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PROTEIN TRANSPLANT: CLONING GENES FOR TWO NtaA PROTEINS FROM *GORDONIA* SP. NB4-1Y

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PROTEIN TRANSPLANT: CLONING GENES FOR TWO NtaA PROTEINS FROM *GORDONIA* SP. NB4-1Y

by

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ABSTRACT

Some telomerisation-based aqueous film-forming foams (AFFF) used to fight forest fires contain 6:2 fluorotelomer sulfonate (6:2 FTS), or precursors that degrade to 6:2 FTS, which has been found to accumulate in groundwater systems and appear in surface waters and in the livers of fish following AFFF deployment. By examining the microbial metabolism of organosulfur compounds such as 6:2 FTS, therein lies potential for development of bioremediation tools. To date, the metabolism of 6:2 FTS has been observed in the soil bacterium *Gordonia* sp. NB4-1Y, an organism that was isolated in Kamloops, British Columbia Canada. When NB4-1Y was supplied 6:2 FTS as the sole sulfur source, the differential production of two nitrilotriacetate (NtaA) monooxygenases was observed when compared to a $MgSO_4$ supplemented culture. This led to the hypothesis that these enzymes initiate the desulfurization reaction used by NB4-1Y for 6:2 FTS biodegradation. To examine this hypothesis, DNA was isolated from NB4-1Y using a modified *Streptomyces* genomic DNA isolation protocol, and this DNA was used as a template to amplify the protein-coding region for each NtaA enzyme by polymerase chain reaction (PCR). PCR primers were designed to introduce restriction enzyme sites to the 5' and 3' ends of the genes to allow for subsequent directional cloning. These genes were then transferred into DH5α competent *E. coli* using a pBluescript cloning vector for sequencing. Once the gene sequence data has been obtained, the gene inserts will be moved into a pMAL-c2 expression vector that will allow for the production and purification of large quantities of the NtaA enzymes for characterization. The enzymes will be purified from an *E. coli* expression host using affinity chromatography by taking advantage of a maltose binding protein introduced to the N-terminal region of the target proteins using the pMAL-c2 expression vector, a

binding protein that has an affinity for amylose. Once purified, spectrophotometric assays monitoring the rate of NADH oxidation will be performed to definitively determine if these enzymes are involved in 6:2 FTS desulfurization in *Gordonia* sp. NB4-1Y, helping us further understand the potential value of *Gordonia* as a bioremediation tool.

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1 INTRODUCTION

The use of aqueous film-forming foams (AFFFs) to fight forest fires, and as fire suppressants by the military and aviation industry, has resulted in their large-scale deployment across natural ecosystems, resulting in the contamination of groundwater (Place & Field 2012). Detectable levels of perfluorinated organic surfactants that are important components of AFFFs have been reported in the livers of fish species found downstream of areas where AFFFs have been used (Oakes *et al*. 2009). AFFFs are known to contain perfluorinated organosulfur compounds that are not naturally metabolized and, as such, the deployment of AFFFs has the potential to result in bioaccumulation of fluorinated compounds within natural habitats.

It is well known that organic sulfonates are important sources of sulfur for soil microorganisms, organisms that contribute greatly to the biogeochemical cycling of sulfur (Boden *et al.* 2011, Li *et al*. 2013). The sulfur cycle allows for the movement of sulfur through living organisms, minerals and the atmosphere. The cycling of sulfur is vital for living organisms, as sulfur is a common component of proteins, cofactors and vitamins.

As the organosulfur compounds present within AFFFs, for example 6:2 fluorotelomer sulfonate (6:2 FTS), are not found naturally and possess a backbone stabilized by strong carbon-fluorine bonds, they are difficult to biodegrade, generating a need for bioremediation tools for ecosystem restoration. *Gordonia* sp. NB4-1Y was isolated in Kamloops, British Columbia from *bis*-(3-pentafluorophenylpropyl)-sulfide enriched vermicompost, and has been found to metabolize a broad spectrum organosulfur compounds including 6:2 FTS (Van Hamme et al. 2013). *Gordonia* spp. are attractive for bioremediation as they have broad

capacity for metabolism of natural and anthropogenic organics (Arenskotter *et al.* 2004). Whole-genome shotgun sequencing revealed that the NB4-1Y genome possesses 72 monoxygenases and 52 dioxygenases. Additionally, using two-dimensional differential in-gel electrophoresis, two putative nitrilotriacetate monoxygenases (NtaA), ISGA 1222 and 1218, were found to be differentially upregulated when NB4-1Y was supplied 6:2 FTS as the sole sulfur source instead of $MgSO_4$. From this, it was hypothesized that these two enzymes may be responsible for the initial desulfonation of 6:2 FTS in the proposed metabolic pathway developed by Van Hamme *et al*. (2013) (Figure 1).

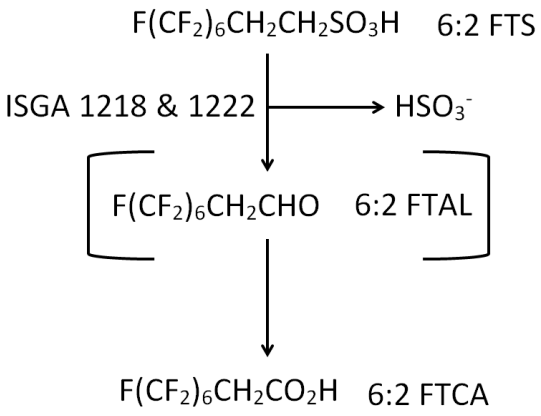

Figure 1 The first two steps in the proposed metabolic pathway for 6:2 fluorotelomer sulfonate degradation by *Gordonia* sp. NB4-1Y, including the desuflonation reaction putatively catalyzed by ISGA 1222 and 1218 (Adapted from Van Hamme *et al.* 2013).

This study is not the first to uncover the degradation of fluorinated sulfonates, however it is one of few studies working with pure culture. Presently, the majority of literature focuses on the microbial degradation of per- and poly- fluoroalkyl substances (PFAS) by mixed cultures (Dinglasan *et al.* 2004; Rhoads *et al.* 2008; Wang *et al.* 2005). As a result, there is a gap in knowledge relating to the biochemistry of microbial metabolism of

such compounds by pure cultures. Currently, only three studies have independently reported microbial metabolism of PFASs by pure culture of *Pseudomonas* species, with only one examining sulfur containing PFASs. Key *et al.* (1998) found *Pseudomonas* sp. strain D2 was capable of using difluoromethane sulfonate, trifluoromethane sulfonate and 6:2 fluorotelomer sulfonate [6:2 FTS; $F(CF_2)_6CH_2CH_2SO_3H$] as sulfur sources for growth. In a different study conducted by Wang et al. (2011) using sewage inocula, 6:2 FTS was found to be degraded to 5:3 fluorotelomer acid [5:3 acid; $F(CF_2)_5CH_2CH_2CO_2H$], perfluorohexanoic acid [PFHxA; $F(CF_2)_5CO_2H$], perfluoropentanoic acid [PFPeA; $F(CF_2)_4CO_2H$] and perfluorobutanoic acid [PFBA; $F(CF_2)_3CO_2H$] via 6:2 FTUCA [$F(CF_2)_5CF_5 = CHCO_2H$], 5:2 ketone [$F(CF_2)_5C(O)CH_3$] and 5:2 sFTOH [$F(CF_2)_5CH(OH)CH_3$]. From this, Wang et al. hypothesized the initial step of the microbial metabolism of 6:2 FTS by sewage inocula was catalyzed by an unidentified bacterial alkane sulfonate-α-hydroxylase.

To determine the function of NtaA monoxygenases found in various bacterial systems, researchers have previously reported using expression vector systems. Kim et al. (2006) determined the function of an NtaA found in *Corynebacterium glutamicum* by cloning and then expressing the gene in *E. coli* prior to purification. They first amplified the protein-coding region for the monoxygenase from chromosomal DNA by PCR using primers that introduced restriction enzyme sites to the 5' and 3' ends before directional cloning into a pPosKJ expression vector. The expression vector functioned to introduce a hexahistidine tag and bacterial haemoglobin at the N-terminus of the NtaA protein, allowing for the purification of the translated protein from harvested recombinant *E. coli* cell lysate through an Ni-NTA column. The eluted fraction was then loaded onto a HiTrap Q anion exchange column, and the bound proteins were eluted, prior to removing the hexahistidine tag removed

from the target protein with a recombinant tobacco etch virus protease. Finally, the proteins were passed through a Ni-NTA column to remove the cleaved hexahistidine tags. Once purified, Kim *et al.* performed *in vitro* NAD(P)H oxidoreductase assays for enzyme activity characterization studies (Eichhorn et al. 1999).

The goal of this project was to clone the sequences encoding the two NtaA monooxygenase proteins from NB4-1Y, namely ISGA1222 and 1218, into competent DH5α *E. coli* to allow for future work to understand the role of the two proteins in the microbial metabolism of 6:2 FTS. The cloning procedure used was similar to that described by Kim et al. (2006) in that PCR primers were designed to add restriction enzyme sites to PCR products to allow for directional cloning into the pMAL-c2 expression vector polylinker. Specifically, an EcoRI restriction enzyme site was added to the 5' end by the forward primer, and an XbaI site was added to the 3' end by the reverse primer. PCR amplicons were identified by size comparison to a molecular weight marker, and were gel purified using a Qiagen II gel extraction kit. The ISGA 1218 amplicon was used for sublconing into a pBluescript vector, which will allow for sequencing of the inserted fragment. Future work with this project will be to express the two NtaA proteins, ISGA 1218 and 1222, using the pMAL-c2 expression vector. Successful incorporation of the protein-coding regions for each protein in the expression vector will result in the addition of a N-terminal maltose binding protein, which like the hexahistidine tag used by Kim *et al.* (2006) will allow for the purification of the target proteins through affinity chromatography. Once purified, the two NtaA enzymes can be used for *in vitro* oxidoreductase assays monitoring the rate of NAD(P)H oxidation to determine their role in 6:2 FTS desulfonation.

2 MATERIALS AND METHODS

All materials were purchased from Invitrogen unless otherwise stated.

2.1 Bacterial Strains and Growth Conditions

A stock culture of *Gordonia* sp. NB4-1Y was prepared from a CyroBank bead harbouring NB4-1Y stored at -80 °C (blue cap, labeled NB4-1Y from sample June 2-14). Using a sterilized loop, the CyroBank bead was used to streak for isolated colonies on nutrient agar. The plate was incubated at 30 °C for four days, after which the plate was sealed using Parafilm to prevent water loss and placed at 4°C.

2.2 DNA Extraction

Two 10 mL liquid nutrient broth cultures were aseptically inoculated using an isolated colony from the prepared stock culture of NB4-1Y. The broth cultures were incubated at 30 °C for three days on a tube roller and the typical clumped white growth characteristic of NB4-1Y was observed. The liquid nutrient broth NB4-1Y cultures were then used to inoculate YEME broth (Appendix B) cultures at 2 % v/v prior to incubation at 30 °C with rotation for seven days.

Cells from each YEME culture (6 mL total) were harvested in microfuge tubes by centrifugation (21 000 rpm, 5 minutes) and a portion of the original culture was used to check for culture purity by streaking onto nutrient agar. The collected pellets were washed twice in 10.3 % sucrose and then resuspended in 500 µL filter sterilized lysozyme buffer (10 mg/mL) including RNAse (50 µg/mL) in TE25S (1.5 M Tris-HCl, 0.5 M EDTA, 0.5 M sucrose, pH 8.0). Tubes were incubated for 1 hour in a 37 °C water bath and vortexed for 10 seconds every 5 minutes.

Following incubation, 250 µL of 4 % SDS (Sigma-Aldrich) was added to each tube, and tubes were then vortexed vigorously. With the addition of SDS into the lysozyme and RNase resuspended cells, a white precipitate appeared in each tube. In an attempt to resuspend the precipitate in each tube, the tubes were heated for 2 minutes in the 37 °C water bath. To remove protein contaminants, 250 µL neutral phenol:chloroform was added and extracted (21 000 rpm, 5 minutes). The upper aqueous layer was transferred into a clean microfuge tube and an additional volume of 250 µL neutral phenol:chloroform was added and extracted (21 000 rpm, 5 minutes). This process was repeated three times. An equal volume of chloroform (500 µL) was added and extracted to remove all phenol present in the sample. This process was repeated a second time, with a volume of 300 µL. A total of 0.1 volumes of 3 M sodium acetate (30 µL, pH 4.8-5.1) and 2 volumes of ethanol (600 µL) were added to each tube followed by gentle mixing by inversion to collect the fibrous DNA pellet. Each pellet was transferred into a 70 % ethanol wash. Pellets were collected (21 000 rpm, 5 minutes) and the ethanol was removed to allow the pellet to air dry. Each pellet was resuspended in 50 µL TE buffer (pH 8.0) and was stored at 4 °C.

2.3 DNA Quantification

The concentration of extracted DNA samples were quantified using a broad-range Qubit Fluorometric DNA Quantification assay.

2.4 Characterizing plasmids

Plasmids pBluescript (pBS) and pMAL-c2 were obtained from Agilent Technologies and New England BioLabs, respectively. Restriction digests were performed on pBS and pMAL-c2 to determine the quality of the provided samples. To determine which restriction enzymes to use to digest the plasmids, published restriction maps were consulted (New England

BioLabs, Addgene). Based on the published restriction sites (Appendix C, Appendix D), pMAL-c2 was digested with BsaI, PstI and EcoRV, and pBS was digested by BsaI and EcoRV to generate fragments of each plasmid of known size, to be used to characterize and identify each plasmid accurately (Table 1). Each reaction was incubated at 37 °C for 45 minutes, and the resulting DNA fragments were separated on a 1 % agarose gel for 1 hour. The sizes of the generated restriction fragments were compared to a λ- HindIII molecular weight marker.

Table 1 Restriction digest performed on pBS and pMAL-c2 to characterize provided plasmids. The single digests performed for pBS were: BsaI, and EcoRV, with a double digest of BsaI and EcoRV. The single digest performed for pMAL-c2 were BsaI, PstI and EcoRV, with double digests between BsaI and PstI, and BsaI and EcoRV.

Digest component	pBluescript		pMAL-c2	
	Single Digest	Double Digest	Single Digest	Double Digest
DNA	66 ng/µL	66 ng/µL	66 ng/µL	66 ng/µL
NEB 2 buffer	3 µL	3 µL	3 µL	3 µL
dH_2O	22.7 µL	21.2 µL	18.8 µL	17.3 µL
Enzyme	3 units/µL	3 units/µL	3 units/µL	3 units/µL
Total volume:	30 µL	30 µL	30 µL	30 µL

2.5 Polymerase Chain Reaction Amplification

2.5.1 PCR Primer Design

Two sets of primers were designed for the amplification of genomic sequences encoding for the proteins ISGA 1222 and ISGA 1218. Restriction enzymes were chosen based on the presence of the restriction enzyme recognition site within the multiple cloning site (MCS) of pBS and pMAL-c2. For directional cloning, the order of the restrictions enzymes (5' or 3') was determined based on the order of the recognition sequences within the pMAL-c2 MCS. Each primer was diluted to a final stock concentration of 100 µM with TE buffer (pH 8.0) and a working solution concentration of 10 µM in TE buffer (pH 8.0).

The first set of primers designed for the nucleotide sequence coding for the protein ISGA 1222 included different restriction enzyme recognition sites at the 5' end than the 3' to allow for directional cloning into pMAL. For the forward primer F1222EcoLS, an EcoRI site (GAA TTC) was added to the 5' end. An additional three-nucleotide extension (GAC) was added to the 5' end to facilitate enzymatic digestion (New England BioLabs 2015). An XbaI site (TCT AGA) was added to 5' end of the reverse primer R1222XbaLS, along with three additional nucleotides (ACA). Following the XbaI site is the reverse compliment of a stop codon TAG (CTA) to trigger the termination of transcription. The second primer set was designed to clone with only one restriction site, XbaI, for non-directional cloning. The forward primer F1222XbaLS included the addition of an XbaI site (TCT AGA) and a three-nucleotide extension (GGT).

The primers designed for ISGA 1218 followed the same design guidelines as those used for the design of the primers for ISGA 1222. For directional cloning, an EcoRI site (GAA TTC) and a three-nucleotide extension (GTA) was added to the 5' end of the primer and a XbaI restriction site (TCT AGA) including a three-nucleotide (TAC) extension added to the 5' reverse compliment for the reverse primer. For non-directional cloning, an XbaI forward primer was designed including the XbaI restriction site (TCT AGA) and a three-nucleotide (ATG) extension. While designing the primers, the length, GC content, melting temperature and 3' end stability were also considered. The sequences of the designed primers are listed in Table 2.

Table 2 Primer sequences for PCR amplification of the nucleotide sequences coding for the proteins ISGA 1222 and ISGA 1218, including Tm values, length, GC content, and melting temperature.

Primer	FEcoRI1218	RXbaI1218	FEcoRI1222	RXbaI1222
Table Sequence	gtagaattcatgaacgtaaacgttg	tactctagatcacgccgcccccac	gacgaattcatggctgatcgagag	acatctagactaaccggtccggcg
Tm	60 °C*	73 °C*	66 °C	71 °C
Length	25	24	24	24
GC content	36%	62.5%	50%	58.3%
Melt temp	52.7 °C	64.1 °C	57.5 °C	62.1 °C

* The Tm difference is greater than the recommended limit of 5 °C

2.5.2 PCR Reaction Setup

PCR reactions were performed using sterile PCR tubes and barrier tips in a biological safety cabinet to reduce the possibility of contamination during reaction setup. Using a Q5™ High-Fidelity DNA Polymerase kit, reagents were added to nuclease-free dH_2O in sterile PCR tubes in the order outlined in Table 3.

Table 3 PCR reaction setup using the Q5 High-Fidelity DNA Polymerase kit.

Component	50 µL Reaction	Final Concentration
5x Q5 Reaction Buffer	10 µL	1x
10 mM dNTP	1 µL	200 µM
10 µM Forward primer	2.5 µL	0.5 µM
10 µM Reverse primer	2.5 µL	0.5 µM
Template Genomic DNA	0.5 µL (100 ng/ µL)	< 1000 ng
Q5 High Fidelity DNA Polymerase	0.5 µL	0.02 Units/ µL
Nuclease-free dH_2O	Up to 50 µL	-

A 50 µL reaction was prepared to amplify both ISGA 1222 and ISGA 1218 for each temperature of the temperature gradient (60-70 °C), and a negative control containing no DNA in the reaction was prepared for each sequence of interest. The samples were vortexed

for 5 seconds before they were placed in a 98 °C preheated thermocycler. The thermocycling conditions for the gradient PCR reaction is listed in Table 4.

Table 4 Thermocycling conditions for PCR amplification of the nucleotide sequences coding for ISGA 1222 and ISGA 1218 in *Gordonia* sp. NB4-1Y.

	ISGA 1218		ISGA 1222	
	Temperature (°C)	**Time (min)**	**Temperature (°C)**	**Time (min)**
Initial denaturation	98	2	98	2
35 cycles	98	0.5	98	0.5
	60-70	0.5	60-70	0.5
	72	1.5	72	1.5
Final Extension	72	2	72	2
Hold	4	∞	4	∞

Following amplification, PCR products were visualized on a 2 % agarose gel (90 volts, 3 hours). PCR products were quantified by comparison to a 1 Kb plus DNA ladder.

2.5.3 Colony PCR Amplification of *E. coli* (DH5α) Using NB4-1Y Specific Primers

A colony PCR reaction was set up to determine whether the primers designed to amplify ISGA 1222 and ISGA 1218 in *Gordonia* sp. NB4-1Y amplify any genes present in *E. coli* DH5α. An isolated colony of DH5α was transferred via a sterile toothpick into 30 µL nuclease-free water and was subject to 70℃ in a thermocycler for 10 minutes to lyse the cells. During the cell lysis, PCR reaction tubes were prepared containing PCR reactions to a total volume of 25 µL (Table 5). Following the completion of the cell lysis, 1 µL of the lysed solution was added to each reaction, and was subject to PCR amplification following the temperature gradient previously described (Table 4). A positive control was prepared using extracted NB4-1Y DNA.

Table 5 Colony PCR reaction setup the Q5 High-Fidelity DNA Polymerase kit.

Component	25 µL Reaction	Final Concentration
5x Q5 Reaction Buffer	5 µL	1x
10 mM dNTP	0.5 µL	200 µM
10 µM Forward primer	1.25 µL	0.5 µM
10 µM Reverse primer	1.25 µL	0.5 µM
Template Genomic DNA	1 µL (Lysed DH5α)	< 1000 ng
Q5 High Fidelity DNA Polymerase	0.25 µL	0.02 Units/ µL
Nuclease-free dH_2O	Up to 25 µL	-

2.5.4 ISGA 1222 & 1218 PCR product gel purification

To isolate the ISGA 1218 PCR product from the non-specific amplicons obtained through PCR amplification, DNA fragments were separated on a 1% agarose gel (90 V, 45 minutes) prior to using a Qiaex II gel extraction kit. To ensure maximum recovery, 15 µL of the PCR product was loaded with 5 µL 5X loading buffer. Bands were visualized using a GelDoc system (BioRad), and the 1473 bp band corresponding to the ISGA 1218 gene was excised using a scalpel and placed into a weighed tube. The Qiaex II gel extraction kit protocol was followed as per the manufactures instructions (Qiagen). The pelleted DNA was resuspended in TE buffer (20 µL, pH 8.0) to elute the DNA into solution. The eluted DNA was then transferred into a new tube, and a second elution was performed. The gel purified first and second DNA elutions were visualized following separation on a 1 % agarose gel at 90 V for 45 minutes.

2.6 Subcloning

2.6.1 pBluescript

The restriction enzyme EcoRV (5'-GAT|ATC-3') was used to linearize pBS to allow for the blunt-ended ligation of the gel purified ISGA 1218 amplicons into the linearized

plasmid. The digest was prepared as per Table 6, and was incubated at 37 °C for 1 hour. The enzyme was then deactivated at 80°C for 20 minutes.

Table 6 Restriction enzyme digest of pBS using the restriction enzyme EcoRV to allow for linearization of the plasmid to insert gel purified ISGA 1218 sequence.

Digest Component	Volume	Concentration
pBluescript	2.8 µL	66 ng/µL
NEB 3 buffer	3 µL	1X
dH_20	22.7 µL	-
EcoRV	1.5 µL	15 units
TOTAL	30 µL	-

Following the deactivation of the enzyme, ligation reactions were prepared (Table 7).

Table 7 Ligation reactions prepared.

Ligation Reaction	i	ii	iii	iv	v
pBluescript	10 ng uncut	10 ng uncut	100 ng linearized	100 ng linearized	100 ng linearized
ISGA 1218	-	-	-	-	10 µL
5X Ligation Buffer	-	2 µL	2 µL	2 µL	2 µL
T4 DNA Ligase	-	1 µL	-	1 µL	1 µL
dH_20	29 µL	21 µL	21.5 µL	20.5 µL	10.5 µL

The four control reactions used in the transformation experiment were prepared as follows:

i. 10 ng uncut pBS (no ligation reaction). From this transformation, the maximum efficiency of the DH5α competent cells can be determined.

ii. 10 ng uncut pBS (ligation reaction). The addition of DNA ligase should reduce the transformation efficiency.

iii. 100 ng linearized pBS (no DNA ligase). From the EcoRV restriction digest of pBS, 100 ng of linearized pBS was used to demonstrate the efficiency of

degradation. As linear DNA is degraded by exonucleases in a bacterial cell, if the digestion by EcoRV was incomplete, the uncut covalently, closed, circular (ccc) pBS will result in recombinant colonies and blue colonies will be visible.

iv. 100 ng linearized pBS (ligation reaction). By adding DNA ligase to the linearized pBS, the ability of the recircularization can be identified.

To allow for the blue-white α-complementation test to detect the successful insertion of an insert into the MCS of pBS, X-gal (20 mg/mL in dimethylformamide) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 20 mg/mL in dH_2O) were added (40 μL each) and spread using spread plating techniques onto ampicillin (100 mg/L) containing nutrient agar plates and were incubated at 37 °C until the liquid evaporated. During this time, DH5α competent cells were removed from the -70 °C freezer and thawed on ice. All transformations were prepared on ice, adding 2.5 μL of either control or experimental ligation reactions into chilled tubes. The thawed competent cells were added to allow for proper dispersal of the ligation reaction with minimal mechanical damage to the fragile competent cells. The competent cells and ligation reaction mix was then incubated for 30 minutes on ice. After incubation, the competent cells were heat shocked at 42 °C for 2 minutes. The competent cells were returned back onto ice, and 1 mL of LB media was added. The solution was placed in the 37 °C incubator for 30 minutes. After incubation, 100 μL of each transformation reaction was aseptically spread onto agar plates containing ampicillin, X-gal and IPTG. The plates were incubated at 37 °C for 24 hours. The number of blue and white colonies on each plate was recorded to determine transformation efficiency.

Successful transformants were identified as white colonies through the α-complementation test. Using a sterile loop, transformants were streaked for isolated colonies

on agar plates containing ampicillin, X-gal, and IPTG. Plates were incubated for 24 hours at 37 °C.

2.6.2 Plasmid DNA Extraction

Plasmid minipreps were prepared using the Qiagen plasmid purification system. Isolated transformant colonies were used to inoculate 4 mL liquid broth tubes containing ampicillin (100 mg/L) prior to incubation for 12 hours at 37 °C while shaking. The plasmid minipreps were prepared as per the protocol described by the Molecular Cloning Handbook. The products were visualized on a 1 % agarose gel (100 V, 45 minutes).

2.6.3 pBluescript + 1218 Restriction Digest

To release the insert from the MCS of pBS, EcoRI and XbaI restriction digests were performed on the plasmid DNA. The PCR primers used to amplify ISGA 1218 gene were designed to introduce a 5' EcoRI site and a 3' XbaI site, and as such, the first restriction digest performed included single digests by EcoRI and XbaI to linearize the DNA and a double digest of EcoRI and XbaI to release the ISGA 1218 fragment from the pBS vector (Table 8). The digests were incubated at 37 °C for 1 hour, and were visualized on a 1 % agarose gel (100 V, 45 minutes).

Table 8 Miniprep restriction digest to release insert from pBS MCS.

Digest component	**pBluescript + ISGA 1218**		
	Single Digest	**Double Digest**	**Concentration**
DNA	2 µg	2 µg	66 ng/µL
Neb 2	3 µL	3 µL	1X
dH_2O	22.7 µL	21.2 µL	-
Enzyme	1.5 µL	1.5 µL (of each enzyme)	15 units (of each enzyme)
TOTAL	30 µL	30 µL	-

After the insert was not observed to be released from the pBS MCS, a second restriction digest was performed. As the insert was introduced into the EcoRV blunt-ended site of the pBS MCS, restriction sites were examined on either side of the EcoRV site in attempt to excise the insert. Using this technique, HindIII and XbaI were used. Additionally, instead of performing two single digests and a double digest including the two restriction enzmyes, a HindIII single digest was performed, and the restriction enzymes and buffers were removed using the EZ-10 restriction enzyme cleanup protocol before the second restriction enzyme digest by XbaI. The EZ-10 protocol was followed as per the manufacturer's instruction (Bio-Basic). The restriction digests and EZ-10 products were visualized on a 1% agarose gel (100 V, 45 minutes).

2.6.4 PCR Amplification of Plasmid DNA

The plasmid DNA samples were subject to PCR amplification using the ISGA 1218 directional primers (5' EcoRI site, 3' XbaI site) used to generate the amplified sequence transformed into pBS. A control sample was run using pBS with no insert as the DNA template to see if the primers amplified any sequence within pBS. Following PCR amplification, the PCR products were observed on a 1 % agarose gel (100 V, 45 minutes).

3 RESULTS AND DISCUSSION

3.1 Genomic DNA isolation

In order to isolate genomic DNA from NB4-1Y to be used as a template for PCR amplification of the target genes, a modified *Streptomyces* DNA extraction protocol was used (Kieser *et al.* 1985). Generally, liquid cultures were grown for 10 days prior to extractions as *Gordonia* and related species have slower growth rates than *E. coli* (Fusconi *et al.* 2006;

Marr 1991). In addition, a high sucrose medium was used as it is believed that high osmotic stress weakens the robust cell wall of these organisms (Van Hamme, personal communication, September 2015) to allow for more efficient lysis. DNA (100 ng/μL) was recovered from 6 mL cultures, and gel electrophoresis revealed high quality, high molecular weight DNA free from RNA contamination (Figure 2). With this DNA in hand, it was possible to carry out PCR reactions.

Figure 2 NB4-1Y genomic DNA isolation, labeled 1 and 2 (1 % agarose gel, 35 minutes at 100 V).

3.2 PCR amplification

PCR reactions were performed to amplify the nucleotide sequences of interest for each NtaA enzyme to subsequently transform into a pBS cloning vector. The Q5 High Fidelity DNA Polymerase was selected for this, as the PCR products formed are blunt-ended (New England BioLabs), allowing for ligation into pBS without causing a frameshift. After taking into account all of the various factors when designing the primers including the length, GC content, Tm values and 3' end stability, the ISGA 1218 directional primer pair (5' EcoRI, 3' XbaI site) exceeded the suggested 5 °C Tm difference; the forward primer had a Tm value of

60 °C, whereas the reverse primer had a Tm value of 73 °C (New England BioLabs). With this large difference in Tm values, the suggested annealing temperature was set at 63 °C. The ISGA 1222 directional primer pair (5' EcoRI, 3' XbaI site) did not exceed this difference, and was suggested to anneal at 69 °C. As each target annealing temperature was found between 60 and 70 °C, a gradient PCR was performed for amplification of each target in an attempt to determine which temperature provided the greatest amount of product (Figure 3).

When examining PCR products for each target, ISGA 1222 and 1218, several non-specific products were visualized under each target band, which may have been attributed to the concentration of the polymerase, the length of the extension time, or the annealing temperature (New England BioLabs). This protocol was repeated using increased concentrations of the template DNA (10 ng, and 100 ng) into the PCR reaction mixture; however the non-specific banding was found to increase with increasing concentration of the genomic DNA template.

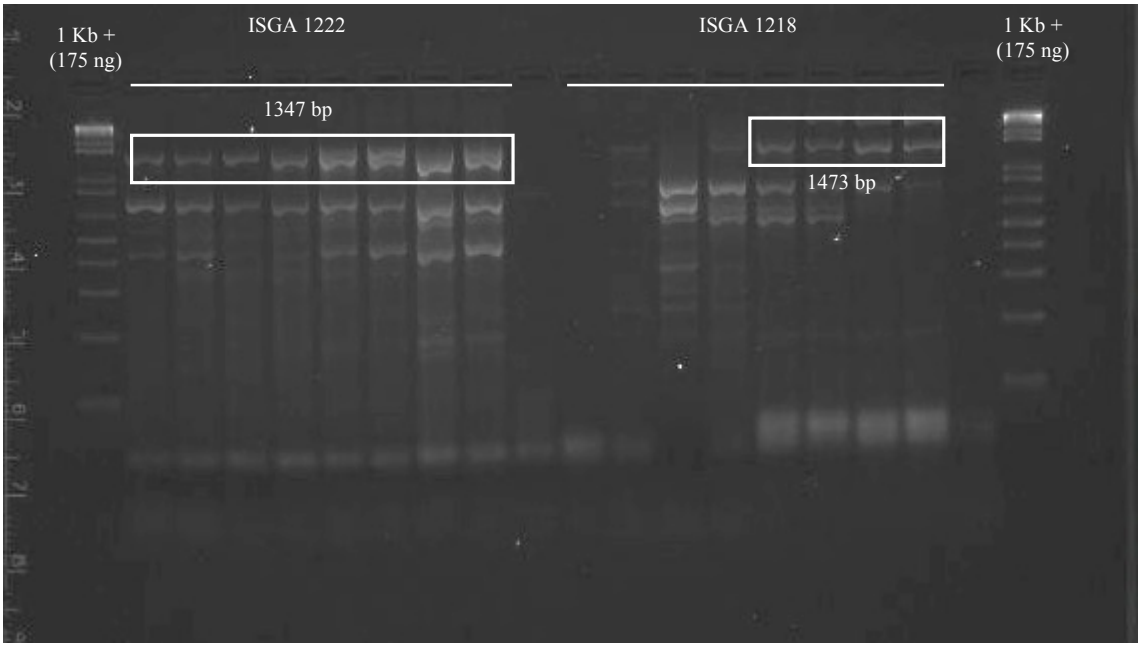

Figure 3 Gradient PCR amplification of ISGA 1222 and ISGA 1218 using PCR primers designed to induce a 5' EcoRI and 3' XbaI restriction site. Each lane contains 5 µL of an individual PCR reaction which was subject to a different thermocycling temperature between 60-70 °C in 5 µL 5X loading buffer.

Once the PCR conditions were optimized to amplify products of approximately the same size as the desired products, a colony PCR amplification was performed to determine whether any sequences in *E. coli* would be amplified using these primers. It was thought that if *E. coli* did not have any sequences amplified by the primers, then a colony PCR amplification of the transformant cells after subcloning would be a suitable method of determining whether the desired insert was ligated into pBS. *E. coli* DH5α cells were used for a temperature gradient PCR and, as seen in Figure 4, there is relatively weak amplification of several targets within *E. coli* using primers for ISGA 1222 and 1218 at all temperatures except (61.2 ºC). At this temperature, not only were there three bands with high concentrations of PCR products, but also one band was approximately the same size as the each target (approximately 1400 bp), and as such this method of determining the presence of the insert was not used. However, with the successful amplification of each target

sequence, the plasmids for which the sequences would later be transformed were characterized to confirm their identity.

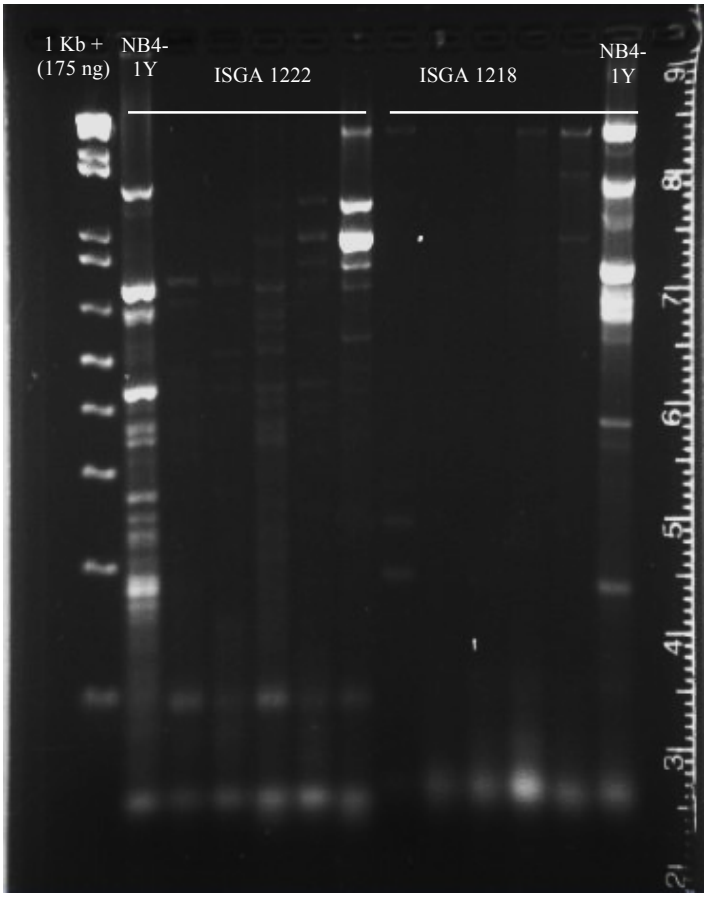

Figure 4 Colony PCR amplification of DH5α competent *E. coli* cells. Genomic NB4-1Y DNA was used as a positive control to determine whether colony PCR amplification of transformant cells was an accurate method to determine the insertion of ISGA 1218 and ISGA 1222 into pBS. Each lane contains 2.5 µL of an individual PCR reaction which was subject to a different thermocycling temperature between 60-70 °C in 5 µL 5X loading buffer.

3.3 Characterization of plasmids

To confirm the identity of the supplied plasmids, pBS and pMAL-c2, the plasmids were characterized using restriction digests by restriction enzymes to compare to published restriction maps (NebCutter) (Appendix C, Appendix D). The restriction enzymes chosen

yielded fragments that could be detected using a 1 % agarose gel, and provided characteristic banding patterns that were used to confirm the purity and authenticity of each plasmid.

Table 9 Expected fragment size of each restriction digest of pMAL-c2 and pBS.

pMAL-c2 (6643 bp)		
Restriction Digest	**Expected fragment size (bp)**	**Observed fragment size (bp)***
BsaI	4101, 1480, 1067	7000, 6000, 3000, 2000
PstI	6643	6700
EcoRV	6643	6700
BsaI + PstI	4101, 1132, 1067, 348	3300, 1400, 1000
BsaI + EcoRV	3386, 1480, 1067, 710	3300, 1400, 1000, 700
pBluescript (2958 bp)		
Restriction Digest	**Expected fragment size (bp)**	**Observed fragment size (bp)***
BsaI	2958	3000
EcoRV	2958	3000
BsaI + EcoRV	1410, 1188	1400, 1300

When analyzing the restriction digest for pMAL-c2 (Table 9) the PstI and EcoRV digest resulted in the expected linearized 6643 bp fragment characteristic for this plasmid. The BsaI single digest was inconclusive, however the BsaI/PstI digest provided four bands approximately the same size as the expected fragments (4101, 1132, 1067, 348bp). Further support for the characterization of pMAL-c2 was provided from the BsaI/EcoRV digest, containing all four expected fragments (3386, 1480, 1067, 710 bp). The combination of the results from the single and double digests allowed for the conclusion of a pure sample of pMAL-c2.

The restriction digests for pBS showed that BsaI and EcoRV single digests resulted in the expected linearized 2958 bp fragment, and the BsaI/EcoRV double digest provided two bands approximately the same size as the expected 1410 and 1188 bp fragments (Figure 5). When looking at the BsaI/EcoRV digest, two additional bands can be seen above the two digested fragments. The larger of the two bands is a result of an incomplete digest, and the

band is remaining ccc pBS, whereas the second band, directly above the two digested fragments is a result of a partial single digest by either BsaI or EcoRV. The confirmed identity of each plasmid through restriction enzyme characterization allowed the progression to subcloning the PCR amplified sequences, first into pBS.

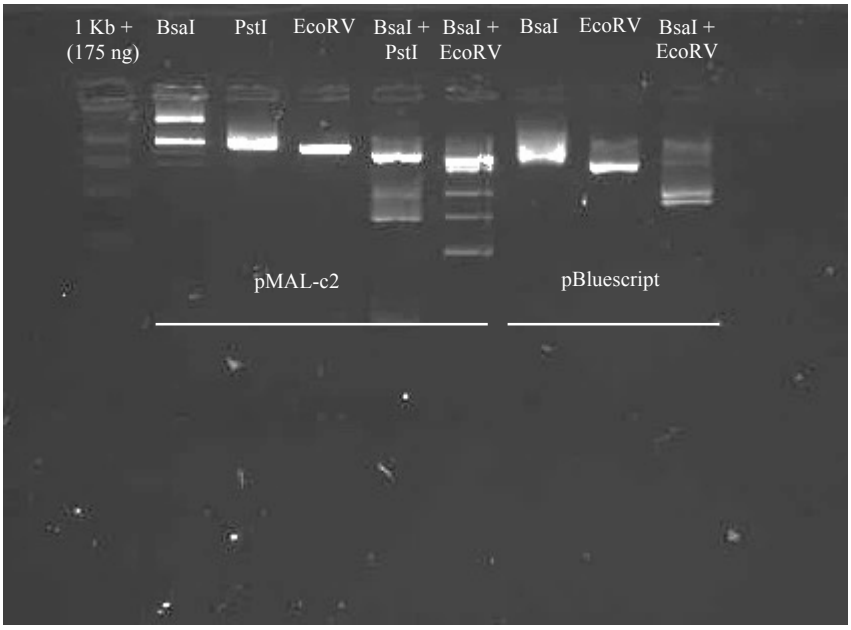

Figure 5 Restriction digest of pMAL-c2 and pBS to characterize plasmids.

3.4 Gel Purification of ISGA 1222 & 1218

Before subcloning into pBS, gel purification was performed due to the high number of non-specific PCR products. By selectively gel purifying the desired product from the non-specific PCR products, a concentrated sample of each target amplicon could be ligated into pBS without competition with unwanted DNA fragments in solution. The Qiagen purification protocol used relies on agarose solubilization followed by the selective absorption of the QiaxII silica beads to the nucleic acids in high salt concentrations provided by the QXI buffer (7M NaPO4, 10 mM NaAc).

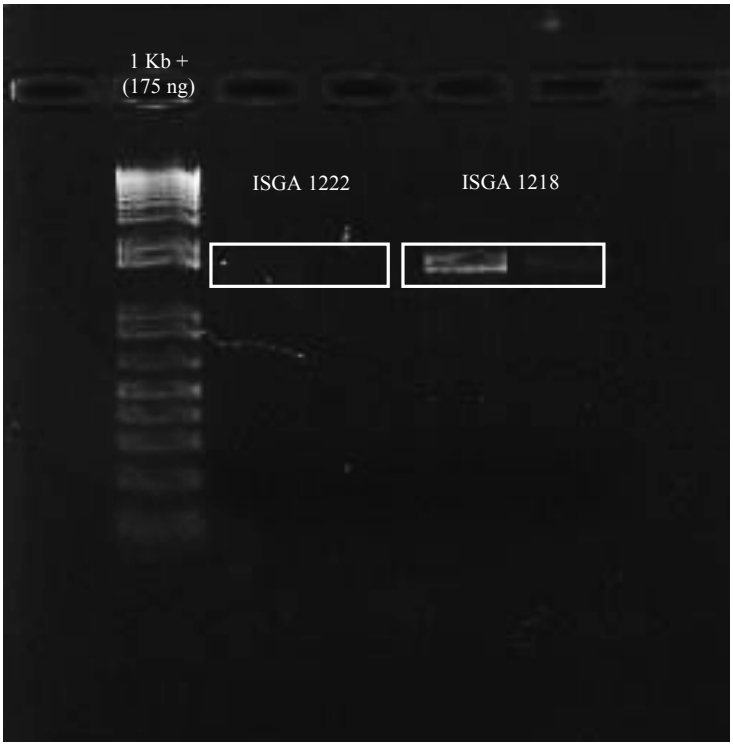

Figure 6 Gel purified ISGA 1218 and unsuccessful recovery of ISGA 1222.

Unfortunately, the ISGA 1222 amplicon was not successfully purified (Figure 6), which can likely be attributed to insufficient binding of the nucleic acids to the QIAEX II silica beads. If the nucleic acids did not bind to the silica beads, DNA would have been discarded in the supernatant. The ISGA 1218 amplicon was successfully recovered, although two bands were seen on the gel due to imprecise excision of the desired band before gel purification. If the procedure were to be repeated, a more precise excision of the band form the gel could have been achieved if the gel was run for an additional 20 minutes, allowing more time for the bands to separate from one another. Despite the presence of two bands, the sample was quantified (5 ng/μL) by comparison to the 1 Kb + DNA ladder and used in subsequent transformation experiments as the lower band, corresponding to ISGA 1218, was a higher concentration than the non-target amplicon.

3.5 Blunt-end ligation of ISGA 1218 into pBluescript

The gel purified ISGA 1218 amplicon was ligated into a pBS cloning vector with the intention of later sequencing the insert to compare to the published sequence data for ISGA 1218. The ISGA 1218 amplicon was ligated into a blunt end EcoRV digested pBS to ensure the insertion of the fragment would not cause a frameshift. The ligation products were then transformed into *E. coli* DH5α cells based on the high transformation efficiency of the strain (Taylor *et al*. 1993). Following incubation, five recombinant white colonies were observed demonstrating the successful transformation of the ISGA 1218 insert into pBS. With the identification of recombinant clones, it was possible to extract the plasmid DNA for further analysis to confirm the presence of the insert.

3.6 Release of ISGA 1218 fragment from pBluescript

Plasmid DNA of the ISGA 1218 + pBS transformants was isolated using a plasmid mini-prep DNA isolation, and was used to excise the ISGA 1218 fragment from the MCS of pBS. A blunt-end transformation of the fragment into pBS was used to allow for the EcoRI and XbaI restriction sites introduced into the fragment by PCR amplification to remain intact. By conserving their sequences, this would allow for restriction digest overhang at these sites, resulting in the sticky ends allowing for directional cloning into the pMAL-c2 expression vector. When the plasmid DNA was digested with EcoRI and XbaI (Figure 7), the plasmid was only found to linearize, and no release of the ISGA 1218 fragment was observed. This result suggested the restriction enzymes were only cutting at the sites present within the MCS of pBS, and not digesting the restriction sites introduced into the ISGA 1218 fragment.

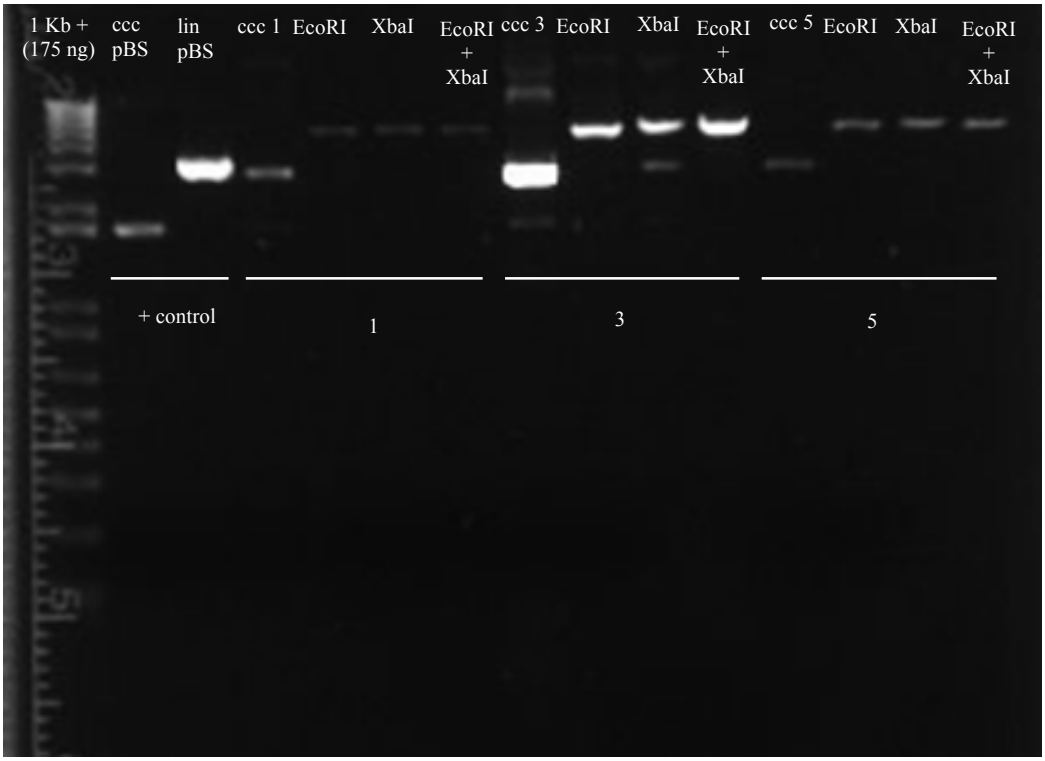

Figure 7 Attempted release of ISGA 1218 from MCS of pBS using EcoRI and XbaI single digests and a EcoR/XbaI double digest. Digests were performed on mini-prep DNA samples (1, 3 and 5). A positive control of EcoRV linearized pBS was used.

A second attempt to excise the ISGA 1218 insert from pBS was performed using EcoRI and XbaI single digests, separated by the removal of the restriction enzyme and their associated buffers. The first single digest was performed using EcoRI, which was observed to linearize the plasmid (Figure 7). Following the incubation of the digest, the restriction enzyme and associated buffers were removed from the linearized plasmid using the EZ-10 Plasmid DNA purification kit. The DNA was selectively isolated in the silica gel-based column, allowing for all of the unwanted products in the restriction digest solution to be washed away. Following the removal of the products, the DNA was precipitated using ethanol and eluted in TE buffer (pH 8.0), allowing for unhindered digestion of the linearized plasmid by XbaI.

Unfortunately, the digestion of the linearized pBS + ISGA 1218 plasmid did not result in the release of the insert; rather, no change in the nature of the plasmid was visualized (Figure 8).

Figure 8 Attempted excision of insert from pBS MCS by restriction digests using EcoRI and XbaI. A single digest of EcoRI was performed, followed the removal of the enzyme and its required buffers using the EZ-10 restriction enzyme removal kit. A second single digest using XbaI was performed on the recovered EcoRI digested DNA.

3.7 PCR amplification of insert within pBluescript

After being unable to release the insert from the MCS of pBS, a PCR amplification of the inserted DNA fragment was performed to ensure the single fragment ligated into pBS was a PCR product of the ISGA 1218 directional cloning primer pair. The PCR reaction containing pBS as the DNA template did not result in any amplification, whereas the gel purified ISGA 1218 fragment acting as the positive control produced two bands (Figure 9). This result was already observed after gel purification of the fragment, having the upper band corresponding to the desired 1473 bp ISGA 1218 fragment. The presence of two bands decreases the chance of the desired band being ligated into pBS. However, when examining

the bands amplified within pBS, the amplified target is the same size as the desired band, and did not contain additional non-specific PCR products. Although the insert was successfully amplified using ISGA 1218 specific primers, because the insert was unable to be removed from pBS, sequencing was not performed. Although the amplified ISGA 1218 sequence was gel purified based on the fragment size, transformation assays consist of the movement of a single molecule into a vector, such as pBS. As such, it is possible an undesirable, or mis-amplified PCR product was introduced into pBS, which resulted in the inability to excise the ligated fragment from the vector.

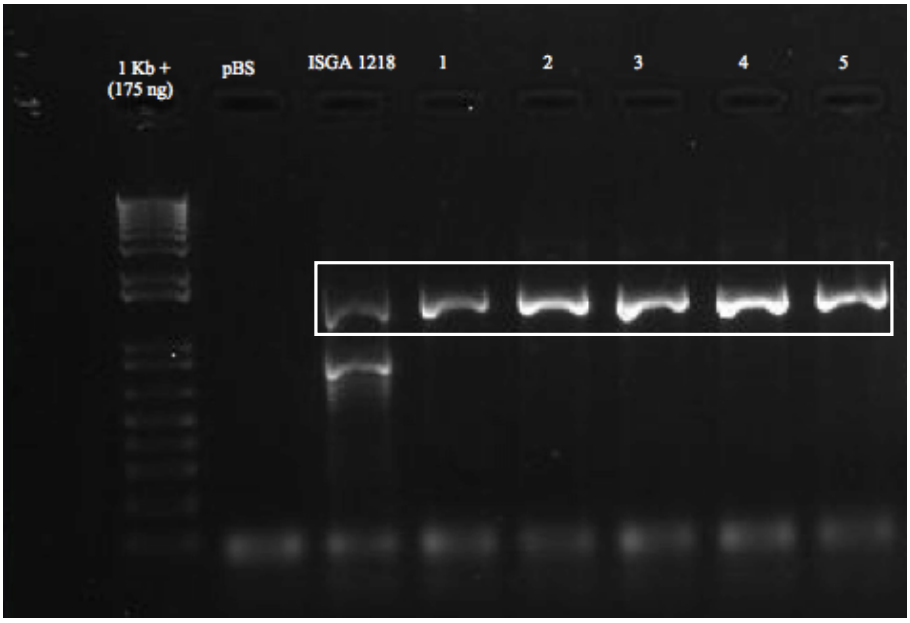

Figure 9 PCR amplification using ISGA 1218 specific primers on mini-prep DNA. Each lane labeled 1-5 contains 5 µL of an individual PCR reaction containing template DNA from pBS, the gel purified ISGA 1218 sample, and plasmid DNA from the five recombinant colonies (listed in order after 1 Kb+ ladder).

4 CONCLUSION AND FUTURE WORK

As the ISGA 1218 insert could not be excised from the MCS of pBS, in future studies a restriction map could be generated from the transformant clone using restriction enzymes whose sites are present in ISGA 1218 to determine if the target sequence was properly

amplified and successfully transformed into pBS. The inability to excise the insert from pBS suggests errors in the amplification of the target sequence. As the purpose of first cloning the insert into the pBS was to visualize the excision of the insert from pBS generating sticky ends to allow for directional cloning, sequencing was not performed on the generated clones, as the ligated insert is of limited use due to its inability to be excised.

For this project to continue, the PCR amplification of the nucleotide sequences coding for ISGA 1218 and 1222 would need to be repeated to generate fresh, amplified targets to be gel purified and used in a pBS transformation assay. Assuming the insertion of a target sequence that can be excised from pBS using the restriction enzyme sites introduced on the 5' and 3' end, the insert could be sequenced using 5' M13pUC-fwd and 3' T3 sequencing primers. The sequencing data obtained can be compared to the sequence data published (see Appendix C) to determine whether or not the sequences align. Any discrepancies in the sequences could result in the misfolding of the protein and inhibit further processing once ligated into the pMAL-c2 expression vector. If, however, the sequence data aligned, and the insert could be digested from pBS, the excised insert could be gel-purified and ligated into an EcoRI and XbaI digested pMAL-c2 expression vector to allow for directional, complementary ligation of the insert into the vector.

Similarly to pBS, the blue-white α-complementation test is also used in the pMAL-c2 expression vector system (New England BioLabs) to determine the presence of an insert within the MCS. In contrast to pBS, pMAL-c2 series also encodes for a maltose binding protein by the *malE* gene, upstream of the MCS to be attached to the 5′ end of the ligated insert. The deletion of the *malE* signal sequence in the –c2 variety of the plasmid results in cytoplasmic expression of the fusion protein, eliminating the possibility of being exported

into the periplasm of the cell. Between the *malE* gene and the MCS of the vector, a spacer sequence coding for 10 asparagine amino acid residues is present to insulate the maltose binding protein from the protein of interest once translated within the cell. Once inserted into the vector, and a white colony is recovered, the purification of the NtaA target proteins can be achieved by generating a large volume of biomass to be subjected to amylose affinity chromatography to selectively bind to the maltose binding protein + NtaA protein complex from all of the other proteins present within *E. coli*. Once all of the unwanted proteins are washed away from the column, the maltose binding protein + NtaA protein can be eluted through a maltose wash. The purification of the NtaA proteins from the maltose binding protein can then occur through proteolysis by a Factor-Xa protease, digesting at a cleavage site present between the *malE* gene and the MCS, releasing the maltose binding protein from the two NtaA proteins. From here subsequent assays determining the function of each NtaA enzyme can be determined, furthering our understanding of the metabolism of 6:2 FTS by *Gordonia* sp. NB4-1Y.

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6 APPENDIX

6.1 Appendix A

ISGA 1222

DNA sequence

EcoRI

5′ GAC GAA TTC ATG GCT GAT CGA GAG 3′ F1222EcoLS

5′ ATG GCT GAT CGA GAG CTC CAT CTG GGC GTC AAT GTC CTC TCG GAC GGT ATG CAC CCA GCC GCG TGG CAG TAT CCG AGT TCC GAT CCG TCG TGG TTC ACG GAT CCG GCG TAC TGG ATT CGT GTT GCG CAG ATC GCG GAG CGA GGA ACC CTC GAC GCG GTC TTC CTC GCC GAC AGT CCG TCG TTG TTC CAG CCG CCC GAC CAG CCG CTG AGT GCG CCA CCG TTG GCC CTG GAC CCG ATC GTG TTG TTG TCG ACA CTG GCA TCG GTG ACC ACA CAC ATC GGA CTC ATC GGT ACG GTG TCG ACC TCG TTC GAG GAG CCG TAC AAC GTC GCC CGC CGA TTC TCG ACG CTG GAC CAC CTC AGC CGG GGT CGT GTG GCA TGG AAC GTC GTG ACG AGT AGT GAT CGG TAT GCC TGG AAC AAT TTC GGT GGT GGT GAA CAA CCC GAC CGC GCT ACT CGA TAC GAG CGG GCC GGC GAG TTC ATC GAA GTC GTC CGG GCA TTG TGG GAT TCG TGG GAC GAC GAC GCA GTT GTC GCC GAC AAG TCC ACG GGT GCG TTC AGT AAG GTC GGT GCG ATA CGA CCG ATC CGG CAT CGC GGT GGG CAC TTC TCG GTG GAC GGG CCG TTG ACT CTA CCC AGA TCC CCA CAG GGG CAT CCG GTG TTG TTT CAG GCA GGC GGT TCC ACC GGC GGG TTG GAT CTG GCG GCG AAG TAC GCC GAC GGG GTC TTT GCG GCA CAG GCC TCG CTC GAG GAT GCG CTG TCC AAC GCG CAG GAG CTG CGG AGT CGG TTG ATC GCG CAT GGC CGT CCC

GCC GAG GCG ATC CGA ATC ATG CCT GGC TTG TCG TTC GTG CTC

GGC AGT ACG GAG GCA GAG GCC AGG TCG CGA AAC GAC GAA TTG

AAC GAG CTC GCC GGG GAT CGA CGC CTG GCA CAT CTG GCT GGT

CAA CTC AGC GTC GAT GTG GCG GAG CTG AAG TGG GAC AAG CCG

CTT CCC GGT TGG CTC CTC GAG GGC GCG GCG CCG ATC AGC GGT

TCC CAG GGA GCT CGC GAC ATC GTC GTC AAC ATC GCT CGG CGG

GAG AAC CTG ACC GTG CGT CAG CTG CTC GAT CGG GTG ATC ACG

TGG CAC CGC TTC GTG GTC GGA TCG CCT GAA CAG ATC GCC GAT

GCC ATC GAG GAC TGG TTC GTT GCG GGC GCT GTC GAC GGC TTC

AAC CTG ATG CCG GAT GTC TTC CCG TCG GGT CTC GAG TTG TTC

GTC GAC CAC GTC GTA CCG ATC CTC CGG GAC CGA GGG TTG TTC

CGG CGG GAG TAC ACA TCG ACG ACA TTG CGT GGG CAT CTG GGC

CTC GAG CGC ACC CCA GAC CGG CCG TCG TCG GGT TCG ATC

CGC CGG ACC GGT TAG 3′

5′ GCG GCC TGG CCA ATC AGA TCT TAG 3′ R1222XbaLS

XbaI

Amino acid sequence

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YERAGEFIEVVRALWDSWDDDAVVADKSTGAFSKVGAIRPIRHRGGHFSV 200
DGPLTLPRSPQGHPVLFQAGGSTGGLDLAAKYADGVFAAQASLEDALSNA 250
QELRSRLIAHGRPAEAIRIMPGLSFVLGSTEAEARSRNDELNELAGDRRL 300
AHLAGQLSVDVAELKWDKPLPGWLLEGAAPISGSQGARDIVVNIARRENL 350
TVRQLLDRVITWHRFVVGSPEQIADAIEDWFVAGAVDGFNLMPDVFPSGL 400
ELFVDHVVPILRDRGLFRREYTSTTLRGHLGLERTPDRPSSGSIRRTG

ISGA 1218

DNA sequence

EcoRI

5′ GTA GAA TTC ATG AAC GTA AAC GTT G 3′ F1218EcoLS

5′ ATG AAC GTA AAC GTT GTT GGC GGA ATC TCT

CAA TGG GAC ACT GTT CAG GCC GAT GCG TGG TGG TCG GCT GCA

GTC GGT CCA GCA ACG AAC GGA GAG ATC TCG ATG TCG GCA CAT

GGG CGT CCA ATT CAC TTG GGC GGG TTT TTG ATT GCA GGG AAT

GTA ACC CAC AGT CAT CCT TCG TGG CGT CAT CCA CGC AGT GAT

CCC GGG TTT CTC ACA CCG GAG TAC TAC CAG CAC CTC GGT AGG

GTT TTC GAA CGC GCG AAA TTG GAC TTC GTC TTC TTT GCC GAC

AAT TCT GCG ACT CCT GCC AGC TAC CGC AAC GAT ATT CGT GAC

CCG CTC GCT CGC GGT ACT CAG AGT GCA GCC GGG TTG GAT CCC

CGC TTC GTC GTT CCT GTC GTC GCG GGT GTC ACG CGC AAC CTG

GGG ATC GTA TCG ACC ACG TCG GCG ACG TTC TAC TCG CCA TAC

GAC CTC GCC CGG AGC TTT GCC ACT CTG GAT CAT CTG ACC CAC

GGC CGC GTC GGT TGG AAT GTT GTG ACT TCC AAT ACG ACC GTC

GAG GCG CAG AAC TTC GGG CTT GCC CGA CAC CTC GAC CAT GAC

GTG CGA TAC GAC CGT GCC GAG GAA TTG CTT GAG GTC GCG TTC

AGG CTG TGG GCC AGT TGG GAC GAT GGA GCT CTG ATC CAG GAT

AAA GAG GCG GGT GTC TTC GCC GAC CCG GAC CTG ATT CAC AGG

CTC GAT CAT CAC GGG GAG AAC TTC GAT GTT CGG GGC CCG CTG

TCG GTT CCC CGC TCA CCG CAG GGA CGG CCG GTC ATC TTT CAA

GCG GGA TCA TCC ACC CGC GGT CGG GAT TTT GCT GCG CGC TGG

GCA GAA GCG ATT TTC GAG ATC GAC CCG ACG TCT GTG GGG CGT

AAG GCC TAC TAC GAC GAC ATC AAG TCG CGA GCC TCC GAC TTC

GGT CGT GAT CCC GAC GGC GTC AAG ATA CTC CCG TCG TTC ATT

CCG TTT GTG GGT GAG ACC GAG TCG ATC GCA CGG GAA AAG CAG

GCG TTC CAC AAC GAA CTG GCC GAT CCG ACC GAT GGA TTG ATC

ACG CTG TCG GTG CAC ACC GAC CAT GAT TTC TCC GGC TAT GAC

CTC GAC GCT GTG ATC GCC GAC ATC GAT GTT CCA GGG ACG AAG

GGG CTT TTC GAA GTC GCT CGG AGT CTG AGT GTG AAC GAG AAC

CTG ACG CTG CGC GAT ATC GGA AAG CTG TAC GCC CAG GGC GTG

TTA TTG CCG CAG TTC GTG GGT ACC GCG GCT CAG GTG GCC GAC

CAG ATC GAG GCT GCC GTC GAC GGT GGA GAG GCT GAT GGG TTC

CTC TTT TCG GCC GGG TAT ACG CCT GGC GGA TTC GAG GAG TTC

GCC GAT CTC GTC ATC CCG GAA CTG CAG CGG CGA GGG CGG TTT

CGT ACG GAG TAC ACG GGT TCG ACG CTG CGT GAA CAT CTG GGT

CTA CCC GCT GAT GCG AAT CTT GTG CCC GTT CCG CGC AAG GCA

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XbaI

Amino acid sequence

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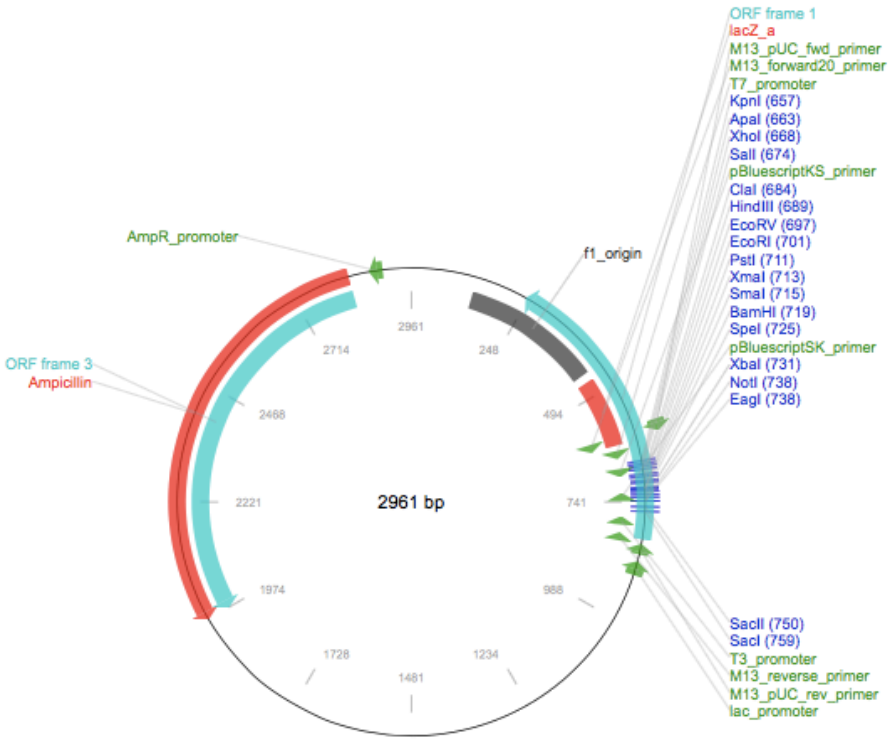
SYRNDIRDPLARGTQSAAGLDPRFVVPVVAGVTRNLGIVSTTSATFYSPY 150
DLARSFATLDHLTHGRVGWNVVTSNTTVEAQNFGLARHLDHDVRYDRAEE 200
LLEVAFRLWASWDDGALIQDKEAGVFADPDLIHRLDHHGENFDVRGPLSV 250
PRSPQGRPVIFQAGSSTRGRDFAARWAEAIFEIDPTSVGRKAYYDDIKSR 300
ASDFGRDPDGVKILPSFIPFVGETESIAREKQAFHNELADPTDGLITLSV 350
HTDHDFSGYDLDAVIADIDVPGTKGLFEVARSLSVNENLTLRDIGKLYAQ 400
GVLLPQFVGTAAQVADQIEAAVDGGEADGFLFSAGYTPGGFEEFADLVIP 450
ELQRRGRFRTEYTGSTLREHLGLPADANLVPVPRKAVGAA

6.2 Appendix B

YEME broth (100 mL)
0.3 g Difco Yeast extract
0.5 g Difco Pepto (bacto)
0.3 g Malt extract
1.0 g Glucose
34.0 g Sucrose
2 mL/L2.5 M $MgCl_2 \cdot 6\ H_2O$

6.3 Appendix C

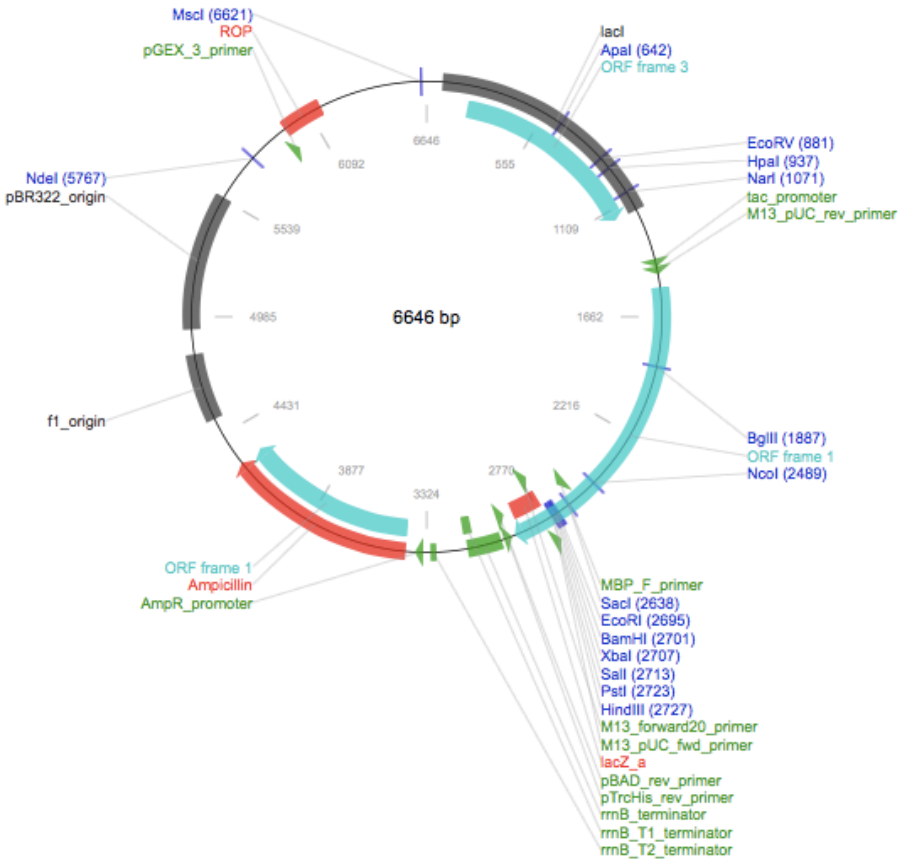
pBluescript



(Addgene)

6.4 Appendix D

pMAL-c2



(Addgene)

6.5 Appendix E

Before transforming the ISGA 1218 gel purified fragment into the EcoRV linearized pBluescript vector, the determination of the amount of ISGA 1218 required to achieve a 3:1 vector to insert ratio was required.

(1473 bp ISGA2 1218) / (3000 bp pBS) = 0.479 ng

0.479 ng * 3 = 1.437 ng ISGA 1218 / 1ng pBS * 100

= 147.3 ng ISGA 1218 required

Due to the poor recovery of the ISGA 1218 gel purified fragment, there was an insufficient amount of DNA to prepare a 3:1 vector to insert ratio. Rather, a 1:1 ratio was used.

(1473 bp ISGA2 1218) / (3000 bp pBS) = 0.479 ng

0.479 ng ISGA 1218 / 1ng pBS * 100

= 47.9 ng ISGA 1218 required

All calculations regarding the transformation efficiency of pBS were performed using the number of blue colonies present on each control reaction. On each control plate, white colonies were present surrounding the blue colonies due the diffusion of β-lactamases expressed from the *amp*^{R} gene within pBS allowing for the growth of competent cells not containing pBS, and the associated *amp*^{R} gene. As such, the white colonies were not included. To determine the fraction of pBS that could be transformed into each competent cell, the number of transformants in the 10 ng uncut pBS control was used. In the sample there was 10 ng of pBS in 25 μL ligation reaction. From this reaction volume, 2.5 μL (1 ng pBS) was transferred into 1 mL of liquid broth, of which 100 μL (0.1 ng) was spread onto ampicillin nutrient agar plates. To determine the number of molecules of pBS transferred, the weight of one molecule of pBS was determined to be 3×10^{-18} g/molecule.

pBluescript = 3000 bp

One base pair = 660 g/mol

3000 bp * 660 g/mol = 2×10^{6} g/molecule

2×10^{6} g/molecule / 6×10^{23} g/molecule = 3×10^{-18} g/molecule

Using the weight of one molecule of pBS, I was able to determine the number of molecules transferred in the 100 μL (0.1 ng) volume to be 3×10^7 molecules. From the number of blue colonies visualized on the 10 ng uncut pBS control, it was determined 1.98×10^{-5} molecules of pBS entered the cell.

1×10^{-10} g / 3×10^{-18} g/molecule = 3×10^7 molecules

The transformation efficiency of the DH5α cells was also calculated using the 10 ng uncut pBS control to be 5940 colonies/ng.

594 colonies / 0.1 ng pBluescript = 5940 colonies/ng

The effects of the ligation reaction on transformation efficiency was determined by using the 10 ng uncut pBS with the complete ligation reaction. To determine this value, the same calculation was used as for the transformation efficiency, using the average counted colonies from the 10 ng uncut pBS (ligation reaction) transformation. In the 100 μL transformation reaction, 316 colonies were counted, giving a transformation efficiency of 3160 colonies/ng. This data suggests that the addition of the ligation reaction does decrease transformation efficiency. The percent of decrease in efficiency was calculated.

(5940 – 3160) / 5940 = 0.468 * 100 = 47 %

In the 100 ng linearized pBS (no ligation reaction) control, the transformation efficiency theoretically should have been observed as 0, as without DNA ligase, the plasmid

would be unable to recircularize and would be transformed into E. coli DH5α as a linear plasmid, to be immediately degraded within the cell. However, 192 blue colonies were observed on this control, suggesting an incomplete EcoRV restriction digest of pBS prior to the ligation reaction. As 100 ng linearized pBS was added to a 25 μL, when 2.5 μL of the ligation reaction was transferred into 1 mL of liquid broth, 10 ng of linearized pBS was transferred. Of which, 1 ng of pBS was plated onto ampicillin containing nutrient agar, resulting in 192 colonies/ng. The efficiency of the digestion was calculated using the transformation efficiency of pBS.

(192 colonies/ng) / (5940 colonies/ng) = 0.032 * 100 = 3.2 %

Using this same calculation, the percent recircularized in the 100 ng linearized pBS (ligation reaction) control could be determined.

(464 colonies/ ng) / (5940 colonies/ng) = 0.078 * 100 = 7.8 % recircularized

The blue to white colony ratio for the 100 μL ISGA 1218 + pBS transformation was 68:1. To improve this ratio, a 3:1 vector to insert ratio should have been used. Due to our poor QiaxII recovery of the ISGA 1218 fragment, the transformations were prepared using a 1:1 vector to insert ratio.

Table 10 Transformation efficiency of control reactions and ISGA 1218 + pBS experimental reaction.

Transformation	DNA form	Blue (# of colonies)	White (# of colonies
10 ng pBS (- ligation reaction)	ccc	594	-
10 ng pBS (+ ligation reaction)	ccc	316	-
100 ng pBS (- ligase)	linear	192	-
100 ng pBS (+ ligase)	linear	464	-
ISGA 1218 + pBS	linear	683	5