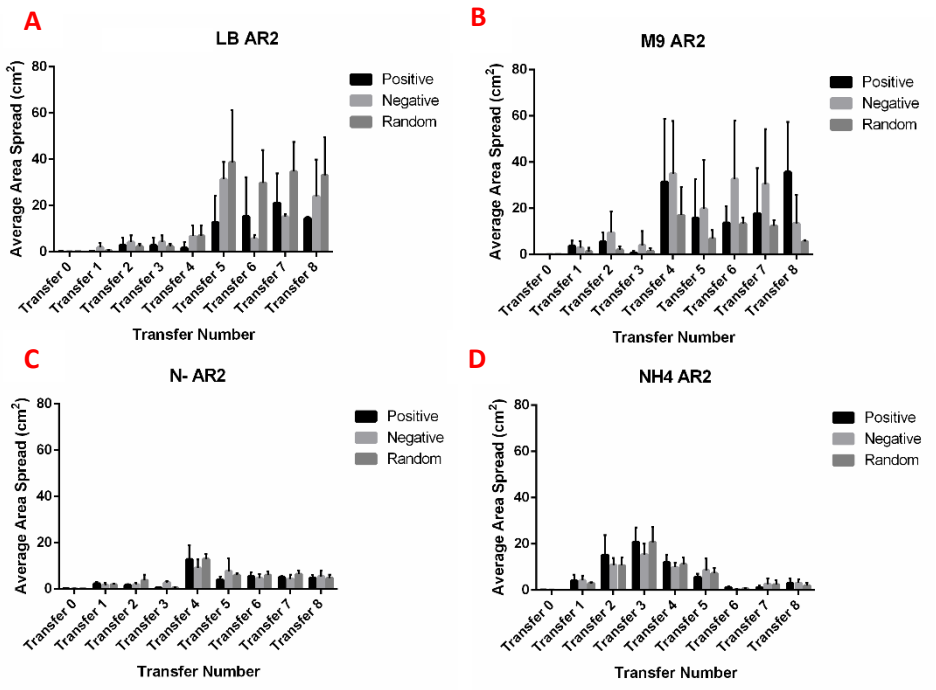

Figure 2: The evolution of the motility of the initially non-motile AR2 strain, measured in area spread. Motility was quickly acquired in all mediums; however the media capped the motile potential of the bacteria. Most successful were mediums were LB (A) and M9 (B) and the least successful were N- (C) and NH4 (D)

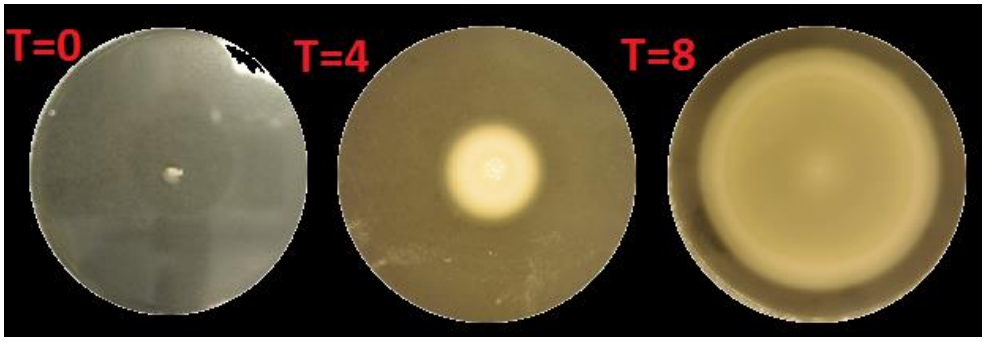

Figure 3: An example of the only motile expression of the AR2 mutants. This examples shows growth on LB media from the ancestral strain at transfer 0, to acquiring motility at transfer 4 and the maximum motile at the final transfer. Although area of spread increased over time, the motile phenotype was not able to adapt to the different environments. This strain was only able to express a smooth phenotypic response to all the mediums evolved in.

The effect of the three motile selections (positive, negative and random) on each strain did not have a significant effect on the wild-type SBW25 ($F_{2,45}$=0.324, p=0.744). The same can be said for the non-motile AR2 strain ($F_{2,45}$ =0.530, p=0.564). When considering the effect of media and selection, the wild-type strain was again insignificant ($F_{6,42}$=0.427, p=0.840). However, the non-motile strain was did have a significance comparing selection with media, ($F_{6,42}$=3.449, p<0.05). The media in which

selection had a significant effect for the non-motile strain was LB ($F_{2,9}$=3.408), p=0.0099), suggesting that when nutrients are plentiful motility strength is more diverse therefore more selectable.

There is a significant difference between the area spread of the wild-type strain and the newly acquired motile strain ($F_{1,95}$=44.90, P<0.05). The wild-type strain, on average, were far more motile than the mutant strain even late in the transfer experiment. Overall, suggesting that acquired motility through reconstructive evolution will not be as successful as the wild-type mechanism.

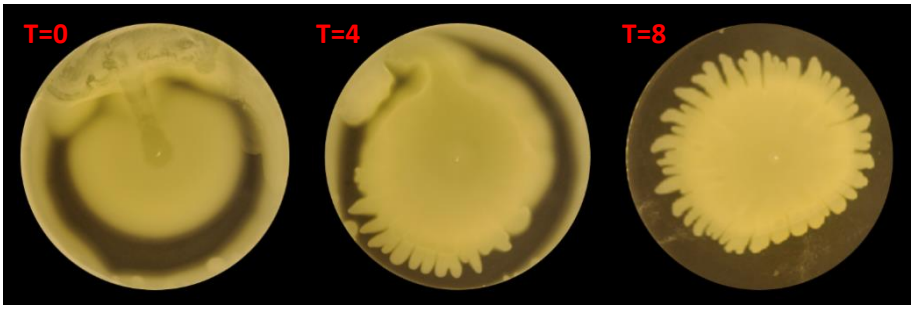

Figure 4: The evolution of a novel 'sun-like' motility overserved in *Pseudomonas fluorescens* grown on 100% LB. Left shows the ancestral transfer, middle is after 4 transfers and right is the motility at the final eighth transfer.

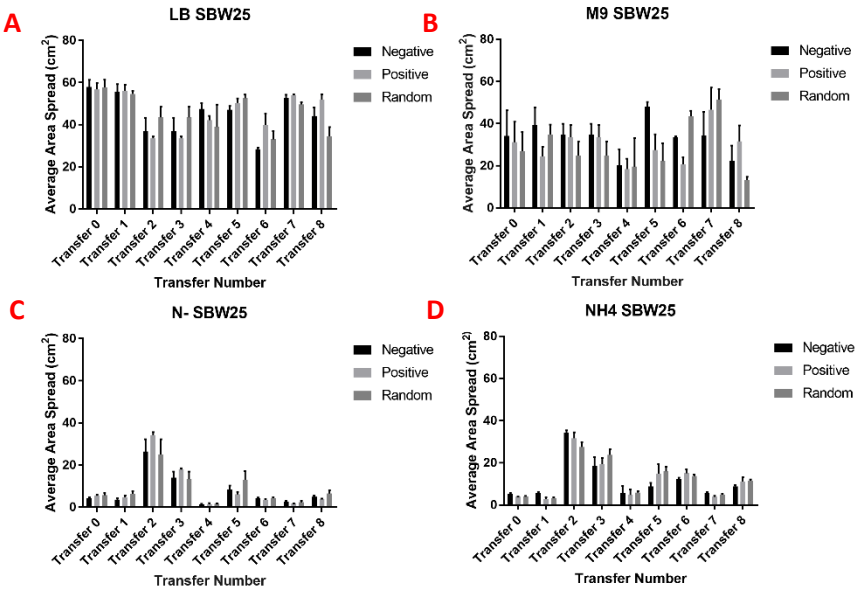

Figure 5: The evolution of motility of the wild-type SBW25 strain, measured in area spread. The motile strength of the bacteria was very media dependent. Most successful were mediums were LB (A) and M9 (B) and the least successful were N- (C) and NH4 (D)

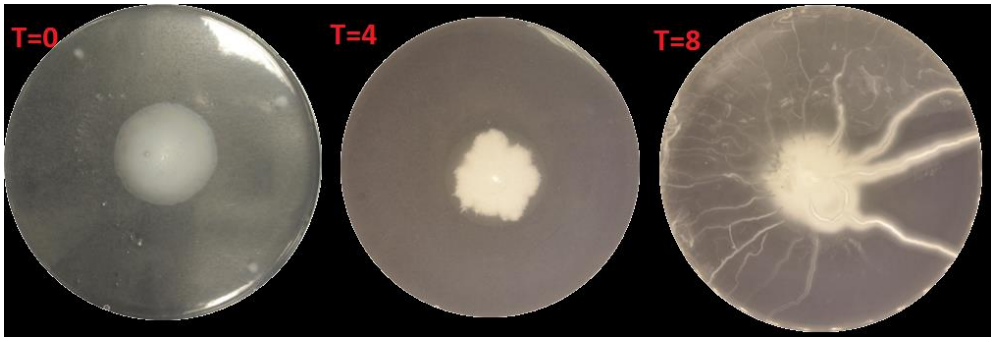

Figure 6: An example of how motile expression diversified in the wild-type SBW25 strain. This examples shows growth on minimal M9 media from the ancestral strain at transfer 0, to transitioning to spidery motility at transfer 4 and at the final transfer. The wild-type strain was able to expressed different motile phenotypes based on the environment the bacteria were presented with.

3.2 Media Gradient

The primary study, on the effect of nitrogen treatment on the evolution of flagellar strength, leads to further research to understand how a gradient of LB mixed with M9 salts will produce a distinct shift in motile strategies. The research from here on out exclusively examined the wild-type SBW25 strain, using the non-motile AR2 mutant only as a negative control for motility. Understandably, the lower the nutrient concentration the slower the bacteria spread. The speed of bacterial spread was measured after two transfers, the first transfer at 24 hours and the second at 48 hours. I found that after 24 hours, only those that were grown on 50% showed any notable motility. However, through the transfers, the 10% mutants began to show increased motility after 24 hours (Figure 7). At 48 hours the motile strategies of the lower LB concentrations can be seen and their area of spread increases significantly compared to 24 hours (Figure 7). The 0% LB bacteria are forced to evolve more efficient motile strategies so they can search for more nutrients in their limiting environment, through the expression of spidery tendrils. Interestingly, the 1% LB mutants only expressed one or two tendrils from the sight of inoculation, this also occurred briefly in the 0% strains, however, the 0% strains were able to evolve beyond this and produce many tendrils. Although the 0% LB strains produced more tendrils than 1% LB, the difference in their area of spread was insignificant (Figure 7), likely due to the small amount of colonisation the 1% LB strains did at the site of inoculation.

The bacteria grown on 10% were the only bacteria that showed any significant change in the area spread throughout the eight transfers at 24 hours (Figure 7). What did change is the motile phenotype expressed (Figure 8) I found that all the ancestral *P. fluorescens* strains used the smooth spreading motile strategy. As the concentration of LB decrease, there is a shift towards spidery spreading.

I also introduced a positive and negative selection into the gradient experiment to see whether the motile phenotype is effect by the distance in which the bacteria travelled. These results prove insignificant ($F_{1,99}$=0.015, p=0.678) and no evidence of increased or decreased motility was observed nor a change in the methods of motility.

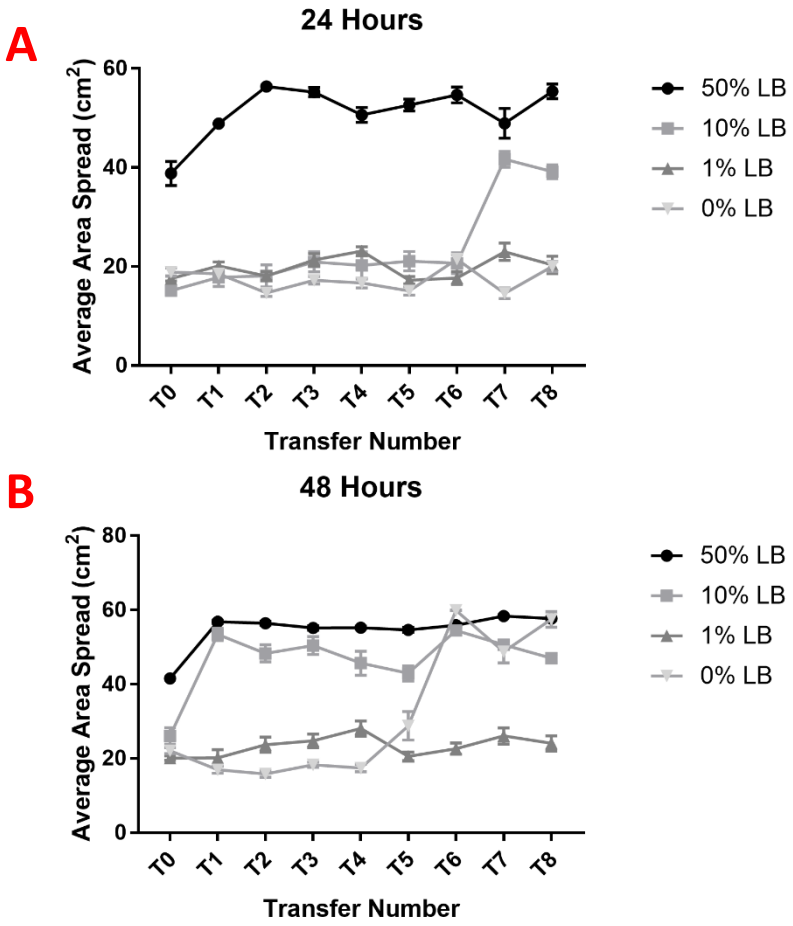

Figure 7: The evolution of flagellar based motility (measured in area of spread) of wild-type *P. fluorescens* on a LB gradient from 50% to 0% mixed with minimal M9 media of the results after 8 transfers at 24 hours (**A**) and 48 hours (**B**). At 24 hours (**A**), little motility is shown in the lower LB concentrations however the higher concentrations show a large amount of motility. At 48 hours (**B**), motility is much more substantial in the lower LB concentrations, and over time the 0% LB mutants are forced to evolve efficient and quick swarming phenotypes, eventually competing with 50% LB for the most motile bacteria.

3.3 Phenotypes

I observed a large diversity of motile phenotypes in *P. fluorescens* species throughout the cross media experiment. I have categorised these into three groups; smooth, spidery and a new novel expression I have named 'sun-like' (Figure 8). The ancestral spreading is exclusively smooth. However, after the bacteria have been effectively expressing motility throughout many generations, they are able to freely express different motile strategies based on their environment (Figure 9). At high LB concentrations smooth spreading is common and the lower LB concentrations spidery spreading in favoured. The sun-like expressed occurs when the bacteria have evolved towards a low LB environment and then introduced to a higher LB environment. In the case of the sun-like motility it appears to be a combination of smooth and spidery spreading which are competing, resulting in its distinctive expression.

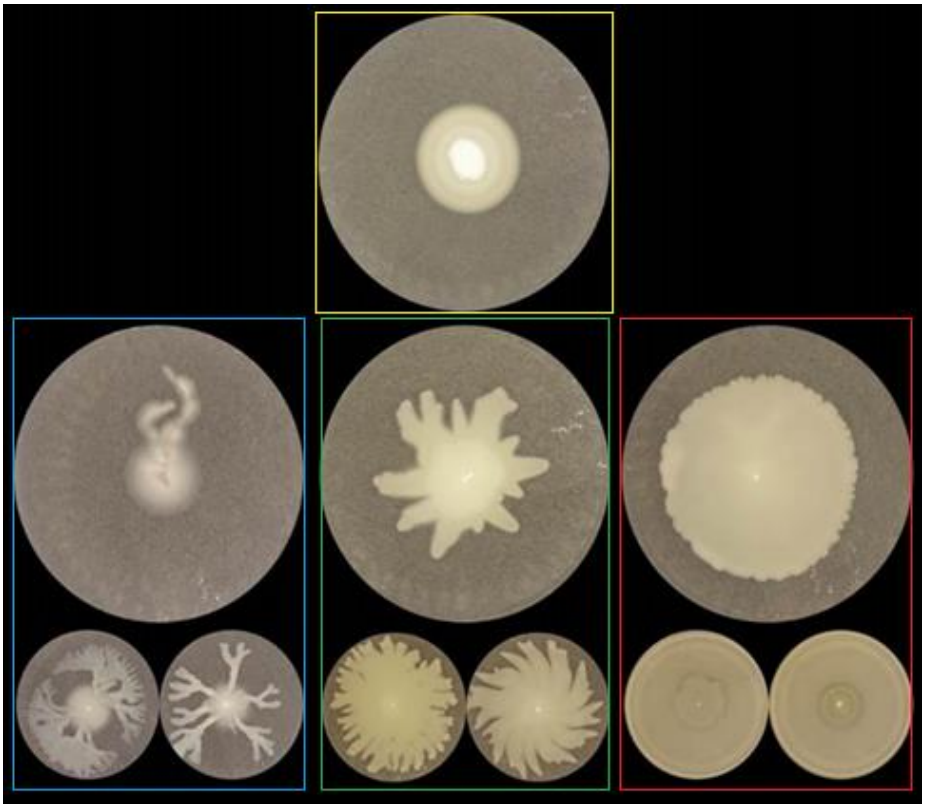

Figure 8: The evolution of the three distinct methods of motile observed through the nutrient gradient of LB mixed with M9 salts. LB increase from left to right. Yellow shows the typical ancestral growth and how to evolve to utilise different motile strategies based of nutrient environment. Blue shows the evolution of spidery motility under minimal nutrients where the bacteria must search for a more variable nutrient source. Red shows the evolution of smooth spreading when nutrients are not an issue. The bacteria utilise a greater surface area relying on surfactant production to take advantage of the plentiful nutrients available to them. Green shows the evolving of 'sun-like' phenotype, where there is an equally selection for spidery and smooth spreading so the bacteria benefit from combination of motile expressions.

Media History	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Environment
50LB	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	
10LB	3	3	3	1	3	3	3	1	1	3	1	2	3	3	3	2	3	3	3	3	50LB
1LB	3	1	3	2	2	1	1	1	1	1	1	3	3	1	3	3	1	3	3	1	
0LB	3	3	1	3	3	3	3	1	3	3	3	3	3	1	3	3	3	3	3	3	
50LB	4	4	4	4	4	2	4	4	4	4	4	1	4	4	1	1	4	1	1	4	
10LB	1	1	2	1	1	1	2	4	1	2	1	2	1	4	4	2	1	2	2	4	10LB
1LB	4	1	1	1	4	4	4	4	4	4	4	4	4	4	2	4	4	1	4	2	
0LB	4	4	2	2	4	4	4	1	4	4	4	4	4	1	4	4	4	4	4	4	
50LB	4	4	4	4	4	4	4	4	4	2	1	4	1	4	2	4	2	4	4	4	
10LB	2	1	4	4	1	4	4	4	2	4	1	4	1	4	4	2	4	2	1	4	1LB
1LB	2	2	2	2	2	2	1	2	1	4	2	2	4	4	4	4	4	4	4	4	
0LB	2	2	1	1	4	4	2	4	2	2	2	2	4	1	2	2	2	4	2	2	
50LB	2	2	4	4	2	4	4	2	4	2	2	2	4	2	4	2	2	4	4	4	
10LB	2	2	2	1	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	0LB
1LB	4	4	4	4	4	4	4	2	2	2	2	2	2	2	2	4	1	4	4	4	
0LB	2	2	1	2	2	2	2	1	2	2	2	2	2	1	2	2	2	1	2	2	

1	Smooth
2	Spidery
3	Sun-Like
4	Indistinguishable

Figure 9: The morphotypes of every strain tested on all four mediums during the cross media experiment. This sable shows all ten replicates of the Positive (P) and the Negative (N) selections. The two most stable mediums are the two extremes (50LB and 0LB) suggesting the strongest selection towards a motile phenotype when nutrients are plentiful (smooth) or sparse (spidery). Many phenotypes are indistinguishable in the middle LB concentrations of 1LB and 10LB suggesting an unstable competition between different motile methods. The only generalist evolution, which does not appear to be media specific, is that those that have evolved on the lowest LB concentration, will perform the novel 'sun-like' motile when introduced to a more generous nutrient environment.

There are examples where the motile phenotype of the strain is strong and so the dramatic change in nutrients plays no affect in influencing the phenotype, for example 50LBN10, 10LBP6 and 0LBP4.

3.4 Cross media area spread

The purpose of this experiment was to understand whether motility fitness is media specific or an evolved trait of the bacteria grown under the specific mediums. Every T8 strain was measured on every medium used in the gradient experiment. The bacteria were point inoculated and area spread measurements taken at 24 hours and 48 hours. 48 hours gave time to allow the bacteria grown in nutrient limiting environments to express a comparable amount of motility.

3.4.1 Area spread

The first observation is that it is clear that evolving strains of *P. fluorescens* towards motility over a 16 day period significantly increases their motility. All strains outperformed the ancestral wild-type of which they evolved from on all mediums, even after 48 hours of growth (Figure 10 and 11).

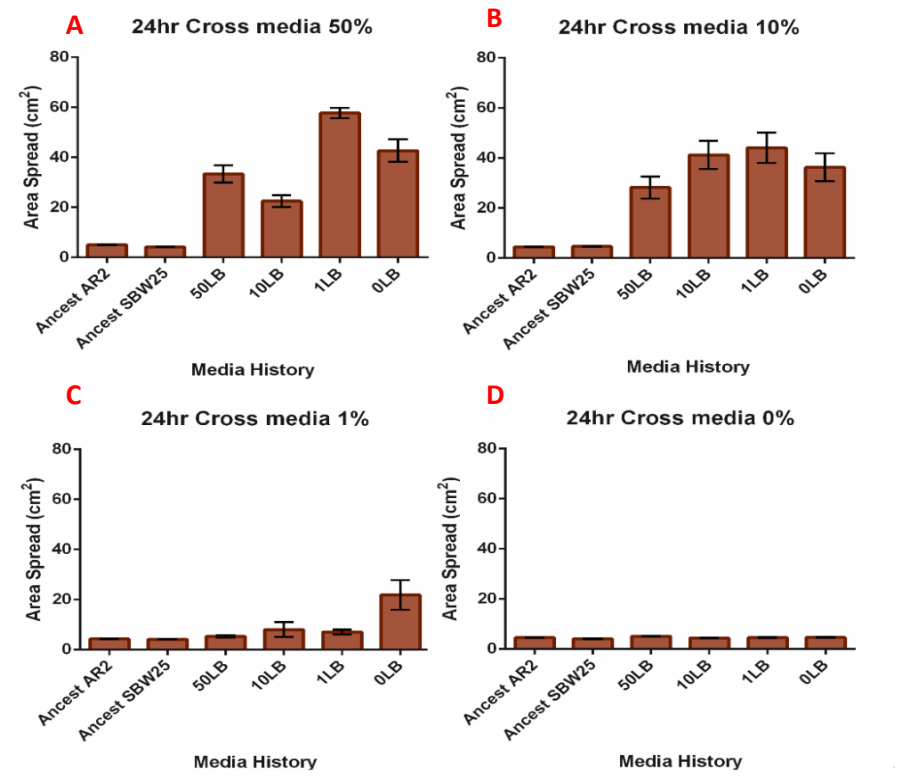

Figure 10: Graphs of the cross media experiment at 24 hours carried out on 50% 10% 1% and 0% LB (A, B, C and D respectively).

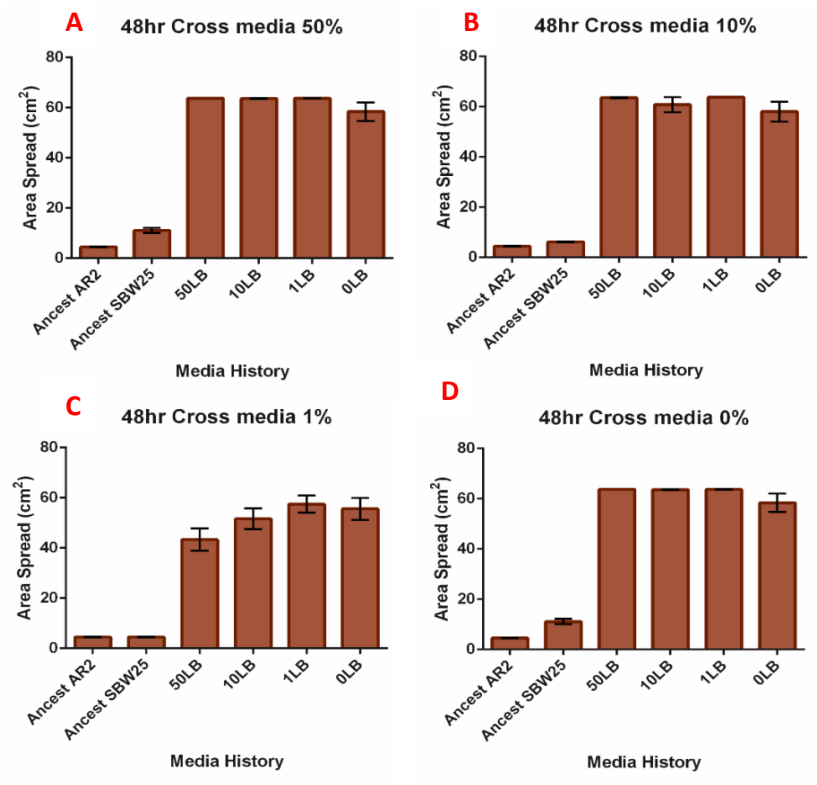

Figure 11: Graphs of the cross media experiment at 48 hours carried out on 50% 10% 1% and 0% LB (A, B, C and D respectively)

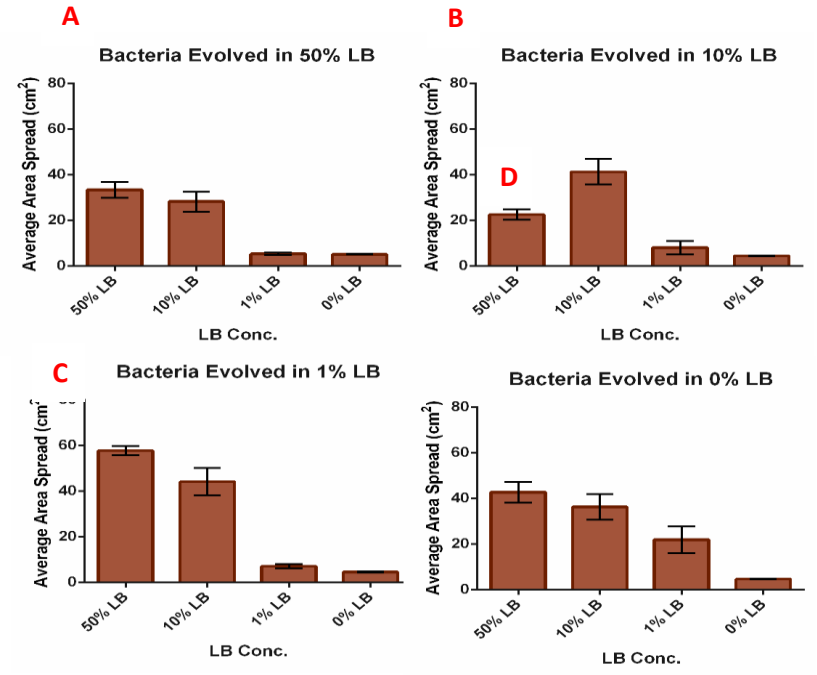

Figure 12: The Cross media graphs separated into media history (the evolved environments of each mutant) at 50%, 10%, 1% and 0% LB (A, B, C, D respectively). Shows how 50% and 10% mutants become specific to their evolved environment whereas 0% and 1% mutants evolve generalist strategies to increase motile spread based on their nutrient availability.

A second observation is that those which have evolved on the lower LB concentrations (1% and 0% LB) appear to on average have a slight motile advantage over the higher LB concentrations (Figure 12). The statistics for this experiment show that the high LB mediums (50% and 10%), which both predominantly use smooth spreading are not significantly different to each ($F_{1,80}$=0.745,P= 0.842) other but are significantly different to the lower two LB concentrations ($F_{1,160}$=0.543, P<0.05). Suggesting the shift in area spread occurs between 10% and 1% LB.

3.5 Biosurfactant assay

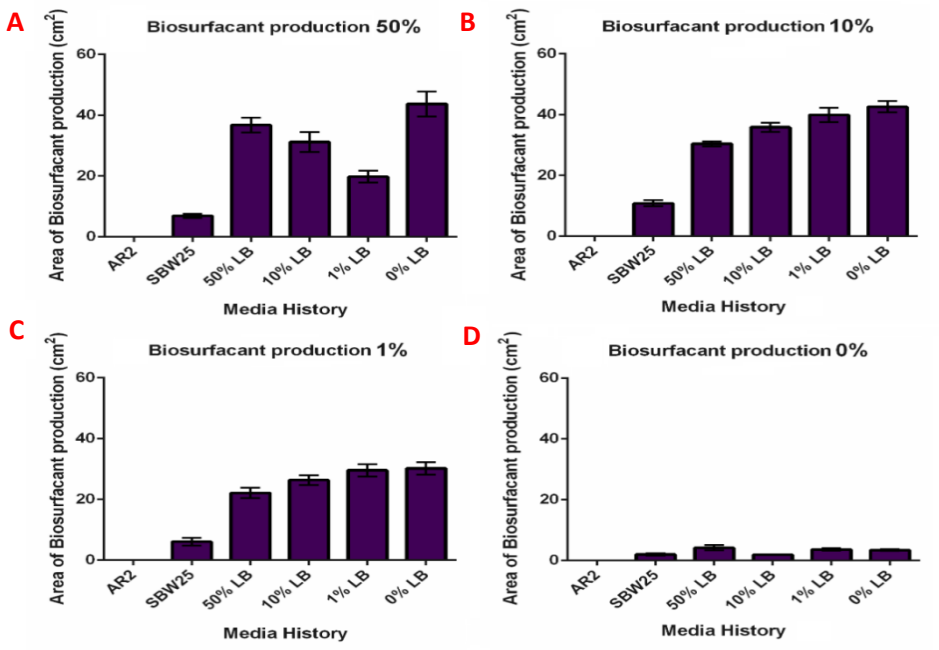

Figure 13: The biosurfacant production graphs separated into media history (the evolved environments of each mutant) at 50%, 10%, 1% and 0% LB (A, B, C, D respectively).

The surfactant assay aimed to prove that biosurfactant production increases in smooth spreading to provide a less resource intensive method of motility. However, the results seemed to prove the opposite. It appears that biosurfactant production is greater in the bacteria evolved in the lower LB concentrations (Figure 13). What does seem to happen is that biosurfactant production is media specific ($F_{1,3}$=122.60, P<0.05), as the complete absence of LB results in a significant loss of biosurfactant production (Figure 13). All the bacteria produced far more biosurfactant than the ancestral SBW25 strain in biosurfactant production, meaning that evolving towards flagella dependent motility increases biosurfactant production.

4.0 **Discussion**

4.1 Evolved Motility of *fleQ* Knockouts

4.1.1 Environmental Effect

As found by Taylor *et al* (2015), the non-motile mutant of *P. fluorescens* achieved motility through time and selection on all four mediums tested. The results follow the idea that an active nitrogen regulation network is required. The no nitrogen control showed limited bacterial growth throughout; this is likely due to a reduced survival rate because of the low nutrient environment. However, it is peculiar that the bacteria evolved motility in this negative control. This is likely due to presence of trace nitrogen either from the agar, or possibly from the volatile ammonia media which was created alongside the nitrogen void medium.

Taken altogether, an active nitrogen regulation system appears to have a great influence on the acquisition of motility in non-motile *Pseudomonas spp.* The nitrogen condition with the highest nitrogen levels (LB) achieved the greatest spread of growth closely followed by the minimal M9 media. This would suggest that active nitrogen regulation is required. However, an abundance of nitrogen allows the bacterial motility to thrive.

In the ammonia environment, where the nitrogen source is exclusive, motility took longer to establish and was never as substantial as the LB or M9 environments. Therefore motility can still occur in non-motile *P. fluorescens* in an exclusive nitrogen environment, but its success and benefits are reduced. This could be due to a greater dependence on the nitrogen regulating genes, thus evolving these genes to express motility is less favourable.

The comparison between the effective acquisition between the M9 and ammonia environments is curious. Given that the M9 environment nitrogen source is much lower than the ammonia nitrogen source, it may be expected that the bacteria adapt quicker towards motility in the ammonia environment. However, ammonia is toxic to bacteria

(Müller *et al* 2006), meaning the non-motile *P. fluorescens* had to compete with the toxic selection pressure. The resulting consequence considerably reduced the adaptation towards motility, suggesting that the toxic environment selects against motility because the mutation that confers motility would increase ammonia intake into the cell to toxic levels. Therefore motility is constrained within these environments. Thereby, in order for motility to evolve in ammonia the bacteria must have first built a resistance to its toxic environment. This gives reasoning as to why motility was able to establish in the M9 medium more readily than ammonia.

4.1.2 Selecting Motility in Non-motile Strains

The evolved AR2 mutants on LB were the only samples to have any effect on the positive and negative selection, where the positive selection significantly increased motility over the negative selection. I believe this to be the result of a combination between increased motility diversity at a high nutrient environment (as there is no consequence for slow motility) and also the fragile nature of acquiring motility through rewiring another regulatory network. The unstable flagella regulation, coupled with an ample nutrient environment, reduces a natural push towards increased motility and so population can be easily manipulated artificially.

4.1.3 Phenotypic Effect

The non-motile AR2 mutants only expressed smooth spreading across all the mediums, whereas the wild-type expressed smooth and spidery with the additional of the novel sun-like motility. This lack of diversity in the AR2 motility could be due to flagellar strength. An evolved flagellar regulatory system from homologous genes may not be as reliable or as strong as a wild-type mechanism; therefore the strength of the product may not behave as well. In other words, the AR2 mutants may not have a robust flagellar regulating mechanism, therefore, the flagellar number is reduced and mutations within the operon could result in a brittle protein structure. For spidery motility, flagella appear to play a vital role, due to its common appearance in hypermotility (van Ditmarsch *et al.,* 2013)**.** Although the non-motile mutant also has a transposon inserted into the viscosin producing gene *viscC,* I still predict a role of biosurfactant in the smooth spreading of the

AR2 mutants. This can be the result of a different biosurfactant, or the loss the transposon.

My reasoning behind a reliance of biosurfactant in the AR2 motility stems from the exclusive motile method (smooth), and a theory of a weakened flagella. The neat circle spread from the site of inoculation observed in the AR2 mutants suggests that they are relying on biosurfactants to move therefore reducing their flagella production; this can be cost effective, due to the intensive production of flagella and constant metabolic fuelling of the flagella (Zhao *et al.,* 2007 and Martínez-García *et al.,* 2014)**.** If this is the case, the evolutionary trait may only be useful *in vitro*, as flagella offer more than just motility to bacteria, in their natural environment *P. fluorescens* must compete for nutrients, location and with pathogens in all of which the flagella plays a role (de Weert *et al.,* 2002, Capdevila *et al.,* 2004 and Péchy-Tarr *et al.,* 2005). Undoubtedly, if the non-motile strain was introduced into the wild, it would quickly be outcompeted and become extinct purely as motility is such an important trait *in vivo* (Martínez-Granero *et al.,* 2006). The smooth spreading also occurs in the wild-type strain if there is an abundant nutrient environment**.** This theory suggests that with an abundance of nutrients and no competition, *P. fluorescens* is better suited to a less flagella intensive motile morphology and relies on biosurfactant to more effortlessly glide across their environment to acquire nutrients. This approach gives the bacteria substantial motility with the minimum amount of energy needed.

4.1.4 Non-motile Versus Wild-type

Overall, the wild-type strain was much more successful in colonising an environment than the mutants. The area spread by the wild-type strain was, on average, much more than the mutant strains, even once motility was widely expressed in the mutants. Therefore it seems that the resurrection of flagella regulation, from the rewiring of past evolution, will not be as successful as the wild-type.

This is also true for the diversity of motile expression. As I have previously explained, the AR2 mutant was restricted to one motile phenotype (smooth) and thus removed the

benefits of the spidery phenotype when exposed to a less nutrient rich environment. These limitations could drastically impair the bacteria in the wild and leave them exposed to competition and predators.

4.2 Motile Phenotypes of Wild-type *P. fluorescens*

The most striking observation found in the cross media and gradient experiments is the large diversity of motile phenotypes across the *P. fluorescens* species. These motile phenotypes have been categorised into three groups; Smooth, spidery and a new novel expression named 'sun-like' (Figure 8). The smooth and spidery spreaders and well recognised across the *Pseudomonas* genus, however, I believe the observed sun-like method is a new expression that occurs when there is no strong selection for spidery or smooth motility at high nutrient levels.

4.2.1 Smooth Spreading

Smooth motility occurred most commonly with a strong presence of LB when nutrients are rich (Figure 9). The benefit of smooth spreading is the increased surface area for the bacteria to colonise, which increases exponentially. I theorised at the beginning that smooth spreading was the result of a substantial biosurfactant production mechanism, causing the smooth spreading by the colony being held together by the surface tension of the biosurfacant, rather than venturing their own paths through the media. Although, the cross media experiment showed that those which evolved in an environment which selected for spidery spreading produce more biosurfactant if nutrients become more favourable (Figure 13). This suggests that the bacteria evolved more effective motility methods if starved of nutrients, meaning that when nutrients are dramatically decreased, bacteria become more motile efficient in comparison to those which have adapted to a more nutrient-rich environment. In other words, when bacteria evolve to adapt motility in a stressful environment, it can enhance their motility in a stable environment.

Rather than smooth spreading being a general evolved trait of a bacterial community, it appears to be media specific. As even the bacteria which adapted towards spidery motility were able to immediately adapt when conditions are favourable to smooth spreading (Figure 9). Suggesting that smooth spreading mechanisms can be turned on and off depending on the specific environment. I would also argue that smooth spreading is synonymous with increased biosurfactant production but again it is media dependent. Very little biosurfactant was produced in the low LB environments (Figure 13) which is where the least smooth spreaders occurred (Figure 9). Although it is clear there is a relationship between smooth spreading and biosurfacant production on a high nutrient environment, I cannot state the direction of the relationship, i.e. whether a high LB concentration selects for smooth spreading or increase biosurfactant production. However, I am sure that whichever is selected for, the other one will be apparent.

It is also worth considering that plentiful nutrients mean the selection for finding nutrients is removed and thus there is no need to fuel such resource intensive flagellar system. In this case, reliance on biosurfacants could be more cost-effective and gives greater evidence to the directionless circle of growth in smooth spreading. The weakened flagellar systems means less independent movement and the bacteria spread as a colony head together by the surface tension from the viscosity of biosurfacants. The impaired flagella function in motility could translated to other flagella roles i.e. biofilm formation. However, the numerous benefits and the general bacterium's dependence of flagella mean that its role is quickly restored when needed, even in the case of a catastrophic deletion of the flagellar regulating gene *fleQ* where other genes can be rewired from previous homoglous adaption (Taylor *et al.,* 2015).

4.2.2 Spidery Spreading

Spidery spreading is highly associated with a low LB environment (Figure 9)**.** The prominent tendrils of spidery motility are often associated with hypermotility (van Ditmarsch *et al.,* 2013) and thus, the spidery spreaders I observed are likely to be the most efficient movers. These spreaders occurred most when biosurfacant production was at its lowest (Figure 13)**,** therefore it is possible that spidery spreading only occurs

when biosurfactant is not selected for. But again, it is difficult based on the current results to conclude definitively whether the environment is selecting primary for spidery spreading or reduced biosurfactant production. However, it is likely that one is selected for, which causes the other.

Another reason for occurrence of spidery spreading at low nutrients is the balance between exploration and colonisation. Spidery spreaders move a greater distance but at a cost to an overall surface area, and so are more cable of exploring an environment than colonising it. At low nutrients, exploration would be favoured to find a more nutrient rich source and so spidery motility would a more successful alternative to smooth spreading. At low nutrient levels it is not favourable for the bacteria to stay and colonise the current environment as this could be fatal in the long-run for the community. Therefore the bacteria spread directionally using spidery tendrils to actively search for more nutrient rich environments.

It is also possible that the cost of producing biosurfacants is high and therefore an unnecessary expense to the bacteria when nutrients are low (Makkar and Cameotra 1999). So, by avoiding wasting nutrients on biosurfacant production, provides a benefit. Although I do not know how much of a selection resource conservation is, as increase flagella number and intensity also come at a high cost (Zhao *et al.,* 2007) so I postulate that the gain may not out way the cost when replacing biosurfactant with flagella usage in spidery spreading.

4.2.3 Sun-like

The Sun-like phenotype occurred only at the highest LB concertation (Figure 9). I believe this motile phenotype to be a combination of both spidery and smooth spreading. The high nutrient environment creates an opportunity for a highly diverse bacterial community, as there are fewer stressful selections the bacteria must adapt to. Therefore, it is not unreasonable to expect that that many motile phenotypes are being co-expressed. In this case however, I am speculating that this motile phenotype was a community of co-operators and cheaters. Here, the pioneering spidery spreaders pave

the way through the media, almost like roads through a forest, and the smooth spreaders hitch-hike through the roads and spread using biosurfacants to colonise the nearby area. Creating the large smooth growth surrounded by thick tendrils, which I have named sun-like.

I believe the reason the sun-like phenotype only occurs in higher LB concentrations, is the absence of the cheaters when nutrients are less accessible. As I mentioned, these cheaters are likely to be the expression of smooth spreaders which my results have shown to not be selected at lower nutrient concentrations (Figure 8 and Figure 9). AT low nutrient levels, the community must constantly explore a harsh environment, as attempts to colonise an area will quickly use up to local resources thus causing the extinction of the colony. Therefore, any cheaters which aim to piggy back off the spidery explorers at low nutrients levels will not survive long enough for effective reproduction, so the characteristic sun-like phenotype does not occur.

For this reason, I propose the sun-like expressions is the result of the environment and phenotypic diversity in the population, where both colonisers and explores (i.e. smooth spreaders and spidery spreaders) can exists as there is no negative selection for one. The expression occurs when a bacterial strain has adapted towards efficient motility, which allows the community to flourish when a motile strength is advantageous. The abundant nutrients reduce the selection towards a specific phenotype and thus they are co-expressed. The nature of the spidery phenotype means they can explore greater distances, and so if the smooth spreaders are able to adhere to the spidery spreaders they can take advantage of these distances and increase the speed in which they colonise area. As the smooth spreaders are more efficient in colonising a large surface area, their population dramatically increases whereas the spidery spreaders population can only increase through the paths they travel. Sometimes, when sun-like expression is observed on a petri dish, after 48 hours the only evidence of spidery spreaders is a very faint pale white line showing the paths they travelled, the rest of the plate appears to be one giant smooth spreader.

Alternatively, the sun-like spreaders may be the causes of a high nutrient environment allowing specialisation very quickly. In this case there would be no selection for cheaters,

just increased motility. So in this situation, the environment allows for a highly diverse population and for very quick motility to cross the media. The bacteria diversify between ancestral (smooth) spreading and hypermotile (spidery) spreading bacteria, which co-exist having no effect on each other's success.

4.3 Biosurfactant

4.3.1 LB Interaction with Biosurfactant

There is a significant difference between the production of viscosin and the presence of any small amount of LB (Figure 13). This suggests a minimal amount of LB can act as a catalyst for viscosin production. Or the LB nutrients favour protein formation allowing for increased viscosin and an exclusive M9 minimal media prolongs protein expression. There have been successful adaptions of protein measurements from minimal M9 media which increase protein production through the addition of LB (Cai *et al.,* 2016 and Paliy *et al.,* 2003), therefore it is likely the absence of LB is significantly impairing viscosin production possibly due to a lack of resource to produce a useable amount of viscosin. Using this point of view you could argue that the media selects for biosurfactants, which the presence, or absences of, selects between smooth or spidery motility respectively.

4.3.2 Evolving Biosurfactant Usage

What is definitely clear is that viscosin production, regardless of the motility strategy, increases in quantity if the bacteria are selected for motility**.** This is evident by the fact that every selected strain of SBW25 produced far more viscosin than the ancestral strain. Therefore, this suggests that viscosin supplies a strong benefit to motility, so much so that selecting for motility has a direct effect on viscosin production. Although viscosin is not essential for motility, its aids can undeniably be a deciding factor when faced with competition and increasing the community fitness.

5.0 Conclusion and Further Research

Overall, evolved motility in the non-motile strain of *P. fluorescens* is not as effective as the wild-type strain. The non-motile mutant cannot adapt to environments as well as the wild-type nor is it able to colonise an area as well as the wild-type. To further prove my theory that the AR2 mutants have weaken flagellum, I would like to assess the number through electron microscopy, or the speed in which the single cells are able to move through a microfluidic devices. Molecularly, I could assess the number of point mutations in the *fleN* gene to get an understanding of flagellar number (Dasgupta *et al* 2000)

What would be interesting is to see whether other flagella involving functions are able to resurrect flagella regulation in non-motile *P. fluorescens,* for example biofilm formation. I could investigate whether substantial biofilms can be formed by evolving *ntrC* and *ntrB* in non-motile *P. fluorescens*. If flagella function is repaired I can delve further and see whether the evolution can induce hypermotility. If the non-motile strains do not evolve any flagellum then it raises an interesting consideration between the importance of motility verses biofilm formation in the evolution of flagella.

The smooth and spidery motile phenotypes are clearly dependent on nutrient availability and I believe spidery motility to be induced in times of stress where the bacteria must explore for a less stressful environment. If spidery motility is a stress response, it may also be employed when the community is exposed to competition or predators. So I could test whether specific motile phenotypes are preferred when competing against other bacteria, or in defence of a predator on agar.

Smooth spreading is seen when the bacteria are in a comfortable environment. I believe a major factor behind smooth spreading is an increase in biosurfactant production, however I am unsure whether the environment selects for smooth spreading which induces increased biosurfacants, or vice versa. To test this I could grow the bacteria in a liquid broth, rather than agar, where swarming motility is not as essential. Biosurfactant production can be detected by inoculating and incubating varied LB concentrations at 27^{o}C overnight. After shaking for 10 seconds and then being allowed to rest for 5 minutes

at room temperature, the biosurfactant presence can be measured (Alsohim 2010). A positive result for biosurfacant is frothy broth at the air-liquid surface. A measurement here between different nutrient concentrations would give an indication whether biosurfacant alone is affected by nutrient levels.

To give further understanding whether my theory of the sun-like motility is a combination of both spidery and smooth spreading, it would be useful to have a 48 hour time-lapse of the bacterial spread on agar. This will give evidence as to whether the spidery tentacles are indeed pioneering through the environment, allowing the smooth spreaders to take advantage to these paths by spreading out using biosurfactants. I could also test whether the population has a large diversity of flagella number, as the spidery spreaders should express a greater number of flagella, whereas the smooth spreaders express fewer. This would result in a varied flagella number within population. Testing for flagella number can be done using electron microscopy or through sequencing the *fleN* gene for point mutations.

What is most essential is to supply ecological significance to this research. A plant assay to understand whether smooth or spidery spreading aids colonisation of plants would prove vital to accompany this research. By measuring whether a particular motile method favours rhizosphere occupation, or has the potential to aid plant growth, could be of huge benefit agriculturally. To understand this it is possible to induce motile phenotype through LB concentration and have a plant root in the centre of the medium. The bacteria can be inoculated away from the root and after incubation; the levels of bacteria on the plant root can be measured, through a serial dilution on nitrofurantoin medium. Another method to assess this is to take natural isolates from stressful environments, i.e. a phage infected population or naturally low nutrient environment. These isolates may favour a particular spreading method and thus give evidences to whether stressful environments select for spidery motility. The same experiment can be done from areas of high colonisation, to see if these natural isolates favour smooth spreading. If a significant difference is found this would be useful in terms of biofertiliser or biocontrol.

The *Pseudomonas spp.* is known to have many colony morphologies, each of which holds important adaptions for the bacteria. For example, the wrinkly spreaders have strong adhesive properties, allowing adhesion to each other and surfaces creating a self-supporting mat (Rainey and Travisano 1998), or the trade-off in fuzzy spreaders between resistance to bacteriophage and poor biofilm formation (Ferguson *et al* 2013). Moreover, previous studies have connected the changes in bacterial motility with antipredatory-defence (Friman *et al*., 2008) and fitness advantages in the presence of phages (Taylor and Buckling 2013). While, these patterns might not be universal **(**Koskella *et al.,* 2011) it is possible that the motile phenotypes I have underpinned may also have yet undefined ecological significances in wider context of bacterial fitness.

6.0 References

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