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Role of the Orphan CRISPR 2 in *Enterococcus faecalis*

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Summited to The Department of Biology in fulfillment of the requirements for Honors in Biology and graduation from the University Honors College
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Acknowledgements:

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Abstract:

Enterococcus faecalis is a Gram-positive aerotolerant anaerobic bacterium primarily found as a member of the human and animal intestinal flora. *Enterococcus faecalis* along with 27 other species comprise the genus *Enterococcus*. Out of the whole genus *E. faecalis* and *E. faecium* are the second most common nosocomial cause for urinary tract and wound infections. The treatment of the infections is made even more complex due to *E. faecalis* resistance to last-resort drugs like vancomycin. An inverse relationship has been observed in the species between antibiotic resistance and presence of the CRISPR-*cas* locus. CRISPR-Cas (clustered, regularly interspaced short palindromic repeats and associated Cas proteins) systems function as an adaptive and specific immune response for many Bacteria, including *Enterococcus faecalis*. The CRISPR-Cas system incorporates a small segment of foreign DNA into the spacer regions in the bacterial chromosome, which allows for future targeting and defense against the phage. Three CRISPR systems have been identified in *E. faecalis*, two of them with corresponding Cas proteins (CRISPR1-Cas and CRISPR3-Cas) and one without (CRISPR2). CRISPR2 is assumed to be an orphan locus and is thought to be inactive due to the lack of associated Cas proteins. As it is presumed to lack a beneficial function, a reason for the high level of conservation of CRISPR2 loci in *E. faecalis* remains unknown. One hypothesis is that CRISPR2 can interact with Cas proteins from other CRISPR types (CRISPR1 or CRISPR3), which can insert spacer DNA from lytic phage into its genome. To test this hypothesis, *E. faecalis* lytic bacteriophage was isolated from an activated sewage sample obtained at Deer Island Water Treatment Plant and used to challenge *E. faecalis* strains containing different arrangements of CRISPR-Cas systems (CRISPR1-Cas/CRISPR2 and CRISPR2 only). Bacteriophage-resistant strains were generated by mixing a high concentration of phage with *E. faecalis* strains containing different CRISPR system combinations. Strains surviving the phage challenge were further tested to ensure that they were resistant to the phage. CRISPR arrays in both the original and the resistant bacterial strains were PCR-amplified. The wild type and the mutant array PCR products were sequenced and examined for new spacers, indicating CRISPR adaptation for phage resistance of the mutant strains. No spacer acquisition was observed in the mutant strains compared to the original, indicating that CRISPR2 was not involved in phage-resistance.

Introduction:

Enterococcus faecalis is a Gram-positive aerotolerant anaerobe bacterium primarily found in human and animal intestinal flora. It is part of a larger group of Enterococci bacteria that thrive in environments with a broad range of pH, salinity, and temperatures. *Enterococcus faecalis* along with *Enterococcus faecium* are two of the 28 known enterococci species that find their niche in a human gut (Fisher & Phillips, 2009). The bacteria can also populate environments such as soil, animals, plants, water, and food (Da Silva et al., 2006). *E. faecalis* is a virulent bacteria as a result of its ability to adhere to many extracellular matrix proteins, urinary tract epithelia, and epithelia of the oral cavity (Franz et al., 1999). It is for that reason that the bacteria are the second most common nosocomial cause for urinary tract and wound infections and third most common cause of bacteremia (De Fátima Silva Lopes et al., 2005). Until 1984 *Enterococcus faecalis* was thought to belong to streptococci genus but as a result of 16s rRNA sequencing it, along with eight other species, were moved to *Enterococcus* genus (Foulquie Moreno et al., 2006). The treatment of the infections is made even more complex due to *E. faecalis* resistance to last-resort drugs like vancomycin (Palmer & Gilmore, 2010).

CRISPR-Cas functions as a specific immune response for many Bacteria and stands for clustered, regularly interspaced short palindromic repeats and associated Cas proteins. CRISPR-Cas system incorporates a small part of the bacteriophage DNA into the spacer regions on the bacterial DNA, which allows for future identification and defense against the phage (Barrangou, 2007). An inverse relationship has been observed in the species between the antibiotic resistance and presence of CRISPR-cas locus by targeting the DNA (Marraffini & Sontheimer, 2008). Three CRISPR systems have been identified in *E. faecalis*, two of them with a corresponding Cas protein (CRISPR1-*Cas*1 and CRISPR3-*Cas*9) and one without (CRISPR2). The relationship

found indicated that antibiotic resistance is highest in *E. faecalis* that do not contain a Cas protein because of the lack of target region by the antibiotic (Rath, Amlinger, Rath, & Lundgren, 2015). CRISPR loci contain repeat sequences of roughly 36 basepairs and are separated by different spacers. Spacers are unique sequences of roughly 30 basepairs derived from foreign DNA such as viruses or plasmids. The general mechanism of how CRISPR-Cas system works can be grouped into three stages as seen in Figure 1. The first stage is known as the adaptation, in which new spacers are acquired by the bacterium into their CRISPR locus. During the second stage the expression of the *cas* gene by transcription of the CRISPR into precursor CRISPR RNA (pre-crRNA) occurs. The pre-crRNA is then processed to produce CRISPR RNA with Cas proteins. The third stage is known as the interference stage, which uses an almost exact complementary strand of the protospacer-crRNA known as the protospacer adjacent motif (PAM) sequence to distinguish between the bacteria and an invader and ensure the CRISPR system cannot attack its own locus (Mojica, Díez-Villaseñor, García-Martínez, & Almendros, 2009). After all three stages have taken place, the targeted nuclei acid of the invader can be attacked and degraded by actions of Cas proteins and CRISPR RNA (Garneau et al., 2010).

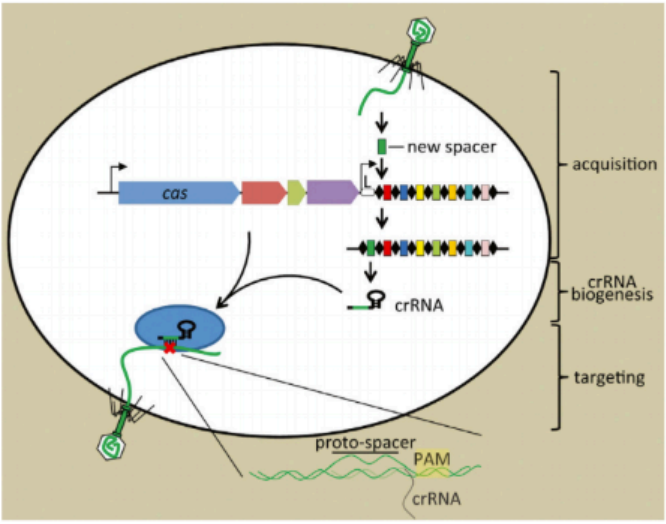


Figure 1. The Three Stages of CRISPR Immunity
CRISPR loci contain clusters of repeats (black diamonds) and spacers (colored boxes) that are flanked by a "leader" sequence (L) and CRISPR-associated (*cas*) genes. During adaptation, new spacers derived from the genome of the invading virus are incorporated into the CRISPR array (Barrangou et al., 2007) by an unknown mechanism. The synthesis of a new repeat is also required. During crRNA biogenesis, a CRISPR precursor transcript is processed by Cas endoribonucleases within repeat sequences to generate small crRNAs (Brouns et al., 2008). During targeting, the match between the crRNA spacer and target sequences (complementary protospacer) specifies the nucleolytic cleavage (red cross) of the invading nucleic acid (Garneau et al., 2010; Gasiunas et al., 2012; Jinek et al., 2012).

CRISPR 2 is termed an orphan locus and is thought to be inactive due to the lack of an associated Cas protein (Palmer & Gilmore, 2010). However, the question then arises why

CRISPR2 locus is so conserved if it does not have any beneficial function. Without a Cas protein, CRISPR2 should not be able to incorporate a phage DNA into its genome. The hypothesis is that CRISPR2 can interact with Cas proteins from other CRISPR types (CRISPR1 or CRISPR3) and thus insert spacer DNA from lytic phage into its genome. The hypothesis that different CRISPR systems interact when present together will also be simultaneously tested. To test the hypotheses, three different strains of *E. faecalis* were used each with a different CRISPR-Cas combination. The three strains were AS035 (CRISPR/Cas 2), MWRA22 (CRISPR/Cas 1 and 2), and MWRA37(CRISPR/Cas 1) as shown in Figure 2.

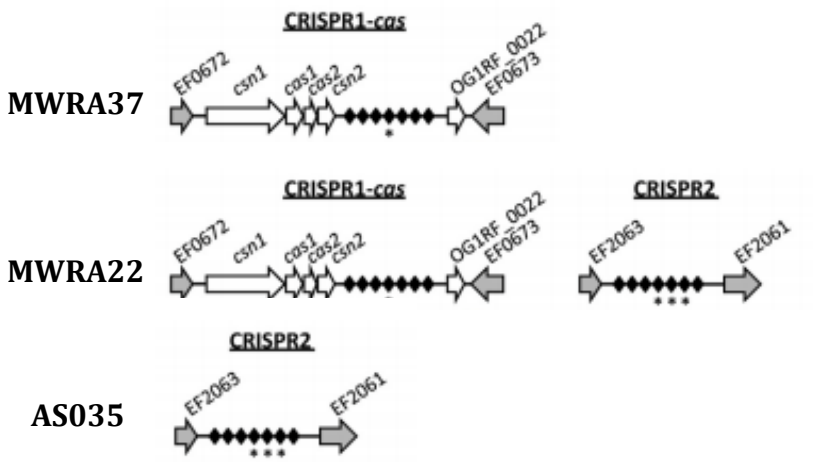


Figure 2: CRISPR loci present in *E. faecalis* isolates: Homologues genes are shown as grey arrows, CRISPR locus genes are shown as white arrows and CRISPR spacers are shown as black diamonds with possibilities of spacer acquisition are starred. Figures modified from (Palmer and Gilmore, 2010).

Methods:

The three strains of *Enterococcus faecalis* used in the study were previously isolated from sewage samples at Deer Island Water Treatment Plant located in Winthrop Massachusetts. The virus with which the three strains were infected was also isolated from another sewage sample from the same facility following the modified bacteriophage isolation

protocol (Otawa, Hirakata, Kaku, & Nakai, 2012). An archived sample of AS003 bacterial strain was inoculated in 2ml of tryptic soy broth (TSB) at 37°C overnight. One percent inoculum of the overnight culture into new TSB was performed and incubated for four hours in a 37°C incubator to achieve log-phase growth. 1ml of the log-phase host culture was then added to 5ml sample of activated sludge from Deer Island Water Treatment Plant and 5ml of TSB. The mixture was then inoculated at 37°C for 8 hours. This enrichment was then centrifuged at 13,000 rpms on a bench-top centrifuge for 10 minutes. The supernatant was filtered through a 0.2-µm pore-size membrane filter.

0.8ml of the lysate (filtrate) was then inoculated with 0.8ml log-phase host culture for 10 min at 37°C. The lysate and bacterial culture mixture were serially diluted by ten-fold dilution to a 10^{-6} dilution. Dilutions (0.9ml) were added to 1.6ml 0.65% soft trypticase soy agar (TSA) separately, inverted, mixed and poured on top of a 1.5% TSA plate. The plate was incubated overnight at 37°C. A single isolated plaque was sampled with an inoculation loop and mixed into 10 ml of TSB and 1ml log-phase host culture and incubated at 37°C overnight. The overnight culture was then centrifuged at 13,000 rpms for 10 minutes and filtered through a membrane filter to remove large bacteria.

Lysate test was then performed by mixing 100 microliters of log-phage bacteria with 1.5 ml of TSB and incubating for 10 minutes at 37°C. 1ml of the mix was then added to 1.5ml of 0.65% TSA and layered over 1.5% TSA plate and allowed to cool on bench top for 15 minutes. After solidification of the top layer, 10 microliters of phage containing lysate was dropped on one side of the plate while 10 microliters of ΦKKAS003 phage was dropped on the other side as positive control. The plate was then tilted and lysates allowed to run down. The plate was then

incubated at 37°C overnight to observe clearing in the bacterial lawn indicative of phage lytic activity.

Titer for ΦABAS003 was conducted by first diluting the phage in ten-fold dilutions until reaching a concentration of $1*10^{-6}$. Phage resistant (mutant) AS003 bacterial lawn was created once again over 0.65% TSA plate. 10 microliters of ΦABAS003 dilutions starting from stock to $1*10^{-6}$ dilution was dropped on the phage resistant AS003 plate and incubated overnight at 37°C.

A loop-full of archived *Enterococcus faecalis* strains AS035, MWRA22, and MWRA37 were inoculated into 2ml TSB and incubated overnight at 37°C. All the same steps were followed as previously to generate log-phase bacterial strains. 100 microliters from each of the three log-phase containing bacterial strains were mixed with 1.5ml TSB and incubated at 37°C for 10 minutes after which the contents were added to 1.5ml 0.65% molten TSA. After mixing the contents they were layered over 0.65% TSA plate and allowed to cool for 15 minutes. 10 microliters of undiluted ΦABAS003 were dropped one each of the three plates three times and incubated at 37°C overnight to check whether the phage will lyse the bacteria.

100 microliters of each of the three strains were then mixed with 100 microliters of undiluted ΦABAS003 and incubated at 37°C for 10 minutes. 100 microliters of each mix were then mixed with 900 microliters TSB and then 2.5ml 0.65% molten TSA. Each tube was mixed and layered over 0.65% TSA plate and incubated at 37°C overnight to generate phage-resistant bacterial growth for each strain.

One colony of each of the three mutant strains resistant to ΦABAS003 were streaked on enterococcel agar plates and incubated at 37°C overnight to rule out contamination, since only *Enterococci* can grow on the plates.

A loop-full of bacterial lawn from each of the three phage-resistant plates was then incubated in 2ml TSB to generate log-phase bacteria and create ΦABAS003-resistant bacterial lawn for all three *E. faecalis* strains following previously explained technique. 10 microliters of undiluted ΦABAS003 were dropped one each of the three plates three times and incubated at 37°C overnight to verify phage resistant bacteria.

Genomic DNA extraction was conducted by picking up a single colony from each of the plates containing both phage-resistant and sensitive bacterial cultures and inoculating it into 1.5ml TSB and incubating at 37°C overnight. The DNA of the overnight culture was then extracted with the MoBio Laboratories, INC. UltraClean® Microbial DNA Isolation Kit following the manufacturer's protocol.

Polymerase Chain Reaction was then used to amplify the CRISPR2 regions for both the phage sensitive and resistant strains containing CRISPR2 (total of four) following PCR for *Enterococcus* 16S rRNA protocol (Muyzer & Uitterlinden ,1993). Platinum Hot Start PCR 2X Master Mix purchased from ThermoFisher Scientific was used for the PCR mastermix. The CRISPR2 array primers used were designed by Palmer and Gilmore, 2010 and obtained from ThermoFisher Scientific.

Gel electrophoresis was then conducted using a 0.9% gel to separate and confirm the presence of CRISPR2 regions in the two original strains as well as then two phage-resistant strains (MWRA22, MWRA22mut, AS035, and AS035mut).

The DNA of the four strains of *E. faecalis* containing CRISPR2 arrays were then prepared according to Eton Bioscience Protocol and sequenced by Eton Bioscience in Charlestown, MA. The sequences were then curated and analyzed.

Results:

After isolation of a bacteriophage containing lysate, the overlay method generated plaques indicative of phage lysing *E. faecalis* strain tested (Figure 3). A plaque generated on a $1*10^{-3}$ dilution plate was used for re-inoculation.

Lysate screening conducted for both ΦKKAS003 and the newly isolated ΦABAS003 showed phage lytic activity as a result of clearing in the bacterial lawn as seen in Figure 4.

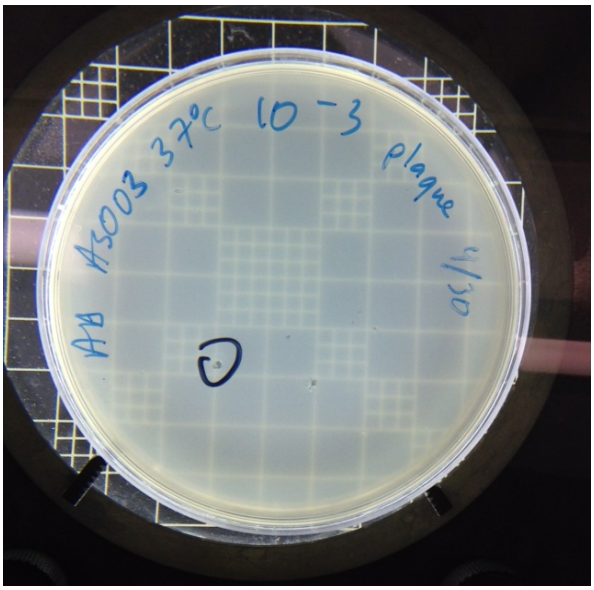


Figure 3. ΦABAS003 generated plaque

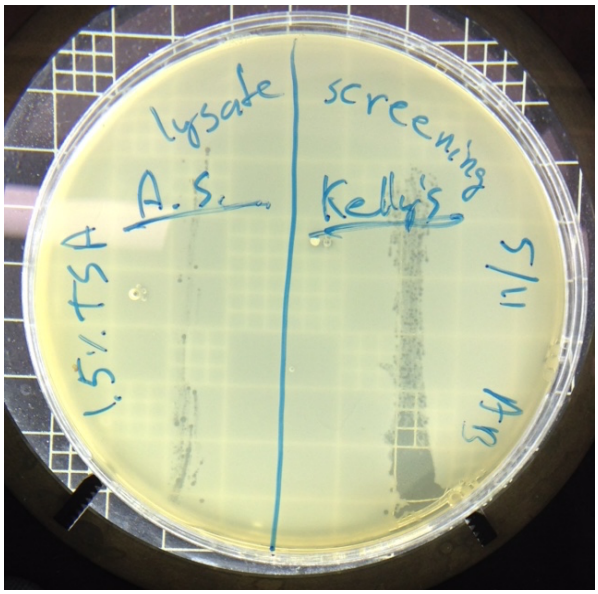


Figure 4. Lysate screening for ΦABAS003 and ΦKKAS003

ΦABAS003 titer was conducted to calculate the number of viruses present in a milliliter of lysate that are capable of forming plaques, known as PFU/ml. Figure 5 shows the titer results where a dilution of $1*10^{-5}$ generated fifteen plaques. By conducting the PFU calculation:

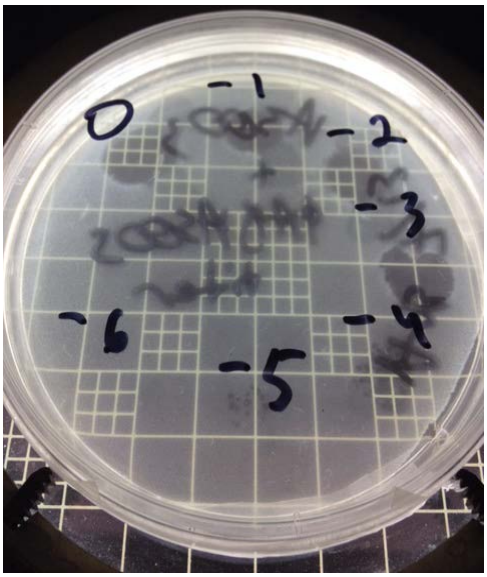


Figure 5. Titer for ΦABAS003

PFU/ml = 15/(0.0000)*(0.01) = 150,000,000 = $1.5*10^8$ PFU/ml the PFU for ΦABAS003 was calculated to be $1.5*10^8$ PFU/ml.

After re-inoculating the three strains containing different combinations of CRISPR/Cas a spot test for each strain was conducted to verify the sensitivity to the ΦABAS003 as seen in Figure 6. All three strains were lysed by the new phage indicative of their sensitivity to the virus.

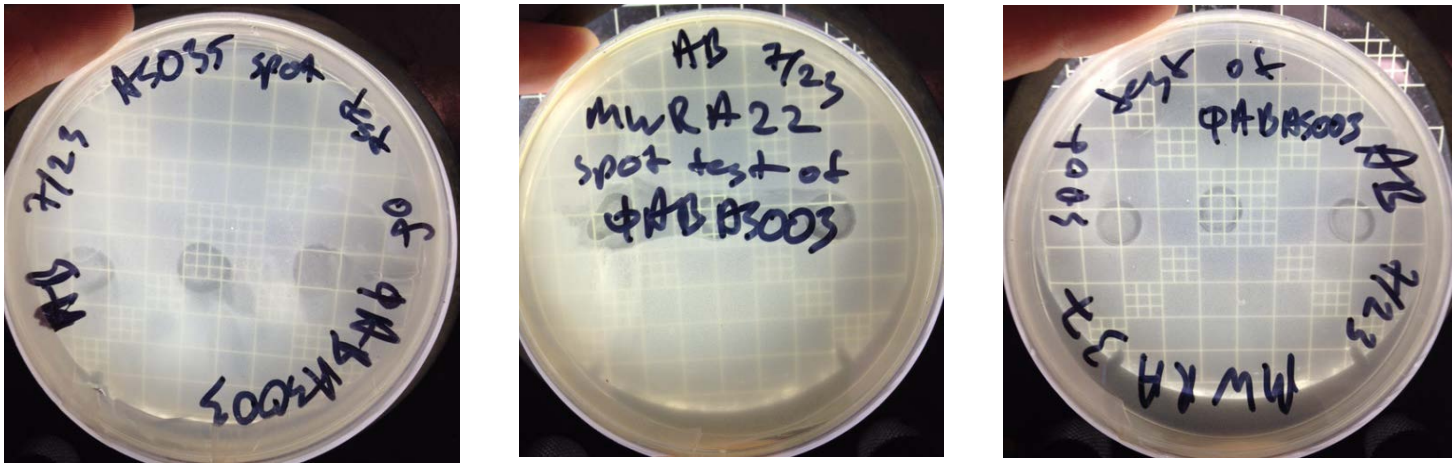


Figure 6. ΦABAS003 spot tests for the three different *E. faecalis* strains

After the generation of ABAS003 phage-resistant strains the bacteria was once again layered over a TSA plate and ΦABAS003 spot test performed again to confirm the resistance to the phage. All three spots on each plate where the phage was dropped grew a bacterial lawn without any lysing indicating that the bacteria was in fact resistant to the newly isolated phage (Figure 7).

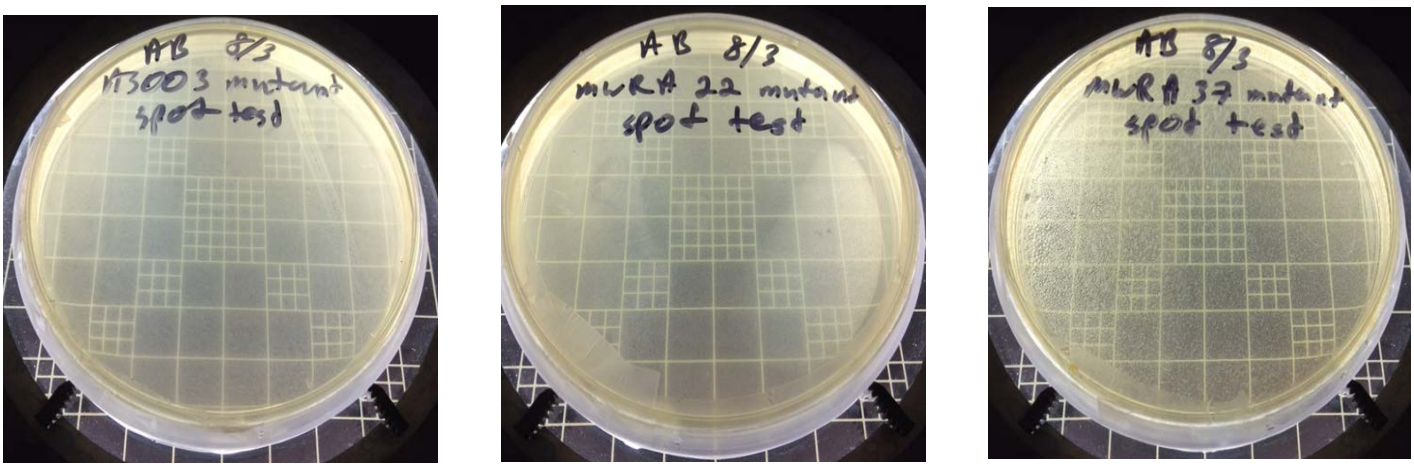


Figure 7. ΦABAS003 spot tests for the three different phage-resistant *E. faecalis* strains

Polymerase Chain Reaction conducted to amplify the CRISPR2 array in both the sensitive and phage-resistant AS035 and MWRA22 *E. faecalis* bacteria used CRISPR2 primers obtained from Invitrogen, ThermoFisher Scientific with the primer sequences according to Table 1.

CRISPR2 Forward Primer	CTGGCTCGCTGTTACAGCT
CRISPR2 Reverse Primer	GCCAATGTTACAATATCAAACA

Table 1. CRISPR2 Array primers used for PCR

The four different strains along with a positive control OG1RF all containing a CRISPR2 region in their DNA after having undergone a Polymerase Chain Reaction were separated by size using gel electrophoresis technique. All five of the strains had confirmed the presence of a CRIPR2 array according to Figure 8.

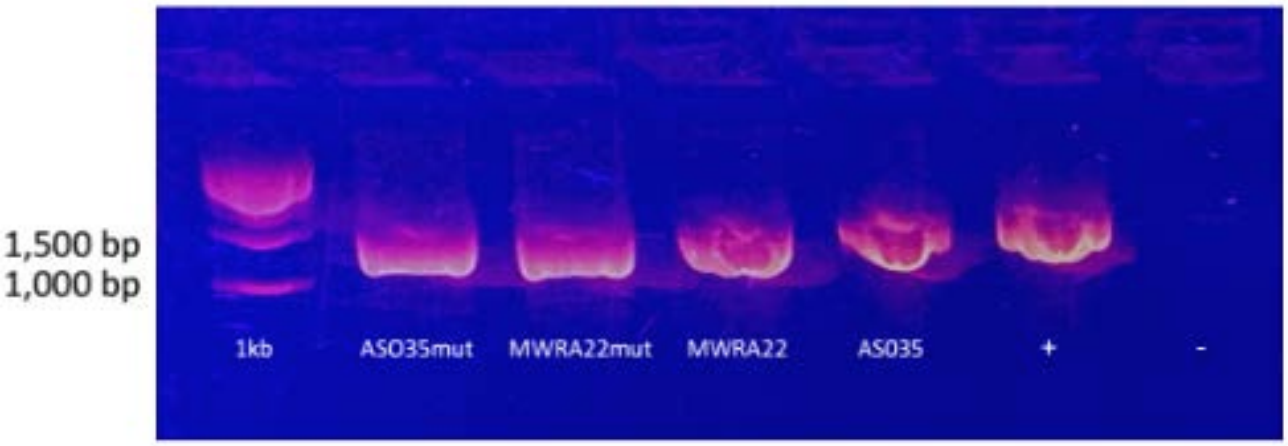
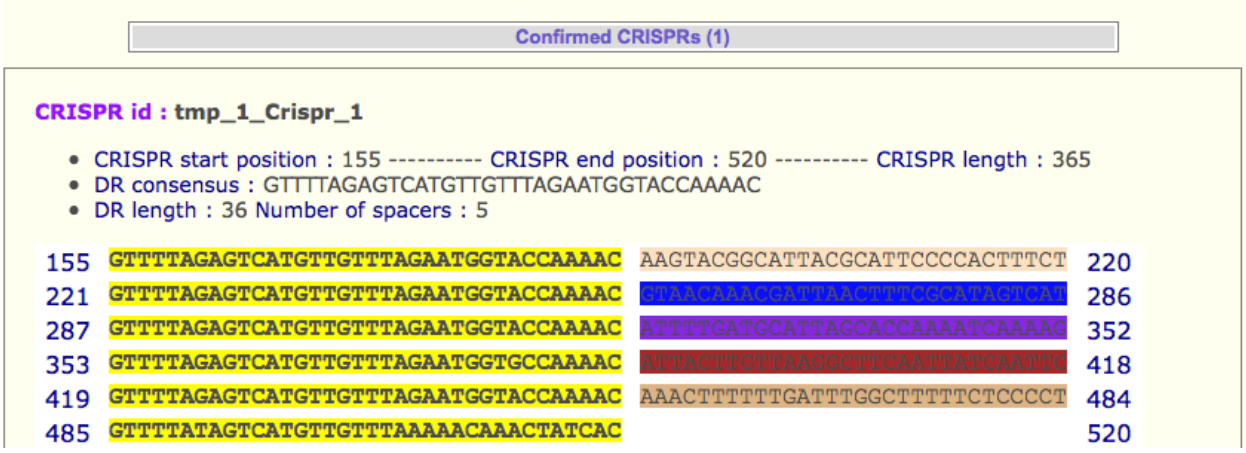


Figure 7. CRISPR2 Array PCR gel electrophoresis. Left to right: AS035mut, MWRA22mut, MWRA22, AS035, positive control (OG1RF), negative control.

The acquired sequences for the repeats and spacers of CRISPR2 array for the four strains using CRISPRfinder showed several one base-pair mutation within the repeat sequence of the original strain that was also present in the mutant strain (Grissa et al. 2007). No new nucleotide acquisition was observed in the spacer or repeat region of the mutant strains as shown in Figures 9 – 12.



Confirmed CRISPRs (1)

CRISPR id : tmp_1_Crispr_1

- CRISPR start position : 155 ---------- CRISPR end position : 520 ---------- CRISPR length : 365
- DR consensus : GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC
- DR length : 36 Number of spacers : 5

```
155 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC AAGTACGGCATTACGCATTCCCCACTTTCT 220
221 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC GTAACAAACGATTAACTTTCGCATAGTCAT 286
287 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC ATTTTGATGCATTAGCACCAAAATCAAAAG 352
353 GTTTTAGAGTCATGTTGTTTAGAATGGTGCCAAAAC ATTACTTGTTAAGGCTTCAATTATCAATTC 418
419 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC AAACTTTTTTGATTTGGCTTTTTCTCCCCT 484
485 GTTTTATAGTCATGTTGTTTAAAAACAAACTATCAC                               520
```

Figure 9. AS035 sequence for the repeat region highlighted in yellow and the spacer region in five different colors.

Confirmed CRISPRs (1)

CRISPR id : tmp_1_Crispr_1

- CRISPR start position : 156 ---------- CRISPR end position : 521 ---------- CRISPR length : 365
- DR consensus : GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC
- DR length : 36 Number of spacers : 5

```
156 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC AAGTACGGCATTACGCATTCCCCACTTTCT 221
222 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC GTAACAAACGATTAACTTTCGCATAGTCAT 287
288 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC ATTTTGATGCATTAGCACCAAAATCAAAAG 353
354 GTTTTAGAGTCATGTTGTTTAGAATGGTGCCAAAAC ATTACTTCTTAACGCTTCAATTATCAATTC 419
420 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC AAACTTTTTTGATTTGGCTTTTTCTCCCCT 485
486 GTTTTATAGTCATGTTGTTTAAAAACAAACTATCAC                               521
```

Figure 10. AS035mutant sequence for the repeat region highlighted in yellow and the spacer region in five different colors.

Confirmed CRISPRs (1)

CRISPR id : tmp_1_Crispr_1

- CRISPR start position : 156 ---------- CRISPR end position : 455 ---------- CRISPR length : 299
- DR consensus : GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC
- DR length : 36 Number of spacers : 4

```
156 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC CGATTGACCTAGGGTTAAGTTACTTACCTA 221
222 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC TAGTGGGGTTTCGGGTTTAAACGTAGCTTC 287
288 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC ACCTTTTAAATTATCTTTGTTAAGACGTAG 353
354 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC GCAAGTTGCTAGCATTACTCAAAAATTAGA 419
420 GTTTTATAGCCATGTTGTTTAAAAACAAACTATCAC                               455
```

Figure 11. MWRA22 sequence for the repeat region highlighted in yellow and the spacer region in four different colors.

Confirmed CRISPRs (1)

CRISPR id : tmp_1_Crispr_1

- CRISPR start position : 154 ---------- CRISPR end position : 453 ---------- CRISPR length : 299
- DR consensus : GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC
- DR length : 36 Number of spacers : 4

```
154 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC CGATTGACCTAGGGTTAAGTTACTTACCTA 219
220 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC TAGTGGGGTTTCGGGTTTAAACGTAGCTTC 285
286 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC ACCTTTTAAATTATCTTTGTTAAGACGTAG 351
352 GTTTTAGAGTCATGTTGTTTAGAATGGTACCAAAAC GCAAGTTGCTAGCATTACTCAAAAATTAGA 417
418 GTTTTATAGCCATGTTGTTTAAAAACAAACTATCAC                               453
```

Figure 12. MWRA22mutant sequence for the repeat region highlighted in yellow and the spacer region in four different colors.

Blasting all four complete genomes of the four strains resulted in a 90% identity with an E-value of zero with *Enterococcus faecalis* ATCC29212 strain whose whole genome has been sequenced. Blasting the five-spacer regions of the AS035 and the identical spacer regions of AS035mutant strains generated a 100% identity with an E-value of $1e^{-06}$ with *Enterococcus faecalis* str. Symbioflor 1 with all five spacers. Blasting the four-spacer regions of the MWRA22 and the identical spacer regions of MWRA22 generated correlation at different identity percentage with either other bacteria or fungi as seen in Figures 13 – 16.

Bacillus cytotoxicus NVH 391-98, complete genome	38.2	38.2	63%	3.5	100%

Figure 14. MWRA22 spacer 2 highest matched to *Bacillus cytotoxicus* bacterium.

displayed in MWRA22 to right the numbers indicate Max score, Total score, Query covered, E value, and identity percentage.

Enterococcus faecalis ATCC 29212, complete genome	60.0	60.0	100%	1e-06	100%

Figure 15. MWRA22 spacer 3 highest matched to a different strain of *Enterococcus faecalis* bacterium.

Leptosphaeria biglobosa brassicae b35_scaffold00016 complete sequence	40.1	40.1	80%	0.88	96%

Figure 16. MWRA22 spacer 4 highest matched to a *Leptosphaeria biglobosa* fungus.

Discussion:

The plaque generated from ΦABAS003 lysing the host AS003 *Enterococcus faecalis* strain indicated the virus is virulent and can be used to infect other strains. The clearings observed for the spot test of the phage on AS035, MWRA22, and MWRA37 lawns indicated that the bacterial strains are susceptible to the virus infection and are lysed as a result. The surviving bacteria post mixing of the phage with susceptible strains generated phage-resistant mutants of the original strains. Spot test confirmed the ΦABAS003 resistance of AS035mutant, MWRA22mutant, and MWRA37mutant.

Successfully conducted PCR for the CRISPR2 array confirms the retention of the CRISPR2 region on the bacterial mutant DNA. Having sequenced the CRISPR2 region of both the original (phage-sensitive) and the mutant (phage-resistant) strains of MWRA22 and AS035 no change has been observed in the sensitive and resistant CRISPR2 arrays. This indicated that the phage-resistance has been gained through a mechanism other than CRISPR2 region DNA acquisition of the ΦABAS003 bacteriophage.

The 100 percent identity match of the CRISPR2 spacers present in both AS035 and AS035mutant strains with *Enterococcus faecalis* ATCC29212 strain demonstrates the highly conserved nature of the locus. It is probable that the two strains either share a common ancestor that encountered environmental pressures which resulted in the spacer acquisition or that the two strains individually encountered the same virus and thus gained identical spacers.

As a result of the CRISPR1 region not being able to be amplified either due to degradation of the region or to other sources of error, it cannot be established whether or not CRISPR1 was involved in the gaining of the resistance to the phage. Other virus defense mechanisms common to bacteria could be responsible for the resistance, such as the restriction-modification system that utilizes bacterial restriction enzymes that recognize and rapidly degrade

unmethylated viral DNA that enters the host cell (Labrie, 2010). Other defense mechanisms such as the prevention of phage adsorption or Sie-system responsible for the prevention of phage DNA entry into the host cell for some Gram-positive bacteria could also be responsible. However, the activity of other defense mechanisms in *Enterococcus faecalis* was not studied in this research.

Conclusion:

A bacteriophage was isolated from a sewage sample that could infect and lyse *Enterococcus faecalis*. Mutant strains were then generated that were resistant to the virus. Upon sequencing and analyzing the CRISPR2 array no new DNA acquisition was observed in the region. This indicates that CRISPR2 locus was not involved in the phage-resistance mutation and may be an orphan locus.

References:

Fisher, Katie, and Carol Phillips. "The Ecology, Epidemiology and Virulence of Enterococcus." *Microbiology* 155.6 (2009): 1749-757. Web.

Da Silva, M. F., Tiago, I., Veríssimo, A., Boaventura, R. A., Nunes, O. C., & Manaia, C. M. (2006). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiology Ecology*, *55*(2), 322–329. doi:10.1111/j.1574-6941.2005.00032.x

Franz, C. M. A. P., Holzapfel, W. H. and Stiles, M. E. (1999). Enterococci at the crossroads of food safety? International Journal of Food Microbiology, 47:1–24.

De Fátima Silva Lopes, M., Ribeiro, T., Abrantes, M., Figueiredo Marques, J. J., Tenreiro, R. & Crespo, M. T. B. (2005). Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. Int J Food Microbiol 103, 191–198.

Foulquie Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L. (2006). The role and application of Enterococci in food and health. International Journal of Food Microbiology, 106:1–24.

Palmer, K. L., & Gilmore, M. S. (2010). Multidrug-resistant enterococci lack CRISPR-cas. *mBio*. doi:10.1128/mBio.00227-10

Barrangou, Rodolphe, Christophe Fremaux, and Philippe Horvath. "CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes." *Science* 315.2819 (2007): 1709-712. Web.

Marraffini, L. A., & Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science (New York, N.Y.)*, *322*(5909), 1843–1845. doi:10.1126/science.1165771

Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*, (April), 1–10. doi:10.1016/j.biochi.2015.03.025

Barrangou, Rodolphe, and Luciano A. Marraffini. "CRISPR-Cas Systems: Prokaryotes Upgrade to Adaptive Immunity." *Molecular Cell* 54.2 (2014): 234-44. Web.

Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J., & Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*, *155*(3), 733–740. doi:10.1099/mic.0.023960-0

Garneau, J. E., Dupuis, M.-È., Villion, M., Romero, D. a, Barrangou, R., Boyaval, P., … Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, *468*(7320), 67–71. doi:10.1038/nature09523

Otawa, K., Hirakata, Y., Kaku, M., & Nakai, Y. (2012). Bacteriophage control of vancomycin-resistant enterococci in cattle compost. *Journal of Applied Microbiology*, *113*, 499–507. doi:10.1111/j.1365-2672.2012.05361.x

Muyzer, G., E. C. deWaal and A. G. Uitterlinden (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. Applied and Environmental Microbiology 59: 695-700.

Grissa I, Vergnaud G, Pourcel C (2007) CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic acids research 35: W52–57. pmid:17537822 doi: 10.1093/nar/gkm360

Labrie, Simon J., Julie E. Samson, and Sylvain Moineau. "Bacteriophage Resistance Mechanisms." *Nature Reviews Microbiology Nat Rev Micro* 8.5 (2010): 317-27. Web.