Master's Thesis

The effects of growth medium on *Flavobacterium columnare* colony morphology and its phage resistance mechanisms.

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The freshwater bacterium *Flavobacterium columnare* is known to exhibit different morphologies when cultured in different media with bacteriophage. This could influence the type of phage-resistance mechanisms the bacteria use, such as CRISPR-Cas system or alterations which reduce bacteriophage adsorption. In this study the different morphologies which arise in different media when treated with bacteriophage was investigated to identify their favoured phage-resistance mechanism. The bacteria were screened for acquisition of new CRISPR spaces and tested for changes in phage adsorption. Results show that the growth medium itself influences the bacterial colony morphology, likely due to the difference in nutrients available. When exposed to bacteriophage, a small proportion of bacteria in HTC medium acquired new CRISPR spacers, however, the preferred phage-resistance mechanism appears to be something other than CRISPR-Cas overall, in SHIEH and HTC. Improved understanding of bacterial resistance mechanisms is useful towards the development of bacteriophage therapies.

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1 INTRODUCTION

1.1 Bacteriophage

Bacteriophage (phage) are bacteria specific viruses (Chanishvili 2012) of which there are estimated to be 10^{31} on the planet (Brussow and Kutter 2005). This is approximately 10 times more than the number of bacteria, suggesting that phage are more abundant than any other known life form. Phage typically follow one of two main life cycles; the lytic cycle and the lysogenic cycle. Lysogenic phage, also known as temperate phage, infect bacteria and either insert their genetic material into the bacterial genome, or exist in the bacteria as an episome/plasmid. This genetic material is then passed on to the daughter cells as the bacteria divides. (Hanlon 2007). Lytic phage attach to the surface of a bacteria and replicate inside the bacteria before causing the bacterial cell to lyse, leading to an increase in the number of phage virions (Delbrück 1940, Luria et al. 1943). Lytic phage are those which are suitable for phage therapy due to their rapid replication and lysis of bacterial cells (Hanlon 2007).

In 2016, antimicrobial resistance was named as the biggest global threat by the United Nations General Assembly (United Nations 2016). The increasing awareness of antibiotic resistance has led to a search for alternative treatments. Phage therapy is one of the proposed alternative therapies, which has been studied for almost 100 years as a treatment against bacterial diseases (Chanishvili 2012). This is due to the many advantages of phage: such as being host-specific, self-amplifying, able to degrade biofilms, and low toxicity in humans (Bourdin et al. 2014, Donlan 2009). Phage therapy is being studied as a treatment for a variety of bacterial diseases of both human and animal origin. One such disease, for which phage therapy is being researched for, is columnaris disease caused by the bacterial fish pathogen *Flavobacterium columnare* (Laanto et al. 2015).

1.2 Resistance Mechanisms against phage infection

As the result of coevolution between bacteria and phage, bacteria have developed many mechanisms to prevent phage infections, including the prevention of phage adsorption, prevention of phage DNA entry, cutting of phage nucleic acids, and abortive infection systems (Labrie et al. 2010). Some of the phage-resistance mechanisms, which relate to *F. columnare* are discussed below.

1.3 CRISPR-Cas

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (cas) genes are one of the 'cutting of phage nucleic acids' phage resistance mechanisms. CRISPRs are loci in bacterial genomes consisting of short palindromic nucleotide repeats interspaced with unique spacers, and genes encoding for Cas proteins. The repeats and the spacers form the repeat spacer array. In 2005 three different studies concluded that the spacers were acquired from phage and other DNA elements. In one study of *Streptococcus thermophilus* the CRISPR spacers were shown to have 75% homology to *S. thermophilus* phage's, and that 20% had shown homology to *S. thermophilus* and *Lactococcus lactis* plasmids (Bolotin et al. 2005). Another study found that, in the spacers of several bacterial genomes studied, 65% of the spacers had homology with bacterial phage's and conjugative plasmids (Mojica et al. 2005). The third concluded that in species of *Yersinia* new CRISPR spacers were acquired from prophage DNA (Pourcel et al. 2005).

In 2007, it was demonstrated that CRISPR-Cas was an immune system of bacteria. This was determined in a study of *S. thermophilus* being targeted with phage and the conclusion that the bacteria attained phage resistance through acquisition of new spacers from the genome of the phage it had been exposed to (Barrangou et al. 2007). This study also concluded that certain Cas proteins provided the immunity due to their enzymatic activity, and that some Cas proteins may be involved in the acquisition of the new spacers.

The CRISPR-Cas system works in three stages to provide adaptive immunity against invading genetic materials: adaptation, expression, and interference. During the adaptation stage a short sequence of foreign genetic material is acquired by a complex of Cas proteins, usually consisting of at least Cas1 and Cas2. This is achieved through the endonuclease activity of the Cas proteins. This genetic material is then inserted into the CRISPR array as a new spacer (Amitai and Sorek 2016). The second stage of expression involves the transcription of the spacer into CRISPR RNAs (crRNA) which will eventually bind with a Cas protein, such as Cas9 (Makarova et al. 2015). Firstly, a precursor crRNA (pre-crRNA) is produced, which is one long transcript of the CRISPR spacer array containing many crRNAs. This pre-crRNA is then cleaved to produce the smaller crRNAs, which contain one spacer and one repeat (Brouns et al. 2008). Processing of the pre-crRNA into crRNAs is generally carried out by Cas proteins with endoribonuclease activity (Li 2015).

In the interference stage, the crRNA works by guiding the Cas protein to the target DNA of a phage or DNA element it has previously been infected with and cuts the DNA where is matches the crRNA. There are many types of CRISPR-Cas systems which can currently be divided into two classes, six types (I-VI), and several subtypes (Makarova et al. 2015). The main difference being that class 1 systems have crRNA effector complexes which are multi-subunit compared with class 2 systems which have a single protein (e.g. Cas9) which carry out all effector complex functions in the interference phase.

1.4 Preventing phage absorption - Surface Modifications

Other resistance mechanisms that bacteria have developed against phage infection include those which prevent phage adsorption. One of these methods includes the modification and/or blocking of the surface receptors and capsules which phage need to attach to. This can be done using phase variation or expression of secondary proteins (Labrie et al. 2010). An example of phase variation employed against phage infections is in the bacteria *Campylobacter jejuni*. *C. jejuni* has a capsular

polysaccharide (CPS) which provides protection to the bacteria. Phage can bind to the CPS to initiate infection. However, mutations in the CPS have resulted in phage being unable to bind. It was found that the identified phage receptor (MeOPN) was phase variable, meaning that the bacteria were able to alter the confirmation of this protein, and that the protein had to be in a specific confirmation for phage to infect the bacteria (Sørensen et al. 2012).

1.5 Flavobacterium columnare

Flavobacterium columnare is a fish pathogen causing substantial losses in fish farms around the world. For example, in the aquaculture of catfish in the USA, it is responsible for up to 60% of the outbreaks of infectious diseases (Olivares-Fuster et al. 2007). In Finland, it is one of the leading causes of fish mortality in the fish farming industry (Suomalainen 2005). The bacteria are gram-negative rod-shaped bacterium belonging to the genus *flavobacterium*, family Flavobacteriaceae (Bernardet et al. 1996). It was first described back in 1922 after being seen infecting fish in the Mississippi river, but it was at the time named *Bacillus columnaris* (Davis 1922). However, it wasn't until 1944 that it was successfully isolated from a disease outbreak in sockeye salmon (*Onchorhynchus nerka*) and was thought to be a *myxobacteria* and was renamed to *Chondrococcus columnaris* (Ordal and Rucker 1944). In 1945, it was isolated again but thought to be a different bacterium, one which was morphologically similar to *Chondrococcus columnaris,* and was named *Cytophaga columnaris* (Garnjobst 1945). Later, in 1986, the bacteria were placed in the family *Cytophagaceae* under the genus called *Flexibacter* and was named *Flexibacter columnaris* (Bernardet and Grimont 1989). It wasn't until 1996, that the bacteria were given the name *Flavobacterium columnaris* after being placed in the genus *Flavobacterium* and the family *Flavobacteriaceae* (Bernardet et al. 1996). The bacteria were given this name after collection of DNA-rRNA hybridisation data. protein profiles and fatty acid profiles (Bernardet et al. 1996). *Flavobacterium* are motile bacteria which use a gliding motion and grow as yellowish colonies on agar

(Bernardet et al. 1996), and *F. columnare* are described rhizoid in shape when grown on agar (Anacker and Ordel 1959).

F. columnare is a freshwater bacterium. It has been shown to thrive in water conditions like the composition of tap water. It does not survive in seawater conditions (Chowdhury and Wakabayashi 1988). Therefore, the disease is only problematic for freshwater fish, in nature and aquaculture.

F. columnare causes a disease known as 'columnaris disease'. The diseases initial visual symptoms are usually as whitish to yellowish spot(s) on the body of the fish. These are lesions which rapidly increase in size and are fatal to the fish (Davis, 1922). It is typical for a fish to first show infection around the fin; however this is not always the case and in many cases the area of initial infection is where fish have suffered a previous injury. The gills of many fish also become infected which appears as white spots (Davis 1922). In various Salmon species, lesions which form around the base of the dorsal fin have been described as having a 'saddle' appearance (Pacha and Ordal 1967). This lesion was termed 'saddleback' disease and was attributed to fish easily receiving injuries in that region of their body. This injury provides an easy route of infection for the bacteria (Cone et al. 1980). The disease gained the names 'columnaris' after it was observed that when placed onto a glass slide, the bacteria would bundle together in what can be described as columns (Davis 1922).

F. columnare has been known to change morphology in laboratory conditions and studies have determined that there is a relationship between rhizoid morphology and bacterial virulence (Kunttu et al. 2011). Studies have also shown that when the bacteria are exposed to phage, they can alter their morphology from a rhizoid morphology to smoother colony morphologies; one of which is named rough type, and another which is named soft type (Figure 1). It was also established that the change in morphology type caused the bacteria to gain phage resistance but become non-virulent (Laanto et al. 2012).

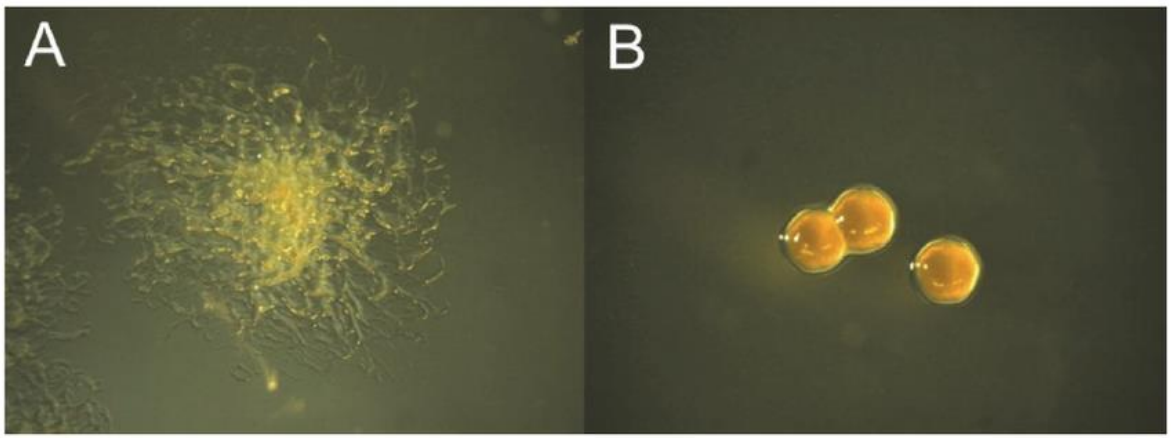

Figure 1: Colony morphologies of *F. columnare* showing the rhizoid type in A and the rough type in B (Laanto et al. 2012)

The strains of *F. columnare* have varying degrees of virulence (Sundberg et al. 2016). Strains of high virulence kill fish more quickly than those of low virulence. Also, fish infected by high virulent strains are less likely to show any physical signs of infection, implying that the bacteria kills them faster than the lesions have time to form. Contrastingly, strains of lower virulence cause more physical lesions to form before the fish die (Pacha and Ordal 1967). This may be due to the longer time taken to kill the infected fish.

Previous studies have concluded that *F. columnare* exhibit a CRISPR-Cas mechanism and it has been revealed that they possess two CRISPR-Cas loci; a type II-C, termed C1, and a type VI-B, termed C2 (Smargon et al. 2017, Laanto et al. 2017). The main difference being that C2 is an RNA targeting system and C1 targets DNA.

1.5.1 CRISPR type II-C

Processing of pre-crRNA is only essential in some type II systems. However, type II CRISPR systems were found to not employ endoribonucleic Cas proteins for cleavage of their pre-crRNA into crRNA. Instead they use a host enzyme called RNase III and a non-coding RNA called trans activating RNA (tracrRNA) (Deltcheva et al. 2011). Type II-C CRISPR are common and they contain only three

cas genes in the locus; cas1, cas2 and cas9. It is thought that cas1 and cas2 are involved in new spacer acquisition (adaptation) (Zhang et al. 2013). Cas 9 has been demonstrated as essential for the interference stage of CRISPR-Cas, as the function of the CRISPR ceases when the Cas9 is deleted or mutated (Zhang et al. 2013). Unlike other type II CRISPR systems, type II-C was demonstrated to not require pre-crRNA processing to effectively carry out interference. However, it does require tracrRNA for effective binding of the crRNA-bound Cas9 to its target DNA. (Zhang et al. 2013) Another feature of type II-C CRISPR-Cas is the discovery of its Cas9 protein being better suited for cleavage of single strand DNA (ssDNA), rather than double stranded DNA (dsDNA). This is attributed to its Cas9, possible, lack of ability to unwind DNA. Therefore, supplementary co-factors may be required to allow for successful dsDNA cleavage in type II-C CRISPR-Cas systems (Ma et al. 2015).

1.5.2 CRISPR type VI-B

Type VI-B CRISPR-Cas loci contains only one effector protein called Cas13b and does not contain common CRISPR-Cas genes cas1 and cas2. Cas13b can cleave its own pre-crRNA into crRNAs and contains the ability to cleave single-stranded RNA (ssRNA) with its HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domain. Cas13b is unique because it only targets RNA. It has been speculated that spacer acquisition in type VI-B locus is achieved by using Cas1 and Cas2 proteins whose genes are encoded on another CRISPR-Cas locus located in the same bacterium. Type VI-B CRISPR-Cas locus almost always occur along with other CRISPR-Cas loci (Smargon et al. 2017).

Many type VI-B systems are accompanied by accessory proteins known as Csx27 and Csx28. Csx28 can enhance the activity of Cas13b, whereas Csx27 was found to repress the activity of Cas13b (Smargon et al. 2017). However, neither of these accessory proteins were identified in the type VI-B CRISPR-Cas of *F. columnare* (Laanto et al. 2017, Smargon et al. 2017).

1.6 Motive of the study

For the purpose of this experiment the study system chosen was the bacterium *F. columnare* strain B185 and the phage FCL-2. FCL-2 is from the *myoviridae* phage family and was isolated from a fishery in central Finland and is known to infect *F. columnare* B185, also isolated in central Finland. (Laanto et al. 2011). It has previously been speculated that *F. columnare* may be able to mutate or decrease the expression of their surface proteins, which are utilized by phage, to prevent infection by phage. Altered expression of the surface proteins utilised by phage was suspected when the colony morphology of *F. columnare* exposed to phage changed from rhizoid to rough and became phage resistant (Laanto et al. 2012).

Preliminary experiments have suggested that culturing *F. columnare* in different media can result in different bacterial morphologies when the bacteria is exposed to phage. Growing the bacteria in SHIEH medium promotes morphology change to rough. However, HTC (High Tryptone Cytophaga) medium allows the cells to retain their rhizoid morphology, even in the presence of phage (Hoikkala personal communication).

The aim of the study is to observe which resistance mechanisms are favoured in different conditions upon phage exposure. It is known that *F. columnare* CRISPR-Cas loci are being utilised in nature (Laanto et al. 2017) and that different morphotypes are known to have varying levels of phage resistance (Kunttu et al. 2011). However, the bacteria's preferential deployment of phage resistance mechanisms has not been studied in laboratory conditions. I hypothesise that the bacteria will remain rhizoid in HTC medium after exposure to phage. It is also hypothesised that the rough colonies from SHIEH are more likely to have developed resistance through a reduction of phage adsorption, whereas the rhizoid colonies in HTC are more likely to develop resistance by acquisition of new CRISPR spacers. Information pertaining to the influences on phage resistance caused by

different medium can be used towards the development of successful phage therapies against *F. columnare*.

2 METHODS AND MATERIALS

The initial experimental set up consisted of making all necessary medium. These included SHIEH medium (Appendix 1) and HTC medium (Appendix 2). The only modification made to these recipes was that formulated water (Appendix 3) was used instead of regular distilled water. 10% medium was created by diluting 100% medium with formulated water. All agar plates used were made from SHIEH agar, by mixing 500 ml SHIEH medium with 5 g of agar powder. Soft agar was made by mixing 100 ml SHIEH medium with 0.5 g of soft agar powder.

An initial culture of B185 was grown, from an existing B185 stock, in 5 ml of 100% SHIEH medium. The culture was left to grow overnight on a rocking platform (120 rpm) in room temperature (RT). The turbid culture was then mixed with glycerol and fetal calf serum (ratio 1:1) and stored at -80 °C. This frozen stock was the source of B185 for all subsequent initial B185 cultures. The FCL2 phage stock used for all experiments was taken from an existing stock made by another member of the research group. The aliquot taken consisted of 10ml of 10^{10} plaque forming units per ml (PFU/ml).

Unless otherwise stated, all incubations throughout the experiments were overnight at RT on a rocking platform (120 rpm).

In some experimental stages *F. columnare* colony forming units (CFU) per ml was inferred by using optical density (OD). This was used in conjunction with a linear equation describing the relationship between CFU/ml and OD which had previously been tested by members of the research group.

2.1 Main Experiment

Using the cryo-stored B185, two 50ml falcon tubes containing 5ml of 100% either SHIEH or HTC growth medium were inoculated. Both tubes were incubated

overnight (~18hours). Using the overnight cultures, the OD of each were analysed (using a Thermo Scientific Multiskan FC set at wavelength 595 nm). Using the OD the CFU/ml was calculated for each of the overnight cultures using a previously determined formula in excel. For both cultures, the CFU/ml was then adjusted to $5x10^4$ CFU/ml. This was done by using equation 1 to calculate the amount of culture and equivalent 10% growth medium needed and mixing them in a fresh tube:

$$C_1 \cdot V_1 = C_2 \cdot V_2 \quad (1)$$

Using the FCL2 phage stock, a dilution series was performed to produce a 10ml stock of 10^5 PFU/ml. The initial experiment was set up in new 50ml falcon tubes in replicates of 4, the set-up of each tube can be seen in Table 1 and all 16 resulting tubes were incubated overnight.

Table 1: Calculated volumes needed for each of the four treatments in the initial experimental set-up.

Treatment (In replicates of four)	$5x10^4$ CFU/ml B185 SHIEH (ml)	$5x10^4$ CFU/ml B185 HTC (ml)	10^5 PFU/ml FCL-2 (µl)	10% HTC in FW (ml)	10% SHIEH in FW (ml)	Total volume in tube (ml)
HTC control	0	1	0	4	0	5
HTC + phage	0	1	50	3.95	0	5
SHIEH Control	1	0	0	0	4	5
SHIEH + phage	1	0	50	0	3.95	5

Multiplicity of infection (MOI) = 1

After incubation, all tubes were vortexed, and the bacterial titre was calculated based on the OD measurements. This information was used to estimate a range of dilutions needed, which would be used to plate the cultures onto SHIEH agar plates, resulting in a countable number of bacteria per plate (between 30-300 colonies).

To determine the bacterial counts a dilution series for each tube was performed, by diluting the cultures in the relevant 10% growth medium, and the three most relevant dilutions were plated onto SHIEH agar plates in triplicate. The tubes were subsequently incubated overnight. This process was repeated daily for another 4 days. The reason for the plating of growth samples was to track the proportions of each colony morphology over the course of the experiment. Using these plates, a more precise CFU/ml was determined later.

On the 5th day, 1ml of each culture was transferred to fresh tubes containing 9ml of the equivalent 100% growth medium. These tubes were then incubated for 1.5 days. On day 7 the same process was performed as the first 5 days to track the colony morphology.

2.2 DNA extraction for population level CRISPR spacer screening

From the day 7 100% medium cultures, 1ml was taken from each tube, and DNA was extracted to perform a sensitive population screening for new CRISPR spacers. The DNA was extracted using Blood and Tissue Kit (Qiagen) following the manufacturer's protocol and using materials provided by the kit.

2.3 PCR and gel electrophoresis of extracted DNA.

Using the resulting extracted DNA from the samples, a polymerase chain reaction (PCR) (protocol, appendix (4)) was performed to amplify both the C1 and C2 spacer section of the CRISPR loci of all the samples. The master mix used for the PCR in shown in Table 2.

Table 2: Volume of substrates needed to make master mix per one PCR reaction used in the experiments.

Master Mix per 1 PCR reaction.	Volume/one reaction (µl)	Final Concentration in the reaction
DreamTaq Polymerase (5 U/µl)	0.2	0.05 U
dNTP mix (10 mM each)	2	1.05 mM
Primer (forward) (10 µM)	1	0.5 µM
Primer (reverse) (10 µM)	1	0.5 µM
Mg2+(50 mM) (for C1 only)	1.6	4.21 mM
H_2O (for C2 add 1.6 µl)	12.2	35.6 M (for C2 40.3 M)

Upon completion of the PCR reactions, 8µl of each reaction was pipetted into the wells of 2% gel electrophoresis gels, 1 gel for the C1 reactions and another for the C2 reactions, and the gels were run for 2 hours (100 v, 100 mA, 2.1 h.m). The electrophoresis gels were made from 125ml of TAE buffer with 2.5g of agarose powder and 3 drops of Ethidium Bromide (EtBr). Upon completion, the gels were imaged (using a ChemiDoc MP imaging system set for EtBr).

The forward and reverse primer sequences used for the PCR reactions are shown in Table 3, designed based on an unpublished genome of B185. They are arranged in a 5′ to 3′ manner and only amplify the variable end of the arrays. The C1 forward primer binds to the second spacer in the CRISPR spacer array and the reverse primer binds to the conserved area downstream of the array. The C2 reverse primer

binds to spacer number five in the CRISPR spacer array and the forward binds to the conserved area upstream of the array.

Table 3: Table containing the sequences of the primers used in the PCR reactions for the amplification of the C1 and C2 CRISPR spacer arrays.

Primer Name	Primer Sequence
C1 Forward	TGTTCTATCGGCGTAAAATAAGTT
C1 Reverse	CCCTAAAGCACCACAACCCA
C2 Forward	GGTCTAAATACAATTGCTCTTTGACATT
C2 Reverse	CATCTGTATAAGTAGCATAGCGTTGT

2.4 Colony Isolation

From the day 5 plates, of the phage exposed treatments, 60 colonies in total were isolated based on morphology (Table 4). Each colony was streaked onto a 5th of an agar plate and inoculated into PCR master mixes for amplification of sections of the C1 and C2 CRISPR spacer arrays. The inoculated PCR mixes were then used to perform a PCR reaction, and the products run on an electrophoresis gel, identical to the protocols performed for the sensitive population CRISPR screening. This was to determine if any of the isolated colonies had obtained new CRISPR spacers.

Table 4: Names given to each isolated colony, including description of its morphology, and which treatment and replicated it was isolated from.

Isolated Colony Names	Morphology	Treatment replicate
A1-5	Rough	SHIEH + Phage Replicate 3
A6-10	Rhizoid	SHIEH + Phage Replicate 3
B1-5	Rough	SHIEH + Phage Replicate 2
B6-10	Rhizoid	SHIEH + Phage Replicate 2
C1-5	Rough	SHIEH + Phage Replicate 1
C6-10	Soft	SHIEH + Phage Replicate 1
D1-5	Rhizoid	HTC + Phage Replicate 1
D6-10	Rough	HTC + Phage Replicate 1
E1-5	Rhizoid	HTC + Phage Replicate 2
E6-10	Rough	HTC + Phage Replicate 2
F1-5	Rhizoid	HTC + Phage Replicate 3
F6-10	Rough	HTC + Phage Replicate 3

The streaked isolated colonies were then taken from the initial plate and streaked onto new plates. This was repeated a total of three times, to ensure that the bacteria were phage-free. After the third streak the colonies were then used to inoculate 1ml of 100% medium, either SHIEH or HTC matching which treatment the colonies had been grown in, on a multi-well plate. The multi-well plates were then incubated overnight on a plate incubator at 120 rpm. The following day the overnight colony

growths were used to created cryo-stored stocks of each isolated colony for further testing. Colony B10 was not viable and therefore a cryo-stored stock was not made.

2.5 Adsorption testing

The method used for all adsorption tests were as follows. The isolated colony to be tested was used to inoculate 1 ml of 100% growth medium, either SHIEH or HTC depending on the medium the bacterial colony was isolated from. The inoculated medium was then incubated on a rocking platform (120 rpm) and periodically vortexed and OD checked. Once an OD of 0.8 was reached it was determined that there were enough CFU/ml of bacteria in the culture to perform the next stage of the adsorption testing. A sample of bacterial culture was transferred to a multi-well plate along with a calculated volume of relevant 100% medium and 0.1 ml of 10^{-5} dilution of FLC-2 phage to contain a total volume of 2 ml containing $1x10^{8}$ CFU/ml. This was done in replicates of three and was a MOI of 10^{-4}.

Simultaneously, 1.9 ml of relevant 100% medium and 0.1 ml of FCL-2 phage was added to 3 other wells as controls. The plate was vortexed then incubated at room temperature for 20 minutes, after which 100µl from each well was separately transferred to tubes containing 1.9ml of relevant 100% medium and 3 drops of chloroform. The tubes were vortexed to allow the chloroform to kill all bacteria in the samples and all tubes were stored on ice.

To determine phage titre in each sample, 100µl subsample of each ice stored tube was transferred along with 300µl of a wild-type B185 culture into tubes containing 3ml of soft SHIEH agar (in a water bath at 47 °C) and vortexed before being poured onto a SHIEH agar plate. All resulting plates were incubated in room temperature for 2 days.

After incubation, the number of plaques on each plate was counted. The control consisted of performing all steps using the wild-type B185. An increase in the number of plaques on a plate compared to the wild-type control would indicate a

reduction in ability of phage to adsorb to bacteria. The plates from the wells containing only phage were used as a control to determine the number phage present in the testing sample.

2.6 Determination of phage resistance

To confirm that all isolated colonies were indeed phage resistant a phage spot test was implemented. Using 1ml overnight growths of each of the 59 viable isolated colonies, 300µl of each was transferred to 3ml of soft agar. They were vortexed and poured onto SHIEH agar plates. Quickly after, dilutions of FLC2 phage was placed as droplets on the agar plates in tenfold dilutions (10^4, 10^5, 10^6 and 10^7 PFU/ml). The plates were then incubated at room temperature overnight. Presence of phage plaques in the areas where the phage droplets were placed would indicate phage sensitivity. Absence of any plaque would indicate phage resistance.

2.7 Data Analysis

The colony morphology and count results were stored in a comma separated value (CSV) file using excel and uploaded to R-studio (version 1.1456) for data analysis. The morphology data was stored as proportion of rhizoid and rough for each replicate and treatment. Analysis was performed by using generalized linear models with proportion as the response variable, and medium, the presence of phage and day as explanatory variables. Due to the proportional nature of the response variable, binomial distribution was used. The significance of each model was calculated using a chi squared (Chi^2) test which provided a p-value signifying statistical significance of the explanatory variables compared to a null-model which lacked these variables. A p-value below 0.05 was considered to be statistically significant. For analysis of total bacterial counts a simple two-tailed T-test was performed, where a p-value of 0.05 was also considered as statistically significant.

3 RESULTS

The plates of each treatment and replicate, which contained between 30-300 colonies, were used to calculated total CFU/ml. Using the same plates the number of each rough and rhizoid colony was counted and this was documented in proportions out of 100%. The results from days one, five, and seven were analysed and compared using R-studio (version 1.1456), exact analysis tests are detailed in methods and materials.

The results of the significance of medium and phage on colony morphology are shown below.

3.1 Day one

On day one (figure 2), the control samples of both SHIEH and HTC contained predominantly rhizoid colony morphology, both with averages of 96% (Standard deviation (SD): 3.8 and 2.8) (Chi^2, 0.0038(6), p=0.9507). In the phage exposed treatments the bacteria remained predominantly rhizoid in both SHIEH, with an average of 82% (SD: 16), and HTC, with an average of 57% (SD: 11). This difference between the percentages of rhizoid was not significant (Chi^2, 0.59(6), p=0.441). Therefore, the medium itself, for both SHIEH and HTC, did not cause any significant differences for day one.

Compared to their controls, the presence of phage had no significant effect on the colony morphology proportions of B185 in either HTC (Chi^2, 2.025(6), p=0.5138) or SHIEH treatments (Chi^2, 0.426(6), p=0.1547). In the SHIEH medium control, the colony morphology was predominately rhizoid, with an average of 96% (SD: 3.8), and with the addition of phage the morphology remained predominantly rhizoid, with an average of 82% (SD:16). In the HTC medium control, the colony morphology was also predominantly rhizoid, with an average of 96% (SD:2.8).

However, with the addition of phage this proportion dropped to an average of 57% rhizoid (SD:11), although this difference was determined not to be significant.

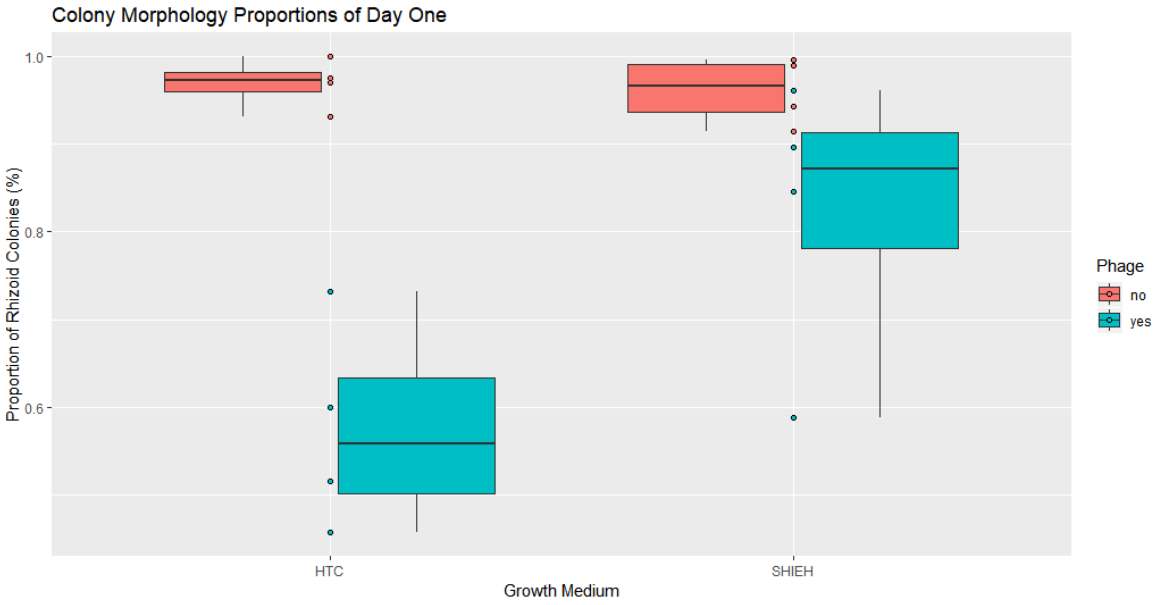

Figure 2: Bar graph showing the percentage of rhizoid morphology in both SHIEH and HTC, with and without phage, on day one.

3.2 Day five

On day five (figure 3), the control samples show that the medium itself had caused a significant difference in the proportions of colony morphology (Chi^2, 8.86(6), p=0.002906). In the SHIEH medium control the proportion of rhizoid colonies was 98% (SD: 1.4), whereas the biggest proportion of colonies were rough in the HTC medium control, with an average of 94% (SD: 3.7). This was not the case between the day five phage treated samples. In the phage treated SHIEH the average was 53% rhizoid (SD: 20) and in the phage treated HTC it was 79% rough (SD: 12.9). Although these values differ, the difference was found not to be significant (Chi^2, 0.92(6), p=0.3366).

Also on day 5, there was no significant difference in the proportions of colony morphology in phage treated SHIEH and its control (Chi^2, 2.77(6), p=0.959) or phage

treated HTC and its control (Chi^2, 0.4366(6), p=0.5087). In the SHIEH medium control the morphology was predominantly rhizoid, with an average of 98% (SD: 1.4). However, in the phage treated SHIEH the percentage of rhizoid colonies varies greatly among the samples, with percentages of rhizoid morphology being as high as 70% and as low as 26% (SD: 20). In the HTC medium control, the samples were predominantly rough, with an average of 94% (SD: 3.7). In the phage treated HTC samples the morphology was also predominantly rough, with an average of 79% (SD: 12.9).

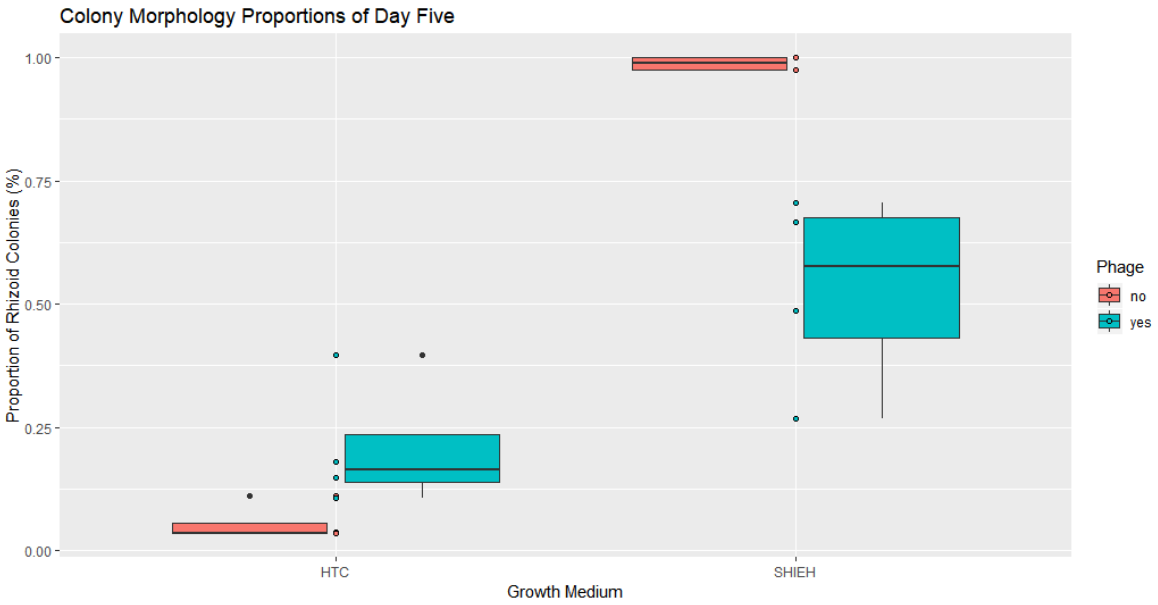

Figure 3: Bar graph showing the percentage of rhizoid morphology in both SHIEH and HTC, with and without phage, on day five.

3.3 Day seven

On day seven (Figure 4) the results show that the presence of phage in SHIEH medium caused a significant change in the proportions of B185 colony morphology (Chi^2, 7.04(6), p=0.007965), when compared to its control. In the SHIEH control samples the colony morphology was primarily rhizoid, with an average of 97% (SD: 4.1). However, the addition of phage to B185 grown in SHIEH caused the colony morphology to become predominantly rough, with an average of 86% (SD: 24).

In contrast, on day seven there was no significant difference in proportions of colony morphology between the HTC controls and phage treated samples (Chi^2, 2.65(6), p=0.1034). In the HTC control samples, the morphology was primarily rough, with an average of 99% (SD: 0.8). In the phage treated samples the morphology proportions differed greatly between samples, from as high as 69% rhizoid and 31% rough, to as low as 25% rhizoid and 75% rough (SD: 19).

Between both the SHIEH and HTC controls of day seven, it shows that the medium itself continued to cause a significant difference in the proportions of colony morphology (Chi^2, 9.947(6), p=0.001611). The morphology of the SHIEH controls were predominantly rhizoid, with an average of 97% (SD: 4.1). However, the HTC controls were predominantly rough, with an average of 99% (SD: 0.8). Similarly to day five, the addition of phage alters the situation to where the proportion is primarily rough in both SHIEH, with an average of 86% (SD: 24), and HTC, with an average of 56% (SD: 19) (Chi^2, 0.94(6), p=0.3323).

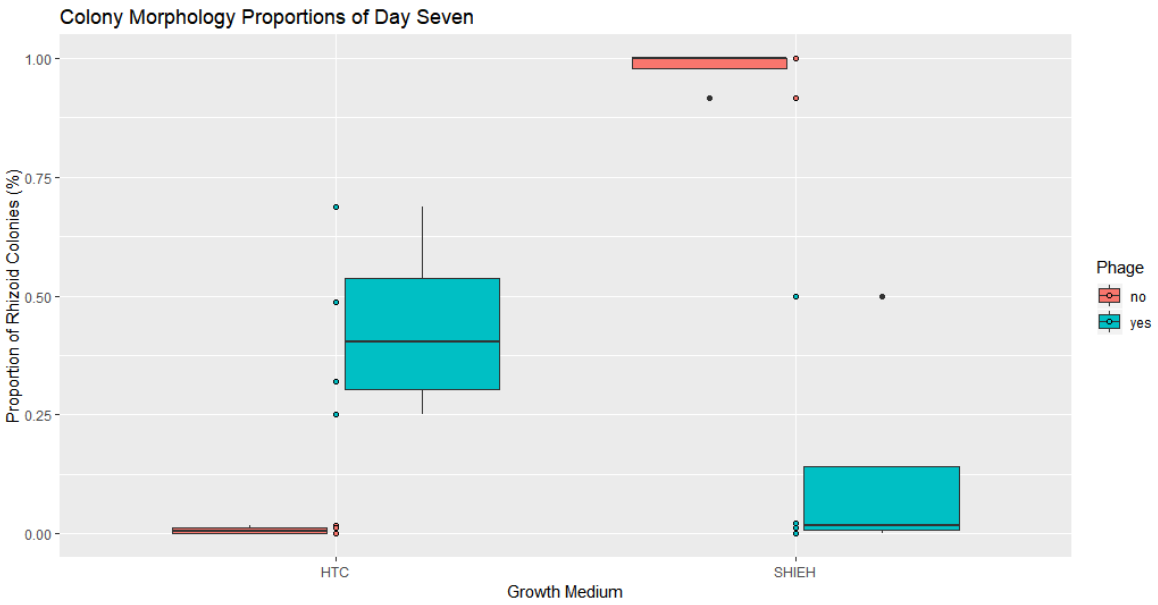

Figure 4: Bar graph showing the percentage of rhizoid morphology in both SHIEH and HTC, with and without phage, on day seven.

To summarise the main significant findings of these results, they show that growth of B185 in SHIEH allowed the bacteria to remain predominantly rhizoid throughout the length of the experiment. However, in HTC it began as predominantly rhizoid but became predominantly rough by day five. The significance of phage in the treated samples caused the bacteria in SHIEH and HTC to become predominantly rough by the end of the experiment. Even though the percentage of rhizoid appears larger in the phage treated HTC compared with the phage treated SHIEH, it was fond not to be statistically significant. A more detailed description of the changes that occur between the days, and their significance is below.

3.4 Significance of day on colony morphology

Between day one and five, there was no significant change in the proportions of colony morphology in the SHIEH control samples (Chi^2, 0.060(6), p=0.8063). There was also no significant change between day five and day seven (Chi^2, 0.0093(6), p=0.923). The morphology started as predominantly rhizoid in day one (96%) and

remained rhizoid over the course of the experiment, 95% on day five and 97% on day seven. In the HTC controls however, there was a significant change in the proportions of colony morphology between day one and day five (Chi^2, 8.30(6), p=0.003964), this change remained from day five through to day seven (Chi^2, 0.17(6), p=0.6798). On day one the HTC control is predominantly rhizoid (96%), but by day five this had significantly changed to predominantly rough (94%) and remained predominantly rough though to day seven (99%).

In the phage treated SHIEH cultures there was no significant change in the proportions of colony morphology between day one and five (Chi^2, 0.801(6), p=0.3708) and days five and seven (Chi^2, 1.50(6), p=0.2203). However, there was a significant difference between day one and day seven (Chi^2, 4.20(6), p=0.04039). From day one the proportion was predominantly rhizoid with an average of 82%. By day five the average had decreased to 53% however, the proportions among day five were quite varied being between 26% and 70% rhizoid. Then by day seven the average had become 86% rough, which was significantly different form the 82% rhizoid on day one. In the phage treated HTC cultures, the proportions of colony morphology were mostly rhizoid on day one at 57% yet varied between 45% and 73%. On day five however, the average proportion changed to predominantly rough, 79%, with varying values from 60% to 89%. This change from rhizoid to rough was calculated to be not statistically significant (Chi^2, 1.176(6), p=0.2782). By day seven the average deceased back down to 56% rough, varying from 31% to 75%. This decrease was also not a significant change (Chi^2, 0.489(6), p=0.4844).

3.5 Total bacterial counts

The CFU/ml of each treatment and replicate, tracked over the course of the seven days of the experiment is shown in figure 5. On day one in SHIEH medium there was no significant difference in CFU/ml between the controls (average $3.24x10^6$) and the phage treatments (average $2.78x10^6$) ($p=0.6$). However, on day one in HTC medium there was significantly less CFU/ml in the phage treatments (average $4.23x10^6$) compared with the controls (average $9.9x10^6$) ($p=0.0009$). On day three there was no significant difference of CFU/ml between SHIEH controls (average $8.43x10^6$) and phage treatments (average $1.73x10^7$) ($p=0.1$). The significance of phage on day three in HTC could not be calculated because the results from HTC controls were absent due to there not being a plated dilution from which CFU/ml could be calculated. On day five there was no significant difference between the CFU/ml of SHIEH controls (average $2.69x10^5$) and phage treatments (average $4.7x10^5$) ($p=0.3$). However, again there was a significant difference between total CFU/ml between HTC controls (average $1.49x10^7$) and the phage treatments (average $9.45x10^4$) ($p=0.04$). Lastly on day seven, there was no significant difference between the CFU/ml of SHIEH controls (average $1.73x10^8$) and the phage treatments (average $3.47x10^8$) ($p= 0.4$). There was also no longer a significant difference in CFU/ml between the HTC controls (average $6.58x10^6$) and the phage treatments (average $1.54x10^8$) ($p=0.3$).

On day one there was a significant difference between the CFU/ml of the SHIEH controls and the HTC controls ($p=0.005$). There was also a significant difference between the CFU/ml of the SHIEH phage treatments and the HTC phage treatments ($p=0.4$). There was no significant difference between the medium of day three. One day five, there was a significant difference in the CFU/ml between the

SHIEH controls and the HTC controls (p=0.04). However, there was not significant difference between CFU/ml of the SHIEH phage treatments and the HTC phage treatments (p=0.2). Lastly, on day seven there was a significant difference in CFU/ml between the SHIEH controls and HTC controls (p=0.03). However, no significant difference between the CFU/ml of the SHIEH phage treatments and HTC phage treatments (p=0.5).

CFU/ml of SHIEH and HTC cultures with and without phage over seven days.

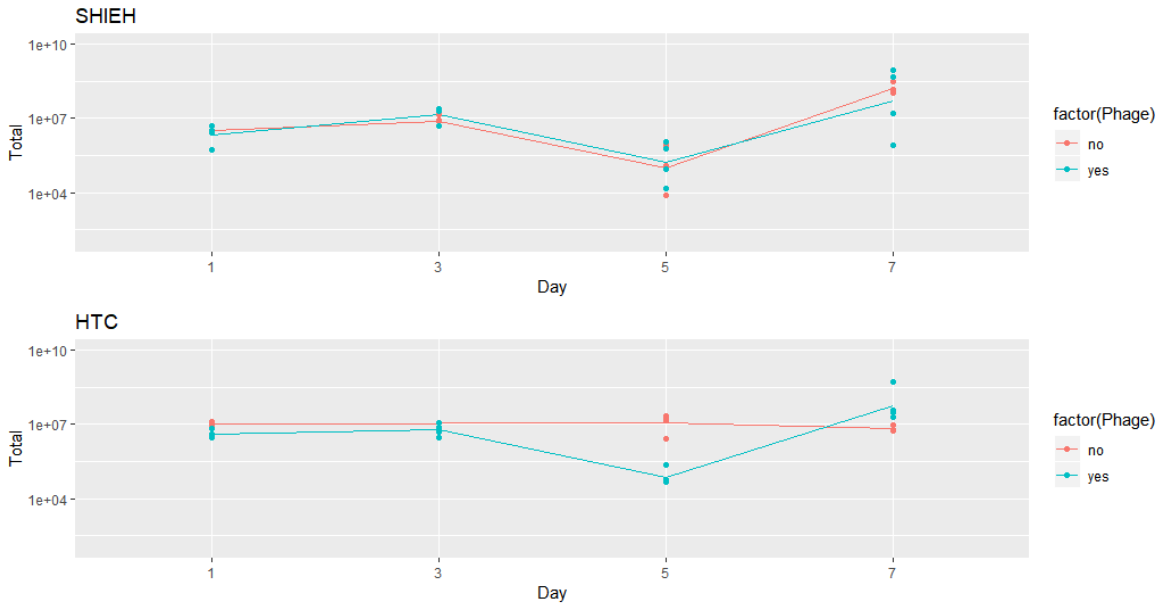

Figure 5: Plots showing the total CFU/ml of each SHIEH (above) and HTC (below) cultures, controls (red) and phage treatments (blue), over the seven days of the experiment.

3.6 CRISPR spacer screening at the population level

In the results from samples extracted from day seven, B185 in 100% medium (figure 6) the only samples to obtain new CRISPR spacers were those exposed to phage when grown in HTC medium. This was determined by the observation of a second band on the electrophoresis gel, located above the wild-type bands which match the distance of positive control. The higher position of the band indicated that it was longer than the wild-type one. However, the band is very faint, indicating that it

was only a small amount of the population that have acquired a new spacer. There was no acquisition of new spacers detected in the SHIEH exposed to FCL-2 treatments.

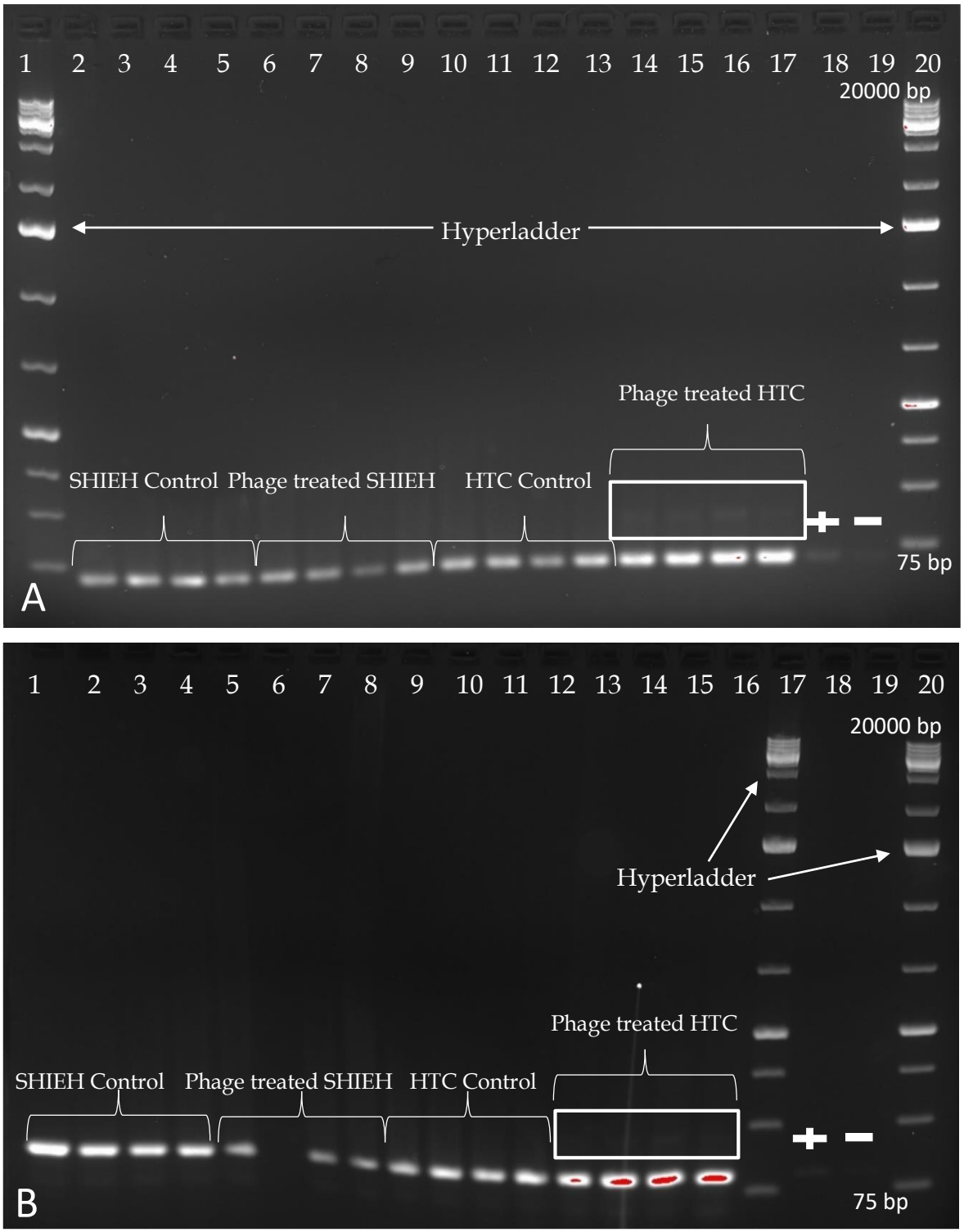

Figure 6: Gel electrophoresis photos showing amplified regions of C1 (A) andC2 (B). The Hyperladder used was GeneRuler 1 kb DNA ladder from Thermo Scientific. Positive controls are shown as + and the negative controls are shown as -. The bands indicating acquisition of a new spacer are highlighter in a white box.

3.7 Isolated Colonies

From the 59 isolated colonies, all were resistant to phage. Several attempts at performing adsorption tests on the isolated colonies were tried, however no attempts were successful. All the isolated colonies grew at different rates, therefore, acquiring equal amounts of CFU/ml for each colony was unsuccessful and it was determined that to distribute the test over several days would not provide for equal and fair testing conditions. Therefore, there were no results regarding their ability to adsorb phage. The results of the PCR amplification of their CRISPR spacers and subsequent gel electrophoresis can be seen in figure 7 (C1) and figure 8 (C2). The results from this determined that there was no presence of newly obtained spacers in any of the 59 isolated colonies. Evidence of a new spacer would present as another band slightly higher than that of the band which represents the wild-type (positive) control. However, none of the bands were higher than that of the wild-type control.

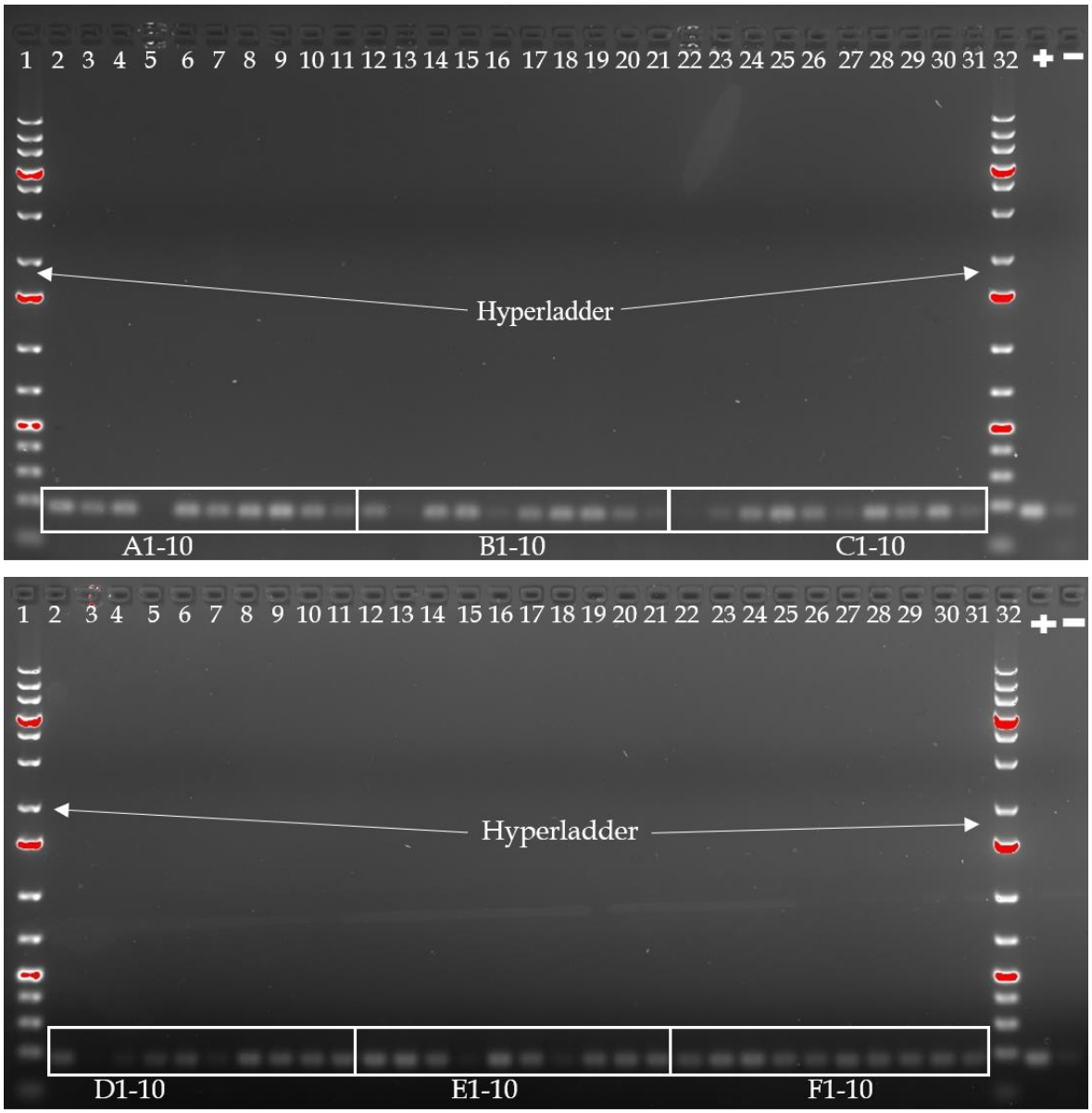

Figure 7: Gel electrophoresis photos showing the amplified region of C1 CRISPR spacers of the 60 isolated colonies. Presented as white bands. Hyperladder used was GeneRuler 1 kb DNA ladder from Thermo Scientific. Positive control shown as +, negative control shown as -.

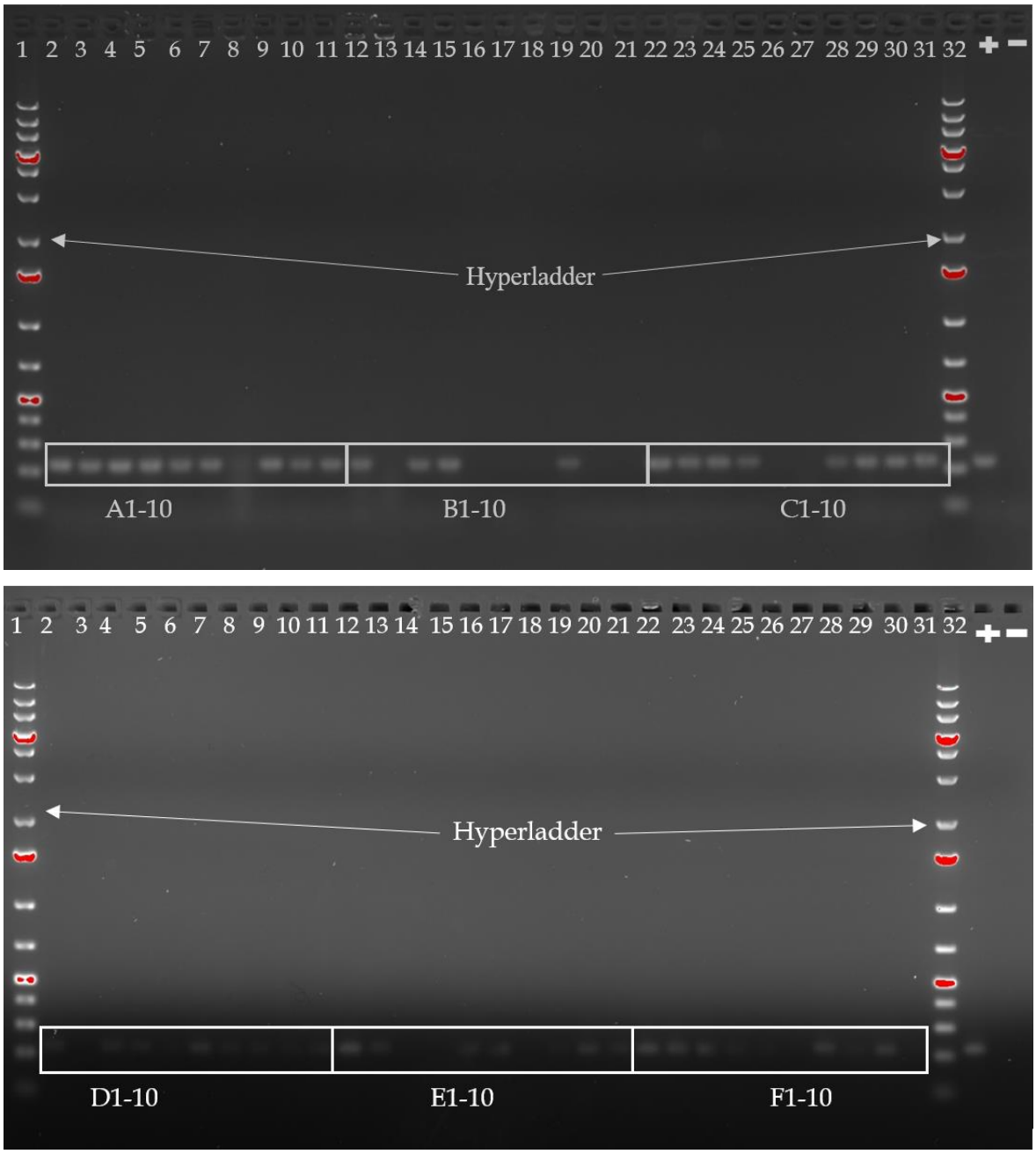

Figure 8: Gel electrophoresis photos showing the amplified region of C2 CRISPR spacers of the 60 isolated colonies. Presented as white bands. Hyperladder used was GeneRuler 1 kb DNA ladder from Thermo Scientific. Positive control shown as +, negative control shown as -.

4 DISCUSSION

In order to put phage therapy into practice, it is important to discover as much information as possible about what could influence its efficacy, including how the type and amounts of nutrients in an environment can influence the mechanisms used and phenotypic properties of bacteria.

Previous studies have indicated that the nutrient levels of medium can affect the type of phage resistance mechanism employed by a bacterium. Westra et al. (2015) found that, when exposed to phage, *Pseudomonas aeruginosa* primarily gained phage resistance through surface modification when cultured together in nutrient-high medium. However, they developed phage resistance through use of their CRISPR-Cas system when cultured together in nutrient-low medium. This could explain the presence of new CRISPR spacers in only the phage treated HTC samples. If the nutrients in HTC medium are not as easy utilised compared with SHIEH, or there are less nutrients to available in HTC to begin with.

The results show that growing *F. columnare* in different media influences the resulting bacterial morphology. During the experiment, significant proportional differences in morphology arose between B185 grown in SHIEH and B185 grown in HTC. *F. columnare* is known to lose its rhizoid morphology when it becomes starved (Laanto et al. 2012). If the bacteria in HTC media are more quickly utilizing the nutrients available to them, it could explain why the morphology changes from mostly rhizoid to mostly rough. In contrast, in SHIEH media the bacteria retain their rhizoid morphology throughout the duration of the experiment, suggesting that the medium provides the bacteria with adequate nutrients. This may be attributed to the differing protein sources in the media. However, to conclude this further study involving alterations of the proteins in the media would be necessary.

Morphology change in *F. columnare* has also previously been linked with gaining phage resistance and losing virulence, in response to being exposed to phage

(Laanto et al. 2012). If changing morphology from rhizoid to rough includes downregulation of surface proteins which make the bacterium rhizoid, then phage resistance may be achieved due to the missing proteins being those normally utilised by phage to adsorb to the bacteria. This resistance mechanism has been detected previously in phage resistant mutants of *E. coli* strain O157:H7, which can gain resistance by silencing the production of the surface protein OmpC, a protein which is used by phage to adsorb to the bacteria (Mizoguchi et al. 2003).

F. columnare and related bacteria have previously been described as having a type 9 secretion system (T9SS) which is related to the secretion of a gliding motility machinery which gives the bacteria their spreading ability (McBride and Zhu 2013). It has been demonstrated that mutations in the T9SS leads to a reduction in virulence in *F. columnare* and deficiency in ability to secrete proteins related to the gliding motility (Li et al. 2017). Studies of *F. johnsoniae* have shown that mutations in the gliding motility machinery genes also leads to the loss of gliding motility and establishes non-spreading colonies, whereas bacteria which express gliding motility machinery are those which form spreading colonies (i.e. rhizoid) (McBride and Nakane 2015). The fact that *F. columnare* changes from a high virulent gliding morphology (rhizoid) to a low virulent non-gliding morphology (rough) and becomes phage resistant suggests that the protein being used by phage to attach to *F. columnare* is likely one that is related to, or secreted by, the T9SS, such as a gliding motility protein (Laanto et al. 2012). This protein is likely to be either SprB or RemA, which are cell surface adhesins demonstrated to be responsible for the gliding motility of bacteria in the *Bacteroidetes* phylum (McBribe and Zhu 2013). SprB and RemA rely on the T9SS for secretion to the cell surface. Therefore, it would make sense that downregulation of the T9SS would be linked with a simultaneous reduction in the presence of SprB and RemA on the cell surface. This would ultimately lead to non-spreading and phage resistant bacteria.

The results of new CRISPR spacer screening at the population level determined that, when exposed to FCL2, B185 in HTC utilise their CRISPR-Cas systems more

compared to being grown in SHIEH. However, the bands indicating new spacers in HTC with phage treatments are faint. This suggests that, although there is evidence of the CRISPR-Cas mechanism being used, it is only being employed by a small percentage of the population. This may explain why, on average, there is a slightly larger population of rhizoid colonies remaining in day seven of HTC with phage, compared to SHIEH with phage. The use of CRISPR-Cas may allow the bacteria to remain rhizoid while still becoming phage resistant. The new spacers in the phage treated HTC cultures are assumed to be from the FCL-2 phage and not self-spacers, due to the absence of new spacers in the control. However, sequencing of the DNA with the new spacers would help to confirm that the spacers are indeed of phage origin.

Another explanation to be considered when discussing the slightly higher prevalence of rhizoid bacteria in HTC with phage could be due to the beef extract in the media. It has previously been noted that, in order to thrive, pathogens must have ability to infect when in the presence of a potential host. However, many virulence factors can be costly for the pathogen, therefore the mechanisms for expressing virulence must be tightly regulated (Penttinen et al. 2016). In this case the bacteria may be tricked into thinking it is infecting, or in the presence of, fish due to the animal-based protein used to make HTC medium, and therefore would prefer to remain virulent when also in the presence of phage. Further research would have to be conducted to confirm these possible links, such as modifications of the medium. However, previous studies have found that the type of resources available to a bacterium can influence the virulence (Ketola et al. 2016). Particularly in the case of plant-based versus animal- based resources, in which it was observed that *Serratia marcescens* became more virulent when exposed to animal-based resources.

One thing to be considered is whether FCL2 phage is obligately lytic or whether it is capable of a temperate life cycle. This would raise the question of whether the bacteria are indeed phage resistant or lysogenic. If the phage is in fact capable of

inducing lysogeny the apparent phage resistance may be explained as a resistance to superinfection. It could be hypothesised that the temperate phage genome may inhibit production of the surface proteins exploited by the phage preventing further infection by phage and resulting in the rough colony morphology. This ability has previously been described in *P. aeruginosa* where lysogenic infection by phage resulted in modifications in production of the O-antigen or pili used by phage to adsorb to bacteria, resulting in resistance to other phage (Bondy-Denomy et al. 2016).

Although the difference in morphology proportions of day seven between HTC and SHIEH with phage were not statistically significant, it does not confirm the absence of biological significance. To confirm whether this difference in morphology proportion is biologically significant or not, further research would need to be conducted. Future research could include using a larger number of replicates to better detect these more subtle differences. Repetition of the whole experiment could also be completed to determine if the results are coincidental.

To conclude the findings of this study it can be said that medium can influence the morphology of *F. columnare*; the addition of phage the medium appears to have less of an influence. Although *F. columnare* may rely on CRISPR-Cas in specific circumstances, the primary form of defence likely arises from morphology change and surface modification. All the isolated colonies being phage resistant, but not containing new CRISPR spacers only emphasises that the preferred mechanism, overall, is something other than the CRISPR-Cas at least in these conditions.

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APENDIX 1. SHIEH medium recipe

Recipe for SHIEH Media	
Ingredient	Percentage of Recipe (%)
Peptone	0.5%
Glucose	0.1%
Na-acetate	0.001%
Na-pyruvate	0.01%
Citric acid	0.001%
Yeast Extract	0.05%
$BaCl_2 \mid H_2O$	0.001%
K_2HPO_4	0.01%
KH_2PO_4	0.005%
$MgSO_4 \mid 7H_2O$	0.03%
$CaCl_2 \mid 2H_2O$	0.00067%
$FeSO_4 \mid 7H_2O$	0.0001%
$NaHCO_3$	0.005%

(Song et al. 1988).

APPENDIX 2. HTC medium recipe

Recipe for HTC Media	
Ingredient	Amount
Bacto-tryptone	0.5g
Yeast extract	0.5g
Sodium acetate	0.2g
Beef extract	0.2g
Water	1000ml
pH	7.2-7.4

(Pate and De Jong 1990).

APPENDIX 3. Formulated water recipe

Recipe for Formulated Water (Per 100 ml H_2O)	
Ingredient	Amount (grams)
NaCl	0.3
KCl	0.1
$CaCl_2$	0.02
$MgCl_2$	0.04

APPENDIX 4. PCR protocol

PCR Protocol	
Temperature (°C)	Duration
95	3 minutes
95	30 seconds
59	30 Seconds
72	1 minute (steps 1-4 repeated 32 times then step 5)
72	15 minutes
4	∞ - until manually finished