4. Experimental Results

In this chapter I will present the results from the experimental isolation of plasmids, the validation of the AMR predictions, as well as the transformation and conjugation attempts. The *in silico* predictions in the previous chapter revealed putative plasmids in one third of the culture collection, and among these predictions 38% are predicted contain mobility sequences, and 68% are predicted to contain AMR genes. Of particular interest are the elements predicted to exist in several phyla across the tree, hinting at the presence of broad host range mobilisable plasmids. Resistance and mobility phenotypes were tested experimentally and the results presented below.

4.1 Plasmid Isolation

Strains with predicted plasmids were cultured for plasmid isolation; strains of particular interest included Firmicutes strains with putative plasmids also predicted to contain plasmid-mobility sequences. Plasmid isolation was completed using two commercial kits; one for plasmids below 10kb supplied by Qiagen, one for plasmids greater than 10kb supplied by MultiTarget. Isolated plasmids were visualised on 0.8 % agarose TAE, to validate the presence of extracted extrachromosomal DNA (Figure 1).

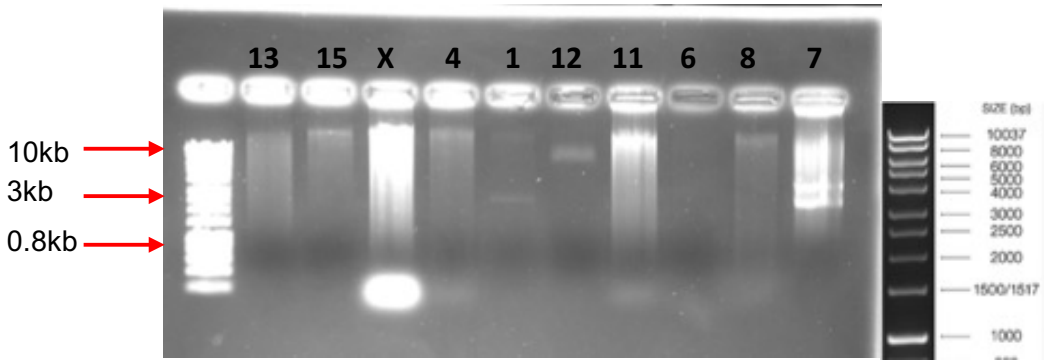

Figure 4.1. *Agarose gel displaying the isolated plasmids greater than 10kb.* DNA gel displaying undigested isolated DNA from plasmids greater than 10kb using MultiTarget's AquaPlasmid, plasmid preparation buffer. Ladder in lane 1 is the 10kb Hyperladder (Bioline) Sample identity is indicated by Table 1 row number, and predicted sizes are as follows:

- **13-** 102023
- **15-** 45070
- **X-** 16S validation failed
- **4-** 6131
- **1-** 12972 & 5302
- **12-** 133666
- **11-** 35136
- **6-** 60425
- **8-** 80318
- **7-** 12278

4.2 Species Validation

To be confident that the bacterial cultures which plasmids were being isolated from were consistent with the species identified in the predictions the identity of each isolate from which plasmid DNA had

been extracted was validated by 16S rRNA gene amplicon sequencing. The 16S rRNA gene sequences were aligned to the database of 16S rRNA gene sequences of the whole culture collection (Table 1). Only one isolate failed the screening and was identified as a different species to its expected identity, this was excluded from further screening.

	Results of the 16S Species Validation					
	Seq ID	**Species Name**	**Isolate Name**	**Matched Seq ID**	**Matched Species Name**	**Validation**
1	18048_2#80	*Erysipelotrichaceae nov.*	fCSP	18048_1#80.contigs_velvet.cleaned.fna|16S	*Erysipelotrichaceae nov. 18048_1#80*	Y
2	20287_6#28	*Blautia nov.*	E79_57	20287_6#62.contigs_velvet.cleaned.fna|16S	*Blautia nov. 20287_6#62*	Y
3	12718_7#90	*Bacteroides uniformis*	H1_6	21673_4#70.contigs_velvet.cleaned.fna|16S	*Bacteroides uniformis 21673_4#70*	Y
4	14207_7#59	*Intestinimonas butyriciproducens*	H5_60	14207_7#59.contigs_velvet.cleaned.fna|16S	*Intestinimonas butyriciproducens 14207_7#59*	Y
5	20287_6#22	*Ruminococcaceae nov.*	E72_40	20287_6#22.contigs_velvet.cleaned.fna|16S	*Ruminococcaceae nov. 20287_6#22*	Y
6	14207_7#7	*Eubacterium rectale*	H4_46	14207_7#7.contigs_velvet.cleaned.fna|16S	*Eubacterium rectale 14207_7#7*	Y
7	20298_2#57	*Bacteroides cellulosilyticus*	D85_115	GCF_000158035.cleaned.fna|16S	*Bacteroides cellulosilyticus GCF_000158035 (DSM 14838)*	Y
8	20427_4#28	*Blautia nov.*	F41_240	20298_3#81.contigs_velvet.cleaned.fna|16S	*Blautia nov. 20298_3#81*	Y
9	13470_2#56	*Lachnospiraceae nov.*	H2_21	13470_2#56.contigs_velvet.cleaned.fna|16S	*Lachnospiraceae nov. 13470_2#56*	Y
10	20287_6#48	*Blautia nov.*	F1_100	20287_6#62.contigs_velvet.cleaned.fna|16S	*Blautia nov. 20287_6#62*	Y
11	13470_2#93	*Lachnospiraceae nov.*	H4 _41	13470_2#93.contigs_velvet.cleaned.fna|16S	*Lachnospiraceae nov. 13470_2#93*	Y
12	20298_3#19	*Enterobacter nov.*	G8_180	20298_3#19.contigs_velvet.cleaned.fna|16S	*Enterobacter nov. 20298_3#19*	Y
13	13414_6#1	*Lachnoclostridium nov.*	H1_38	13414_6#1.contigs_velvet.cleaned.fna|16S	*Lachnoclostridium nov. 13414_6#1*	Y
14	20298_3#39	*Eubacterium limosum*	G14_207	20298_3#39.contigs_velvet.cleaned.fna|16S	*Eubacterium limosum 20298_3#39*	Y
15	13414_6#62	*Ruminococcaceae nov.*	H3_43	13414_6#62.contigs_velvet.cleaned.fna|16S	*Ruminococcaceae nov. 13414_6#62*	Y

Table 4.1. *Results of the 16S species validation.* Columns one and two contain the expected ID number and species name of each isolate. Columns four and five contain the ID number and species name the isolate has been identified as from 16S sequencing. The last column indicates if this is a correct (Y) match. Only one species failed and came back as a different species.

4.3 Plasmid Digests

To further validate that the extracted plasmids were consistent with the predictions the plasmids were linearised to measure their true size using separation by gel electrophoresis and this was compared to the size predicted for each plasmid. Plasmid restriction sites were predicted using SnapGene by GSL Biotech. The plasmids were linearized according to predicted restriction sites; enzymes predicted to have only one restriction site were used. The isolated plasmids under 10kb were all size verified through restriction digest (Figure 2), the larger plasmids experienced degradation upon digest (Figure 3).

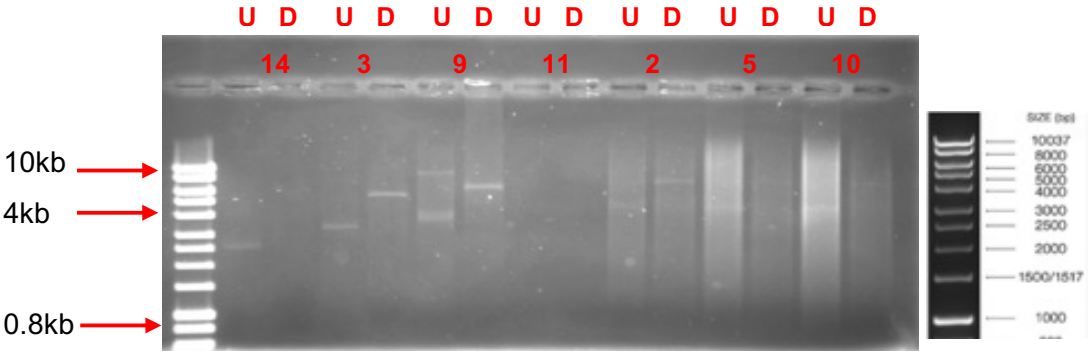

Figure 4.2. *Agarose gel displaying small plasmids in native (U) and digested (D) form.* Plasmids under 10kb were isolated using the QIAprep mini prep kit. The restriction sites were predicted using the SnapGene, the sequences were digested using restriction enzymes, and the linearised DNA run alongside the native DNA to observe the size of the sequences. Ladder in lane 1 is the 10kb Hyperladder (Bioline). Sample identity is indicated by Table 1 row number, and predicted sizes are as follows:

- **14-** 5498
- **3-** 5629
- **9-** 6018
- **11-** 6131
- **2-** 7296
- **5-** 7170
- **10-** 7170

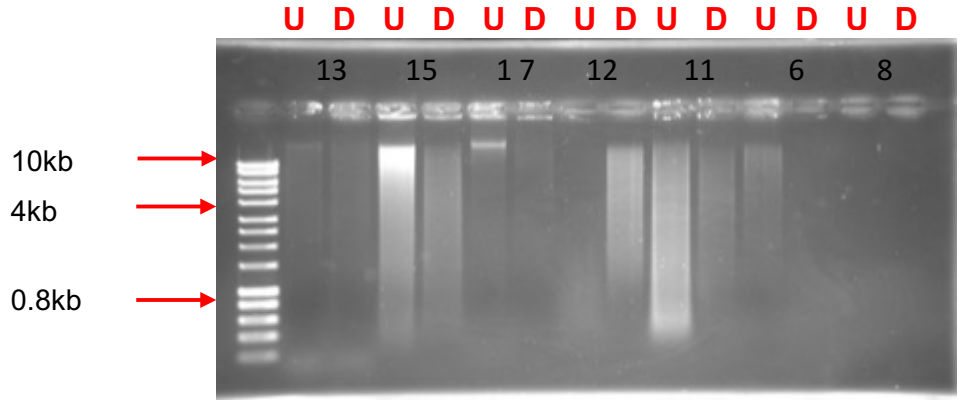

Figure 4.3. *Agarose gel displaying mid size and large plasmids in native (U) and digested (D) form.* Plasmids greater than 10kb were isolated using the AquaPlasmid buffer. The restriction sites were predicted using SnapGene, the sequences were incubated with the identified restriction enzymes, however the digested DNA appears degraded on the gel so the sizes have not been confirmed by restriction mapping. Ladder in lane 1 is the 10kb Hyperladder (Bioline), and sample identity is indicated by Table 1 row number

4.4 Antimicrobial Screening

The antimicrobial resistance predictions from screening with ARIBA (as described in chapter 3) were tested with Biomérieux Etest strips to validate phenotypic antibiotic resistance. The most commonly predicted resistance was tetracycline (tet) resistance, predicted in 45% of the putative plasmid sequences. Erythromycin (erm) sensitivity was also tested to identify selectable markers that could be used in the conjugation system. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values (ECOFFS) were used as guidelines to classify strains as either resistant or sensitive. Suggested thresholds are 2-4mg/L for tet, and 4-8 mg/L for erm; the

levels of resistance are recorded in Table 2 in mg/L. The validations were used to select donors with tet resistance and erm sensitivity, the recipients were selected for the opposing profile. The majority of the predictions were observed to be accurate with 7 out of the 44 being incorrect.

Antibiotic Resistance Levels as Observed with Etest Strips							
			Tet (mg/L)		**Erm (mg/L)**		**Validated**
Seq ID	**Isolate Name**	**Species Name**	**Predicted**	**Observed**	**Predicted**	**Observed**	
12718_7#90	H1_6	*Bacteroides uniformis*	x	0.75	x	8	N
13414_6#1	H1_38	*Lachnoclostridium nov.*	✓	48	x	0.016	Y
13414_6#62	H3_43	*Ruminococcaceae nov.*	x	0.016	x	0.016	Y
18048_2#80	fCSP	*Gemmiger formicilis*	x	0.094	x	0.06	Y
20298_2#57	D85_115	*Erysipelotrichaceae nov.*	✓	48	x	0.5	Y
20298_3#19	G8_180	*Bacteroides cellulosilyticus*	x	2	x	8	N
20298_3#39	G14_207	*Enterobacter nov.*	✓	24	x	256	N
13470_2#56	H2_21	*Eubacterium limosum*	✓	16	x	0.016	Y
13470_2#93	H4_41	*Lachnospiraceae nov.*	✓	16	x	0.016	Y
14207_7#59	H5_60	*Intestinimonas butyriciproducens*	✓	48	x	0.016	Y
14207_7#7	H4_46	*Eubacterium rectale*	✓	12	x	0.016	Y
20287_6#22	E72_40	*Lachnospiraceae nov.*	✓	16	x	0.016	Y
20287_6#28	E79_57	*Ruminococcaceae nov.*	✓	48	✓	256	Y
20287_6#48	F1_100	*Blautia nov.*	✓	24	✓	0.016	N
20427_4#28	F41_240	*Blautia nov.*	✓	4	x	0.016	Y
13414_6#32	H2_22	*Blautia nov.*	x	0.016	x	0.032	Y
20287_6#76	F29_X14C	*Blautia nov.*	x	12	✓	0.016	N
20287_6#41	E92_84	*Blautia nov.*	x	0.016	✓	256	Y
20287_6#56	F9_115	*Blautia nov.*	x	0.016	✓	256	Y
20287_6#49	F2_101	*Blautia nov.*	x	0.016	✓	256	Y
20287_6#32	F83_63	*Blautia nov.*	x	0.016	✓	256	Y

Table 4.2. *Antibiotic resistance levels as observed with Etest strips.* EUCAST ECOFFs were used as a guide to classify strains as either resistant or sensitive. Species predicted to be resistant to the antibiotic are marked with a tick, and those predicted to be sensitive with a cross. The observed level of resistance is recorded in mg/L, and the last column indicates if both of the predictions from ARIBA were correct (Y) or incorrect (N). The validations provided a list of donor and recipient candidates.

4.5 Strain Growth Monitoring

The species used in this work are primarily novel commensals and their growth kinetics have not been previously determined. Bacterial population growth follows an S-shaped curve going through an exponential log growth phase, and forming a plateau in stationary phase. Generation times vary between species and will affect the efficiency of assays such as plasmid harvest and inducing

competency. The bacteria were incubated in YCFA and the growth curves were plotted to observe the growth patterns of commensals compared to well-characterised species, and to try and identify optimal timings for assays (Figure 4). Based on the results of these graphs 48hr cultures were preferred for plasmid harvests rather than overnight cultures. Additionally, knowing the generation times of the recipients allowed more suitable time points for creating competent cells to be identified.

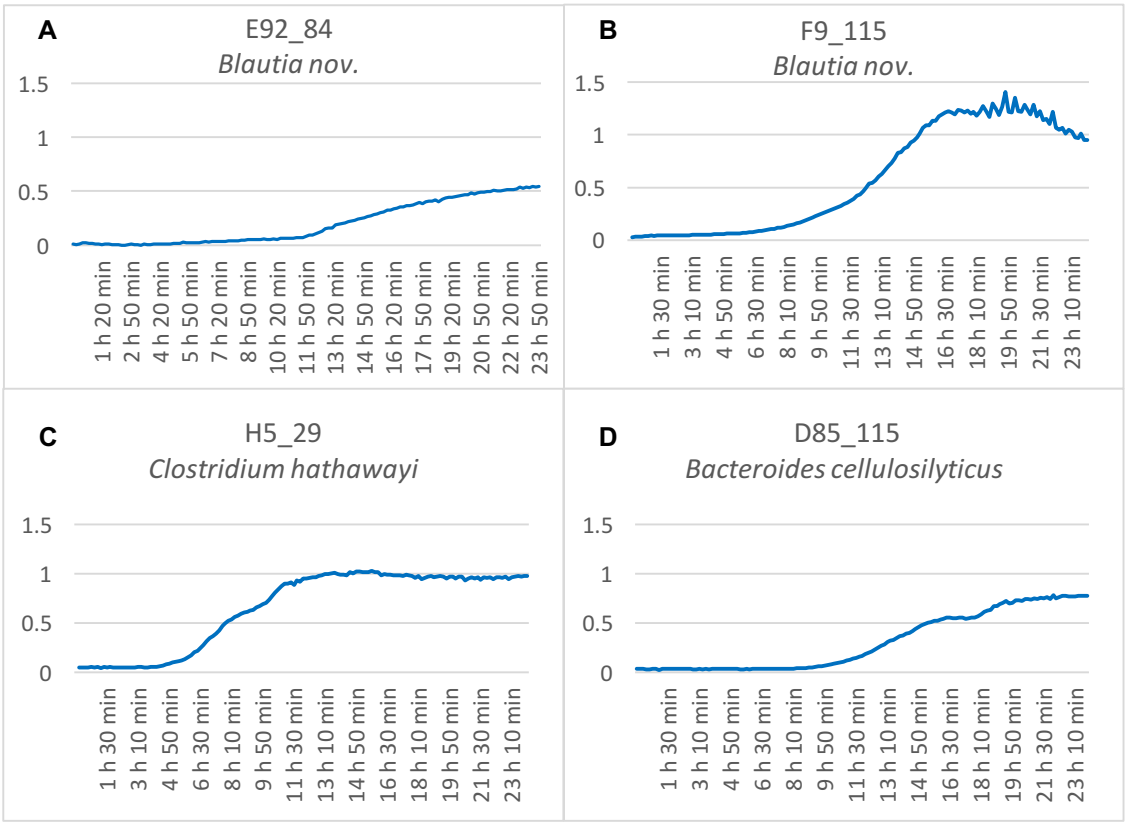

Figure 4.4 a-d. *OD growth curves for selected isolates.* A and B are candidate recipient strains, their growth curves display long lag phases which may be a factor in the creation of competent cells and the ability to participate in conjugation. C and D are plasmid containing strains, their growth curves demonstrate the point that different strains enter late stationary phase at varying times. These differences were used to optimise the timings of plasmid harvest and competent cell creation.

4.6 Plasmid Transfer

Plasmid transfer was attempted using a variety of protocols from the literature of bacteria phylogenetically similar to the selected isolates. Inducing natural competency was attempted first; natural competency is the innate potential of bacteria to uptake DNA from the environment when induced by a variety of stressors including DNA damage and starvation (Huddleston J. R., 2014). Plasmid DNA was isolated from all the strains in Table 1 for the transformation experiments. The recipient candidate was a novel *Blautia* species selected from a branch on the culture collection tree close to several of the donors. In addition, its genome annotation displayed predicted competence genes, and no antibiotic resistance genes; the resistance profile was validated by Etest. All the bacteria were incubated in different percentages of spent media. The spent media consisted of filtered

YCFA from overnight cultures of the same species mixed with normal YCFA broth in incremental ratios from 100% spent to 10% spent. This was to induce varying degrees of starvation, and potentially identify the correct level to trigger competence. The controls indicated that they survived the starvation but there was no growth on the selection plates.

Electroporation was attempted next; electroporation is the process by which bacterial cells are made porous by an electric pulse allowing DNA to move into the cell (Miller J.F., 1994). The recipient candidate was another novel Firmicute species selected experimentally due to its ability to survive the cell wall weakening procedure. Three electroporation buffers were tested, two caused the samples to arc, one allowed the bacteria to be shocked at an appropriate voltage. Once again, the controls indicated that bacteria survived the procedure, but there was no growth on the selection plates. The next attempt used protoplast transformation; protoplasts are formed by the enzymatic digestion of the cell wall (Rattanachaikunsopon P. and Phumkhachorn P., 2009). These protoplasts were then physically perturbed using glass beads and incubated with DNA to be taken up during cell wall repair. The candidate recipient was selected as mentioned above, with the same result– growth on the control plates only.

In addition to the transformation methods, conjugation was also attempted; conjugation is the procedure by which a bacterium directly transmits DNA to a neighbouring bacterium through an appendage called a pilus (Cabezón E., *et al* 2015). Species were selected to be donors from the list in Table 1, the final donors included two containing large plasmids with conjugation genes and two small plasmids containing mobility sequences. This would allow me to look at plasmids encoding their own conjugation machinery and plasmids relying on host conjugation machinery. The species chosen to be conjugation recipients were chosen based on their phylogenetic proximity to the plasmid-containing Firmicute donor species or their profile of selectable markers. The first iteration used antibiotic resistance negative Firmicute recipients that were selected for naladixic acid resistance.

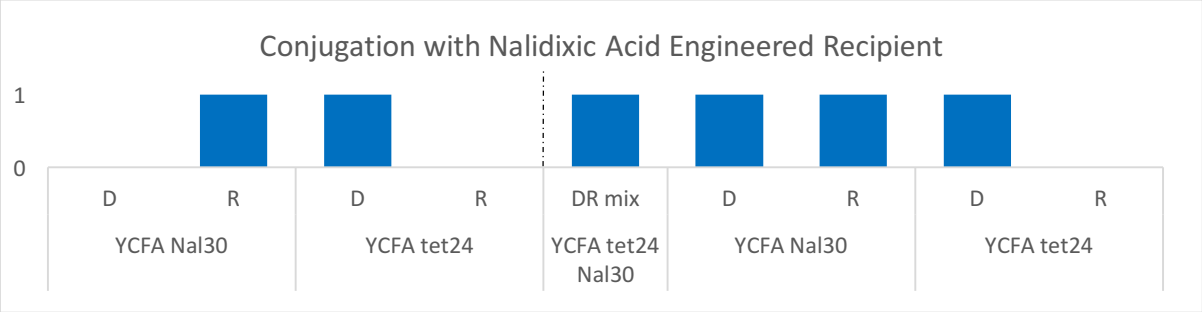

Figure 4.5. *Results of the conjugation between the donors (D) and naladixic acid engineered recipient (R).* 1 indicates growth and 0 indicates no growth. The left-hand side shows the bacterial profile before conjugation, and the right after conjugation. The donor and recipient both grew on the Naladixic Acid selection plate (YCFA Nal30), rendering the selection of trans-conjugants unsuccessful

Nalidixic acid resistance occurs as the result of a spontaneous point mutation in DNA gyrase genes (*gyrA, gyrB)* (Gellert M. *et al.,* 1977*)* When initially screened, the donor cells were nalidixic acid sensitive, however when plated during the test there was growth on the negative control, indicating that they probably acquired the mutation (Figure 5).

The second iteration used a selection of recipient candidates that were phylogenetically close to the donors and contained different resistance genes to the donor. There were several candidate Firmicutes identified that were predicted to contain only *erm* resistance; this however resulted in the exclusion of donors predicted to contain *erm* resistance in addition to *tet* resistance. The eligible donors were carried forward and mixed with the recipients. Growth was observed on the control plates but not the selection plates. In the case of the small plasmids this may indicate that the conjugation process did not occur due to lack of machinery in the host. In the case of both small and large plasmids the plasmids may not be compatible with or replication competent in the recipients (Figure 6).

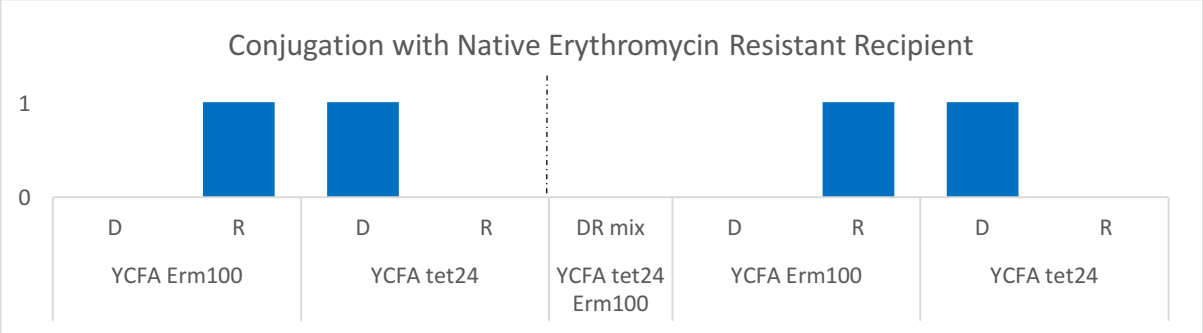

Figure 4.6. *Results of the conjugation between the donors (D) and erm-resistant bacteria (R).* 1 indicates growth and 0 indicates no growth. The left-hand side shows the bacterial profile before conjugation, and the right after conjugation. There was growth on the controls, but no growth on the selection plates (YCFA tet24 Erm100), meaning the conjugation was unsuccessful; this may be due to lack of conjugation machinery or incompatibility with the recipient.

The third iteration used *E. coli* and *Enterobacter cloacae* strains as recipient candidates to use aero-tolerance as a selectable marker. The recipient strains, while phylogenetically distant, were chosen as several of the plasmids showed potential broad host range (discussed in Chapter 3; Forster S. *et al, unpub, not shown*). Additionally, while the recipients could grow both aerobically and anaerobically, the donors were completely aero-sensitive allowing for easy selection between donors, recipients, and transconjugants. Donor and recipient strains were mixed and transconjugants were successfully observed for one of the donors. The levels of tetracycline resistance before- and after conjugation were verified by Etests (Figure 7), and plasmid DNA has been extracted for PCR validation and long read sequencing.

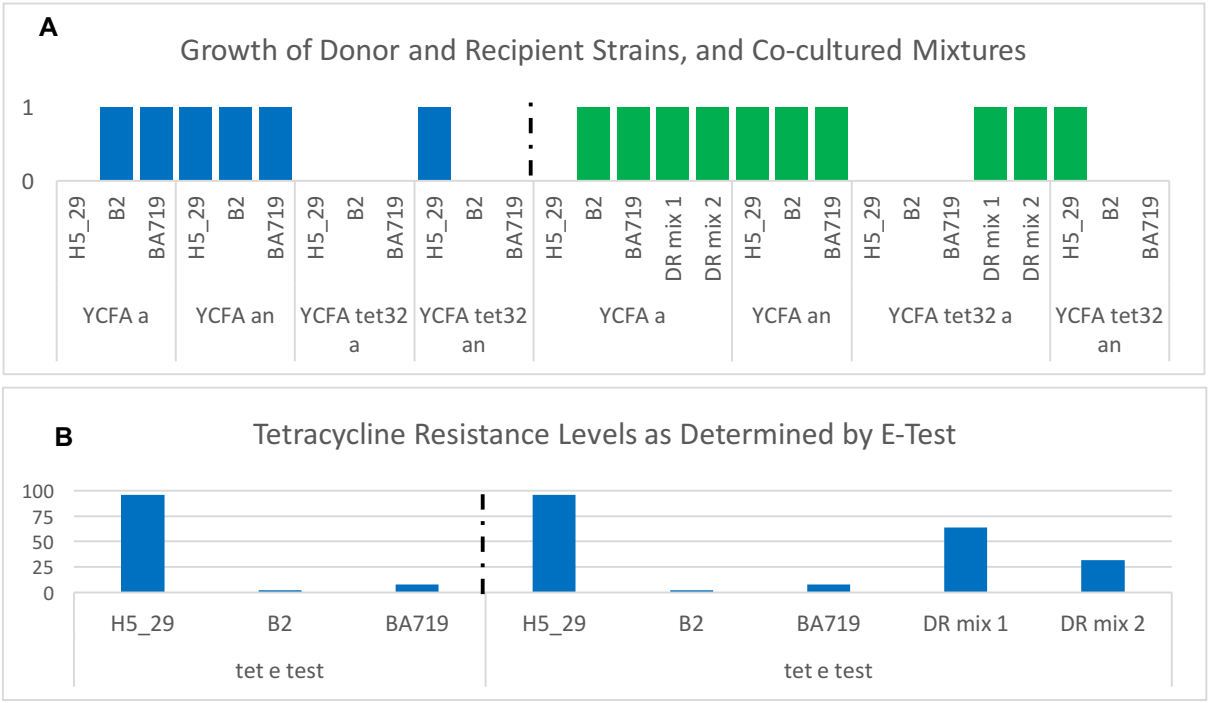

Figure 4.7 a-b. Results of the conjugation between donor H5_29 and aerobic bacterial recipients. The left-hand side shows the bacterial profile before conjugation, and the right after conjugation (a) 1 indicates growth and 0 indicates no growth. Growth of the donor and recipient mixes (DR mix 1 or 2) on the selection plate, YCFA tet32 in aerobic conditions (YCFA tet32 a), and the absence of donor or recipient growth under the same conditions indicates that conjugation was likely successful. (b) The levels of tet resistance in mg/L as determined by Etest before and after the conjugation show that the donor maintains its high level of resistance, the recipients remain sensitive, and the conjugation mixes display a boosted level of resistance.