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PCR-based Construction of CodY Gene Deletion Mutants from *Streptococcus pneumoniae*

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ABSTRACT

Pathogenic strains of *Streptococcus pneumoniae* can cause various pneumococcal diseases such as pneumonia and meningitis. The pathogenicity of *S. pneumoniae* is associated with virulence factors, such as the enzyme hyaluronate lyase that degrade hyaluronan to penetrate the host. One of the regulatory gene for producing this enzyme is CodY gene. CodY structure consists of dimers of metabolite-binding domain (MBD) and DNA-binding domain (DBD). This work is aimed to construct three different CodY deletion mutants of *S. pneumoniae* a full-length CodY deletion mutant, MBD-CodY deletion mutant, and DBD-CodY deletion mutant. Genomic DNA template was extracted from local clinical isolates of *S. pneumoniae*. Primers were designed by using the genomic sequence of *S. pneumoniae* strain TIGR4 retrieved from NCBI website. A single/partial gene-deletion method was chosen to create CodY mutant constructs containing aphA-3 gene (encoding kanamycin resistance protein) replacement cassette by employing recombinant fusion PCR method. Visualization using agarose gel electrophoresis indicates successful construction of all three mutants, with single band of PCR product at expected size. However, only the full-length CodY recombinant amplicon was at sufficient concentration for transformation experiments. The results unveiled no viable CodY mutant post-transformation. The negative transformation results are postulated to be due to the essential nature of CodY regulator. This work demonstrated the feasibility of performing single/partial CodY gene deletion mutants, which will complement the current study of CodY and its domains' regulatory effect on hyaluronate lyase in *S. pneumoniae*.

Key words: CodY, hyaluronate lyase,*Streptoccocus pneumoniae*, virulence, gene-deletion method

1. INTRODUCTION

Streptococcus pneumonia (*S. pneumoniae*) is a Gram-positive pathogen that can cause many diseases that contribute to significant mortality and morbidity rate like pneumonia, bacteraemia, meningitis, otitis media and sinusitis [1]. There are ongoing studies on the regulatory mechanism of invasion of host tissue and penetration of host defense [2]. One of the pneumococcal products that are involved in the invasion of host cell is hyaluronate lyase. Hyaluronan or hyaluronic acid (HA) is a glycosaminoglycan and a major component of extracellular matrix of vertebrate connective tissue. *S. pneumoniae* produces hyaluronate lyase that is capable to degrade HA into disaccharide units [3]. By degrading the extracellular matrix, hyaluronate lyase is proposed to assist in the invasion of *S. pneumoniae* into host tissue and increase the level of tissue permeability [2]. Besides that, *S. pneumoniae* scavenges potential carbon sources at epithelial cells that may also contribute to its virulence and survival in the host [2].

CodY protein has been found in the low G+C Gram-positive bacteria and is significant in determining its pathogenicity in regulating expression of virulence genes[4]. In *Staphylococcus aureus*, CodY structure is shown to be predominantly made up of a metabolite-binding domain (MBD) at its N-terminal end and a DNA-binding domain (DBD) at its C-terminal end. The two domains are joined together by a long helical linker [5]. Han et al [5] has proposed a model of CodY activation based on their solved crystal structure, whereby CodY is prohibited from binding to DNA when nutrient availability is low (inactive state). Consequently, genes normally repressed by CodY are activated. On the other hand, in a nutrient-rich environment, GTP metabolites binds to MBD, and the DBD are positioned to increase its DNA-binding ability. In the presence of branched chain amino acids, DBD of CodY is increased while GTP does not [3]. Thus, CodY is activated and consequently regulates cellular metabolism (active state) [5].

Not much is known however, about the role of CodY in regulation of *S. pneumoniae* hyaluronate lyase (SpnHyl) gene [6]. Pneumococcal CodY gene was discovered to comprise of primarily genes that are responsible in amino acid metabolism carbon metabolism and iron uptake [3]. It is imperative to investigate the function of CodY regulatory genes for SpnHyl in *S. pneumoniae* using molecular methods. To achieve that aim, we attempt to apply fusion PCR amplification technique to produce a linear recombinant PCR amplicon containing partial deletion of CodY-MBD gene from local clinical isolates of *S. pneumoniae*.

2. MATERIALS AND METHODS

This research aimed to construct *S. pneumoniae*CodY mutants by employing a gene deletion construct (in the form of a linear recombinant PCR amplicon) followed by transformation into *S. pneumoniae* of local clinical isolate. The transformation protocol employed competence stimulating peptide (CSP-1) to induce natural competence of the pneumococcus.

2.1 Genomic DNA Sample

Glycerol stocks of *S. pneumoniae* strains from local clinical isolate NP830/94 with recorded positive criteria of invasive capability, presence of capsule (encapsulated) and hyaluronate lyase production was chosen as study sample for the whole research work [7]. Bacterial samples were cultured on the blood agar plates in period of 16-18 hours in condition of 5% CO_2 at 37°C. Fresh colonies were used as inoculum in BHI broth and incubated for 18 hours at 37°C in 5% CO_2. The genomic DNA was extracted and purified using Vivantis' GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies Sdn. Bhd.). The extracted genomic DNA was then visualised in 1.0% agarose gel electrophoresis. The genomic DNA sample's quality was checked and quantified via Nanodrop 2000c (Thermo Fisher Scientific) machine.

2.2Kanamycin Gene Requirement

Gene aphA-3 encoding the kanamycin protein was acquired from *Escherichia coli* (*E. coli*) pSF-OXB14 (OG557) plasmid (Oxford Genetics Ltd). To ensure ready and sufficient amounts of kanamycin gene available for the whole research works, the plasmid DNA were transformed into *E. coli* Subcloning Efficiency DH5αTM electrocompetent cells (Life Technologies, USA) for propagation. The plasmid DNA was then subsequently extracted and purified from the transformed cells using AxygenAxyPrep Plasmid Miniprep Spin Kit (Corning Inc.). AphA-3 gene was then acquired by performing PCR by using the purified plasmid as template.

2.3Primer Design

The primer sets designed for the gene deletion construct procedures were performed based on published gene-deletion techniques [8], [9], which utilise the technique for genome-wide essential gene identification in *Streptococcus sanguinis*. Based on the complete *S. pneumoniae* strain TIGR4 genomic DNA sequence from National Center for Biotechnology Information (NCBI) (Accession number: AE005672, https://www.ncbi.nlm.nih.gov/nuccore/AE005672), three primer sets were designed for each mutant construct and purchased in sterile lyophilised forms from Integrated DNA Technologies (IDT). The compatibility of each primer pairs was analysed using OligoAnalyzer software (http://sg.idtdna.com/calc/analyzer). CodY gene length is 789 bp, the N-terminal MBD range is from 1488816bp (amino acid 3 to 182), the C-terminal DBD range is from 1489320 bp (amino acid 202 to 259).

F1/R1 and F3/R3 primer sets were primarily designed via NCBI Primer-BLAST online software. Manual design was then performed to add 20-25bp adaptor sequences at primer R1 and F3's 5' end that are complementary to the aphA-3 gene. The adaptor sequence at R1 and F3 overlapped with F2 and R2 primers sequence respectively. F1 and R3 were then adjusted by adding sequences based on genome template to ensure melting temperature of all primers to have no difference more than 5°C and GC content not exceeding 55%. GC content for all primers was designed to be low as possible was to ensure feasible priming process.

In order to perform gene-deletion of the full-length CodY gene, F1/R1 and F3/R3 primer sets were designed to amplify approximately 1 kb upstream and 1 kb downstream respectively of the target gene, CodY (Table 1). Length of flanking sequences were limited to approximately 1 kb for both F1/R1 and F3/R3 to ensure the total length amplified from the final recombinant PCR employing F1/R3 primers was not more than 3 kb sequence; 795 bp for aphA-3 gene and maximum 2 kb for both flanking sequences. F2/R2 primer set was designed with slight modification from universal primers amplifying aphA-3 gene encoding kanamycin resistance protein (Km^r) (Table 1). For CodY-MBD mutant, primer set F3/R3 was designed to consider the C-terminal DBD region of CodY as sequence downstream of target gene, since the objective is to delete only the MBD region of CodY gene (Table 1). Likewise, primer set of F1/R1 CodY-DBD mutant was designed to consider the N-terminal MBD region of CodY as sequence upstream of target gene, since the objective was to delete only DBD region of CodY gene (CodY-DBD mutant) (Table 1). Primer sequences were listed in Supplementary Table 1.

Table 1: Primers used in this study

Primer Set	Primer	Length (bp)	GC (%)	T_m (°C)	Amplicon Length
CodY					
F1/R1	F1	45	44.4	65.1	796bp
	R1	45	40	65	
F2/R2	F2	27	40.7	59.5	795bp
	R2	27	44.4	59.2	
F3/R3	F3	45	42.2	66	972bp
	R3	44	47.7	67.4	
CodY-MBD					
F1/R1	F1	Same as CodY F1/R1 primer set			796bp
	R1				
F2/R2	F2	Same as CodY F2/R2 primer set			795bp
	R2				
F3/R3	F3	48	50	68.8	620bp

	R3	45	44.4	66.4	
CodY-DBD					
F1/R1	F1	34	47.1	62.6	762bp
	R1	44	34.1	62.3	
F2/R2	F2	Same as CodY F2/R2 primer set			795bp
	R2				
F3/R3	F3	45	40.0	65.1	944bp
	R3	27	48.1	62.5	

2.4Polymerase Chain Reaction (PCR)

Linear recombinant DNA i.e. gene deletion construct was generally assembled by employing four polymerase chain reactions (PCR). The first three PCR reactions were performed by using normal Taq polymerase (Promega Gotaq® Green Master Mix, Table 2 and Table 3) while the last PCR reaction i.e. recombinant PCR combining all first 3 amplicons to make up recombinant amplicon was performed by using high fidelity polymerase (Clontech Advantage®-GC 2 Kit, Takara Bio Company, Table 4 and Table 5). All four PCR reactions was optimised by first employing gradient PCR and after the optimum temperature was determined for each respective reaction, standard PCR reaction was performed to obtain sufficient concentration of purified PCR amplicons from each reaction to be used as templates for the final fusion PCR reaction. Before purification of amplicons, all PCR reaction results were first visualised and confirmed via agarose gel electrophoresis. Presence of multiple unintended amplicons (visualised as one or more unintended bands) was solved by employing band-stab PCR technique [10]. This technique was implemented to ensure amplification of only one targeted amplicon species. One negative control was also prepared accordingly. The band-stab PCR mix was then re-amplified using the same previous PCR parameters (Table 4) but with less cycles (20 cycles). Using less cycle numbers was justified to avoid presence of unintended amplicons due to over-amplification. All PCR amplicons from in this work was purified by using AxygenAxyPrep PCR Clean-up Kit (Corning Inc.).

Table 2:PCR parameters for Promega GoTaq® Green Master Mix Reaction

Step	Temperature (°C)	Time (minutes)
1. Initial Denaturation	95	2
2. Denaturation*	95	1
3. Annealing*	XX*	1
4. Extension*	72	1
5. Final Extension	72	2
6. Hold	4	

*Step 2 to 4 were repeated for 30 cycles. Annealing temperatures vary according to chosen temperatures in gradient PCR

Table 3:PCR Reaction Mix for Promega Gotaq® Green Master Mix

PCR Component	Initial Conc.	Final Conc.	1 reaction (µL)
Promega Gotaq® Green Master Mix	2X	1X	12.5
Forward Primer	10 µM	1 µM	2.5
Reverse Primer	10 µM	1 µM	2.5
DNA Template	100 ng/µL	10 ng/uL	2
Sterile Distilled Water	-	-	5.5
Total			25

Table 4:PCR parameters for Clontech Advantage®-GC 2 Kit

Step	Temperature (°C)	Time (minutes)
1. Initial Denaturation	94	3
2. Denaturation*	94	30sec
3. Annealing*	XX*	10sec
4. Extension*	68	2
5. Final Extension	68	3
6. Hold	4	

* Step 2 to 4 were repeated for 30 cycles. Annealing temperatures vary according to chosen temperatures in gradient PCR

Table 5:PCR Reaction Mix for Clontech Advantage®-GC 2 Kit

PCR Component	Initial Conc.	Final Conc.	1 reaction (µL)
GC 2 PCR Buffer	5X	1X	5
GC Melt	5M	0.5M	2.5
Forward Primer, F1	10 µM	0.2 µM	0.5
Reverse Primer, R3	10 µM	0.2 µM	0.5
dNTP Mix	50X	1X	0.5
Advantage® -GC 2 Polymerase	50X	0.5X	0.25
DNA Template			
1) F1R1	10	0.5 ng/uL	1.25

	ng/µL		
2) F2R2	10 ng/µL	0.5 ng/uL	1.25
3) F3R3	10 ng/µL	0.5 ng/uL	1.25
PCR-grade Water	-	-	12
Total			25

2.5Streptococcal cell transformation

Approximately 4 µl of 140 ng of Competence Stimulating Peptide-1, CSP-1 (AnaSpec, Inc.) and 4 ul of linear recombinant DNA (approximately 80 ng) was pipetted to a sterile 1.5 mL microfuge tube. The transformation mixture of CSP-1 and recombinant DNA was then pre-warmed at 37°C. 330 µl of *S. pneumoniae* study sample grown to OD660 of 0.07-0.08 was added into the tube containing transformation mixture, mixed and then incubated at 37°C in 5% CO_2 for 1 hour. Negative control was set by replacing recombinant DNA with distilled water in the transformation tube. After 1-hour incubation, the transformation tube was placed in ice and 100 µl of transformants was spread on BHI agar containing 500 µg/mL kanamycin. The agar plates were incubated at 37°C in 5% CO_2 for 2 days.

3. RESULTS AND DISCUSSIONS

This research's long-term aims are to utilize the mutant construct for future studies to determine the essentiality of CodY gene in regulating of hyaluronate lyase production and its pathogenic pathway mechanism.

3.1Genomic DNA extraction and purification

Genomic DNA of study sample (*S. pneumoniae* isolate NP830/94) was successfully extracted and purified for use as DNA template for two PCR reactions (F1/R1 and F3/R3 PCR amplification, Figure 1). Two duplicate samples were chosen as working genomic DNA samples for the whole research works, with A_{260}/A_{280}ratio of 2.16 and 2.15 and DNA concentration of 1812.3 ng/µL and 1450.9 ng/ µL respectively. Both duplicate genomic DNA samples were later diluted into working stocks of concentration 100 ng/µL.

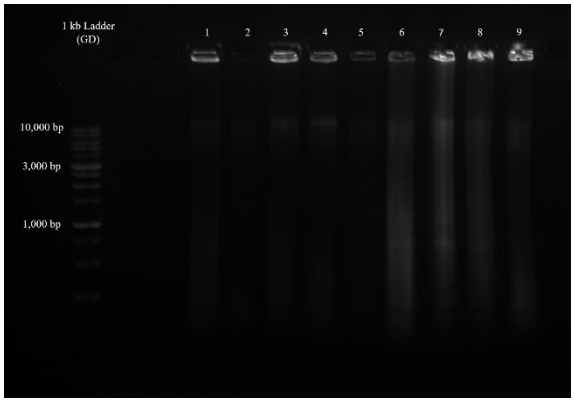


Figure 1: Gel image of genomic DNA extracted from S. pneumoniae sample NP830/94. Well 1 to 9 indicate genomic DNA extracted from bacteria cultured from the same glycerol stock but extracted using separate purification columns (sample 1-9)

3.2Plasmid DNA (pSF-OXB14) extraction and purification

Plasmid DNA (pSF-OXB14) extracted and purified from the transformed DH5α *E. coli* cells yielded 119.57 ng/µL of DNA with A_{260}/A_{280} ratio of 1.62. aphA-3 gene encoding kanamycin resistance isolated and amplified from this plasmid DNA template would be used as template for F2/R2 PCR amplification.

3.3CodY mutant construct

PCR Amplification

PCR amplification for F1/R1 amplicon (~1kb upstream of CodY), F2/R2 amplicon (aphA-3 gene) and F3/R3 amplicon (~1kb downstream of CodY) for CodY mutant construct were successfully amplified (Table 6, Figure 2-4) with sufficient quality.

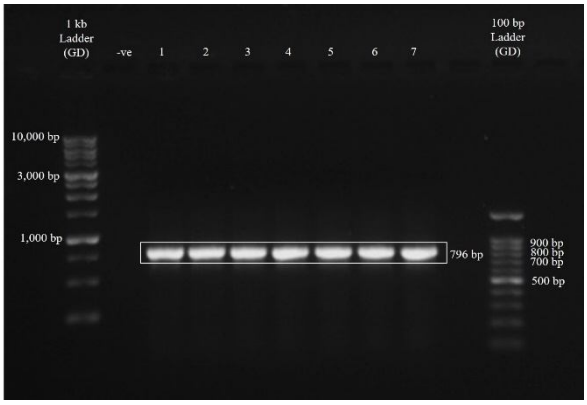


Figure 2: Gel image for CodY F1/R1 PCR amplification reaction. Well 1-7 indicate reactions employing the optimum temperature, 60.5°C. DNA ladders used were 1 kb DNA ladder (Genedirex) and 100 bp DNA ladder (Genedirex)

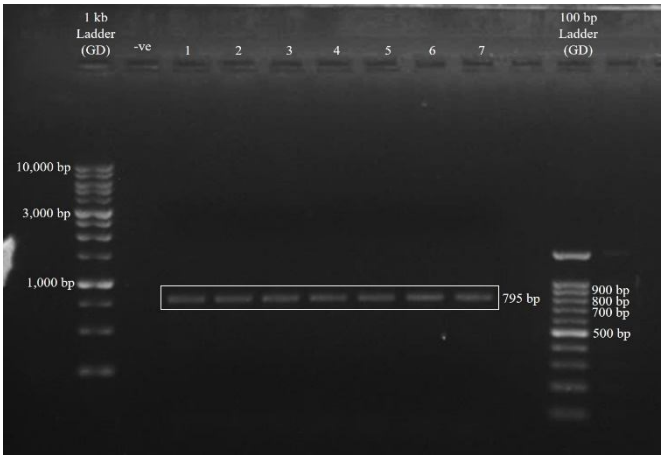


Figure 3: Gel image for CodY F2/R2 PCR amplification reaction. Well 1-7 indicate reactions employing the optimum temperature, 52.5°C. DNA ladders used were 1 kb DNA ladder (Genedirex) and 100 bp DNA ladder (Genedirex)

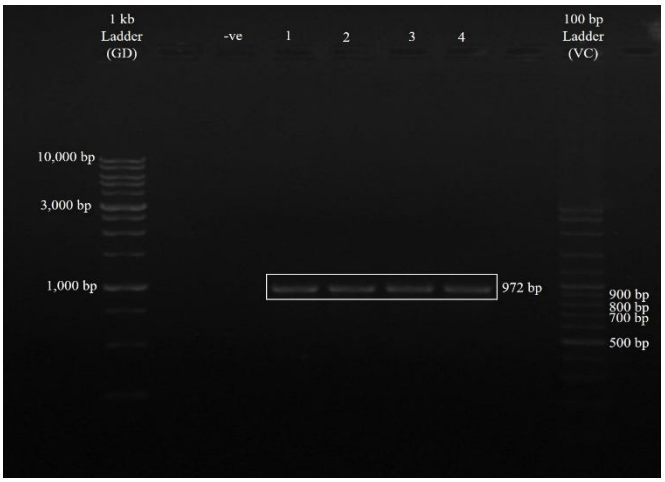


Figure 4: Gel image for CodY F3/R3 PCR amplification reaction. Well 1-4 indicate reactions employing the optimum temperature, 52.5°C. DNA ladders used were 1 kb DNA ladder (Genedirex) and 100 bp DNA ladder (Vivantis)

Table 6: Nanodrop Readings for Purified PCR Products for CodY Mutant Construct

Amplicon	DNA Concentration (ng/µL)	A260/A280	A260/A230
F1/R1	76.1	1.85	1.35
F2/R2	40.0	2.85	0.60
F3/R3	56.2	1.77	0.25

Recombinant Fusion PCR Amplification

This PCR amplification employed the combination of primer F1/R3 and simultaneously used three purified amplicons species previously amplified from three PCR amplifications; F1/R1, F2/R2 and F3/R3 respectively as DNA template. The objective of the recombinant fusion PCR (hereby will be referred as recombinant PCR) was to combine the three DNA amplicons to construct linear recombinant amplicon (linear recombinant DNA) consisting of apha-3 gene (amplicon F2/R2) flanked by amplicon F1/R1 and amplicon F3/R3 respectively. The expected size of the resultant recombinant amplicon was the sum of sizes of all three amplicons used as DNA templates i.e. 2,563 bp. This gene deletion construct was assembled to delete CodY gene from the genomic DNA in streptococcal transformation reaction. Multiple attempts were made to optimize production of single band product from the fusion PCR, but other band species were still amplified (Figure 5). This problem was later solved by employing band-stab PCR technique discussed in the following section.

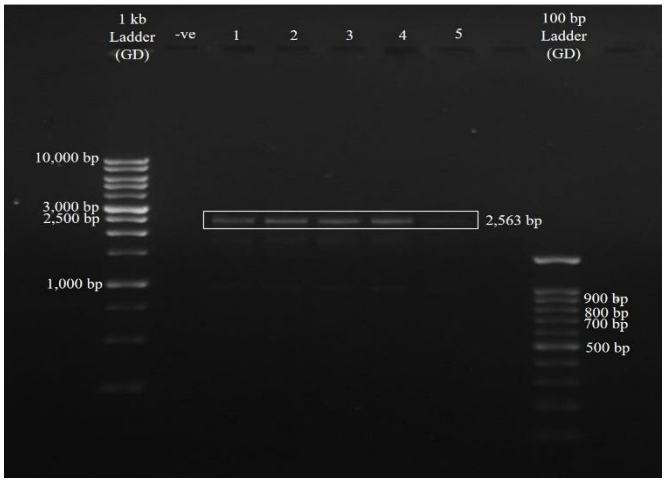


Figure 5: Gel image for CodY Recombinant Fusion PCR amplification reaction. Well 1-5 indicate reactions employing optimum temperature 60.8°C. Negative control was run at the first well. DNA ladders used were 1 kb DNA ladder (Genedirex) and 100 bp DNA ladder (Genedirex)

Band-stab PCR

Band-stab PCR technique was employed to solve the problems of unintended amplicon species presence (unintended multiple bands) after previous PCR troubleshooting failed to yield only one targeted amplicon. Band-stab PCR re-amplified only one amplicon obtained directly from the agarose gel after separating it from other unintended amplicons via agarose gel electrophoresis. The single recombinant amplicon (Figure 6) resulted from the band-stand PCR was immediately purified with yield of 18.6 ng/µL from a 25 µL PCR reaction and A_{260}/A_{280} ratio of 1.6.

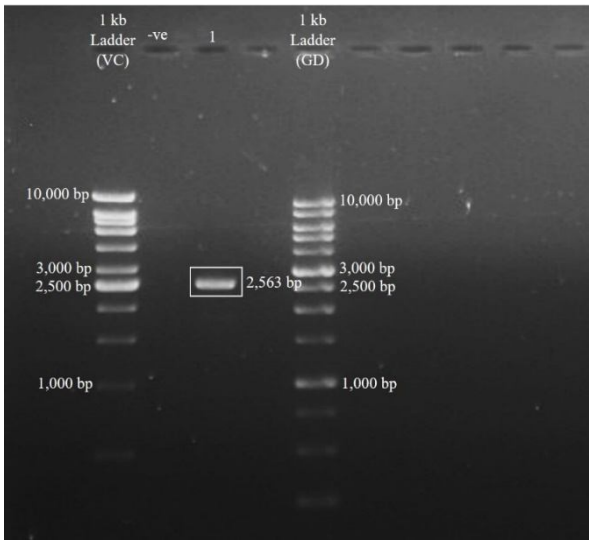


Figure 6: Gel image for band-stab PCR result. Negative control was run at the first well. DNA ladders used were 1 kb DNA ladder (Genedirex) and 1kb DNA ladder (Vivantis)

3.4CodY-MBD mutant construct

PCR Amplification

PCR amplification for F1/R1 amplicon (~1kb upstream of CodY), F2/R2 amplicon (aphA-3 gene) for CodY mutant construct were successfully amplified (Figure 7 and Figure 8). There were multiple band products observed for F3/R3 PCR reaction, and after repeated attempts to optimize this, band-stab PCR was utilized to obtain a single band for F3/R3 PCR amplicon (~1kb downstream of CodY, Figure 9).

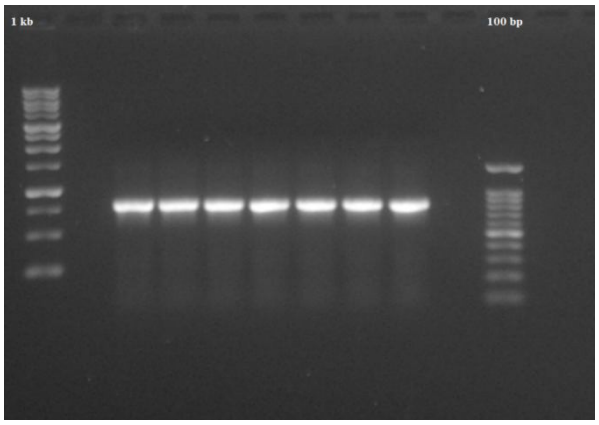


Figure 7: Gel image for CodY-MBD F1/R1 PCR amplification reaction. Lane 1-7 represent PCR replicates samples run at annealing temperature of 60.5°C. 1 kb: 1 kb DNA ladder, 100bp: 100bp DNA ladder. (Expected PCR product size = 796 bp)

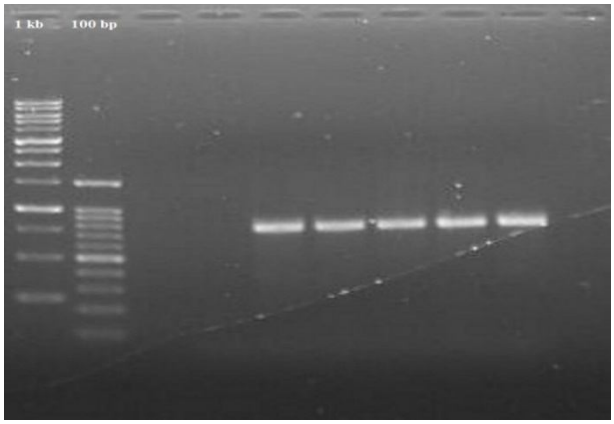


Figure 8: Gel image for CodY-MBD F2/R2 PCR amplification reaction. Lane 1-5 represent PCR replicates samples run at annealing temperature of 52.5°C. 1 kb: 1 kb DNA ladder, 100bp: 100bp DNA ladder. (Expected PCR product size = 795 bp)

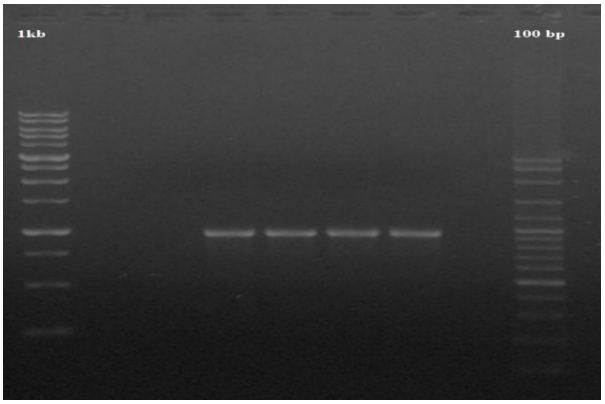


Figure 9: Gel image for CodY-MBD F3/R3 PCR amplification reaction after optimization using band-stab PCR. Lane 1-4 represent PCR replicates samples run at annealing temperature of 62.2°C. 1 kb: 1 kb DNA ladder, 100bp: 100bp DNA ladder. (Expected PCR product size = 620 bp)

Recombinant Fusion PCR Amplification

In fusion PCR, a linear recombinant PCR amplicon will be amplified by 'fusing' the three PCR amplicons from the three PCR amplification reactions (standard gene-specific PCR reactions using F1/R1, F2/R2 and F3/R3 primer sets respectively) in one PCR reaction. The fusion PCR reaction will utilize forward primer F1 and reverse primer R3 for the amplification of the linear recombinant PCR amplicon. This fusion PCR reaction helps to 'fuse' or assemble the upstream region of CodY with the kanamycin resistance cassette replacing the CodY-MBD region, and followed by fusing of the C-terminal of the kanamycin resistance cassette to the downstream region of CodY (including the DBD region of CodY). This will produce a CodY-MBD mutant with a KanR gene replacing the MBD region of the CodY gene. Figure 10 shows that PCR amplification of F1/R3 was successful as single band was formed near expected size of PCR product which is 2210 bp. The gradient fusion PCR reaction was performed using 50 µL reaction (10 µL for each tested

temperatures). After PCR purification, the average concentration is 72.9 ng/µL and the A_{260}/A_{280} ratio of 1.75.

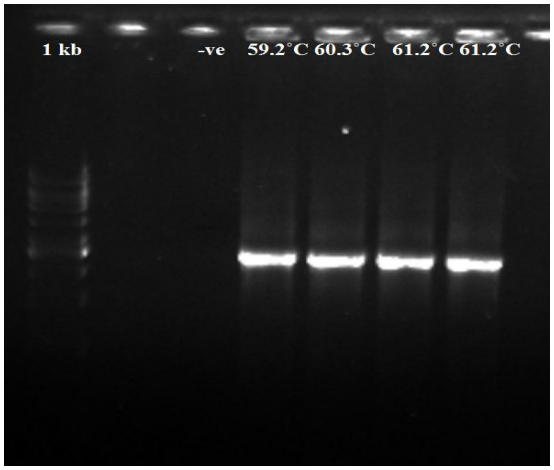


Figure 10: Gel image for CodY-MBD fusion PCR. Wells are labeled with each annealing temperature tested in the gradient fusion PCR. –ve represents as negative control. 1 kb: 1 kb DNA ladder (Expected PCR product size = 2210 bp)

3.5CodY-DBD mutant construct

PCR Amplification

Similar to CodY-MBD mutant construct, single amplicon was observed from F1/R1 and F2/R2 PCR amplification (Figure 11 and Figure 12). Multiple bands were observed for F3/R3 PCR amplification reaction (Figure 13). After multiple attempts of optimization, the other band species was still present (Figure 14). Table 7 indicates the concentration and quality of each amplicons.

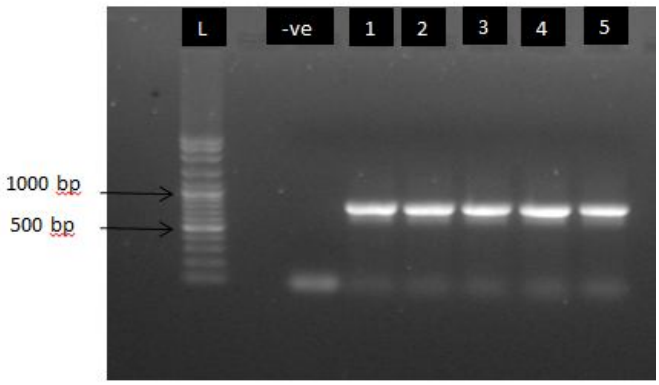


Figure 11: Gel image for CodY-DBD F1/R1 PCR amplification reaction. An intense band was present at ~762bp. Well 1-5 represents PCR replicates. L refers to VC 1kb ladder (Vivantis) while –ve is the negative control

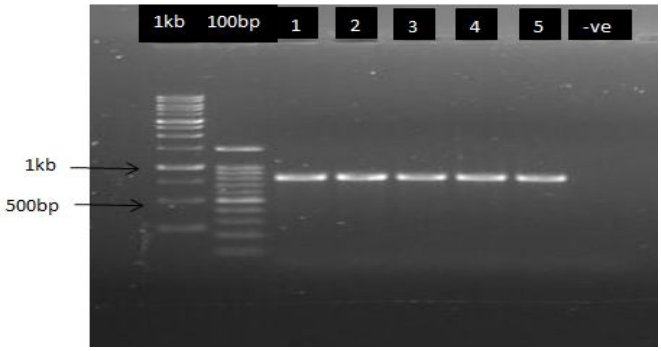


Figure 12: Gel image for CodY-DBD F2/R2 PCR amplification reaction. An intense band was present at ~795bp. Well 1-5 represents PCR replicates. L refers to VC 1kb ladder (Vivantis) while –ve is the negative control. 1kb and 100bp refers to VC 1kb and 100bp ladder (Vivantis) respectively while –ve is the negative control

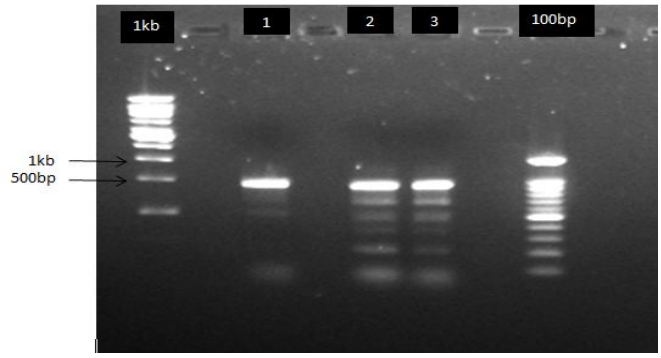


Figure 13: Gel image for CodY-DBD F3/R3 PCR amplification reaction. An intense band was present at ~944bp. Well 1-3 represents PCR replicates. 1kb and 100bp refers to VC 1kb and 100bp ladder (Vivantis) respectively while –ve is the negative control

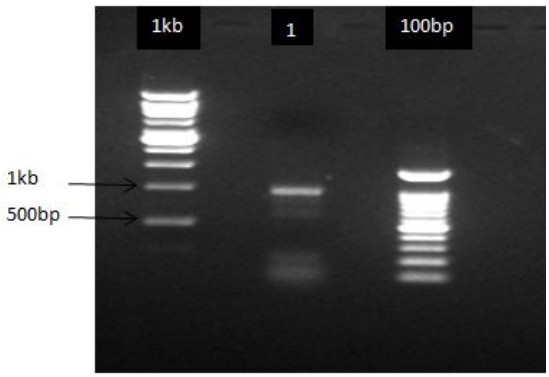


Figure 14: Gel image for CodY-DBD F3/R3 PCR amplification reaction. The presence of multiple bands can be observed under the desired expected band (~944bp band size). 1kb and 100bp refers to VC 1kb and 100bp ladder (Vivantis) respectively. There is only one well used as this was the fourth trial to eliminate the multiple bands after gel purification

Table 7: Nanodrop Readings for Purified PCR Products for CodY-DBD Mutant Construct

Amplicon	DNA Concentration (ng/µL)	A_{260}/A_{280}
F1/R1	115.47	1.84
F2/R2	72.2	1.76
F3/R3	57.5	1.63

Recombinant Fusion PCR Amplification

The fusion PCR reaction will utilize forward primer F1 and reverse primer R3 for the amplification of the linear recombinant PCR amplicon. This fusion PCR reaction helps to 'fuse' or assemble the upstream region of CodY (including the MBD region of CodY) with the kanamycin resistance cassette replacing the CodY-DBD region, followed by fusing of the kanamycin resistance cassette to the downstream region of CodY. This will produce a CodY-DBD mutant with a KanR gene replacing the DBD region of the targeted gene.

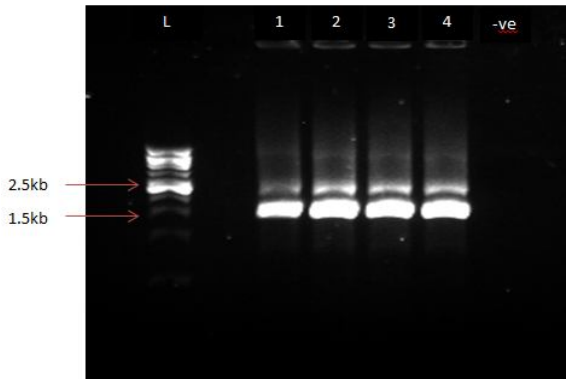


Figure 15: Gel image for CodY-DBD Fusion PCR amplification reaction (expected PCR amplicon size ~2.5kb). Well 1-4 represent PCR replicates sample. L indicates VC 1kb ladder while –ve is negative control.

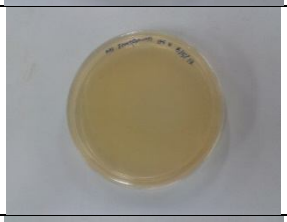
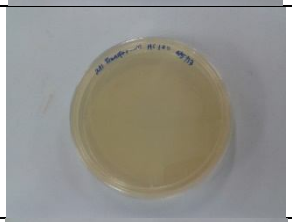
From Figure 15 above, the fusion PCR can be considered a success as there was a mutant PCR product of expected size at 2.5kb indicating that the mutant recombinant gene has been amplified. However, there was another smaller size but more intense band present at 1.5kb. The 1.5kb product may be partially amplified product from any of the DNA template used for the reaction (as the three gene-specific PCR amplicons were combined for the fusion PCR reaction). Unfortunately, due to time constraint, we could not attempt to optimize the PCR condition or components to try to get only 2.5kb band product. The purification of both 1.5kb and 2.5kb products also could not be conducted as well. Thus, the fusion PCR reaction could not be repeated to further amplify the 2.5kb DNA product and purified at sufficient concentration to be sent for sequencing reaction to confirm our findings.

3.6 Streptococcal cell transformation

Construction of CodY deficient mutants was the primary objective of this research work as it is essential to form basis study for future works studying the overall regulatory property of CodY gene, especially its regulatory function over the gene encoding hyaluronate lyase enzyme, Hyl. If CodY mutants are successfully established and cultured, the quantitative difference of Hyl expression between wild type and CodY deficient mutant can thus be studied.

Construction of CodY mutant in this research work was attempted by transforming the previously assembled recombinant PCR amplicon as single-gene deletion construct into competent *S. pneumoniae* study sample. The gene deletion construct aimed to delete CodY gene from the genomic DNA of the study sample by replacing it with apha-3 gene which confers resistance to kanamycin antibiotic. However, the transformation protocols yielded negative results as no kanamycin resistant mutant (CodY mutants) colony evident in all BHI-kanamycin selective plates. Three transformation attempts (repeats) were performed and all attempts resulted in the absence of kanamycin-resistant mutant on the selective plates (Table 8).

Table 8: Transformation Results

Transformant Plate	Transformant Plate Pictures	Observation
Plate 1		No colony
Plate 2		No colony
Plate 3		No colony
Negative Control		No colony

The results of third and final transformation attempt are shown in Table 8. Negative control was set to ensure the wild type of *S. pneumoniae* study sample was not initially contaminated with kanamycin resistance gene. All three transformation plates showed no presence of kanamycin-resistant mutant (no successful transformants). The discussion on the methods employed and the results are presented in the following paragraphs.

Transformation of streptococcal cells in this work employed the streptococcal CSP-1 transformation system. This transformation system made use of natural capability of *S. pneumoniae* to uptake exogenous DNA when in competent state. Streptococcal competent state depends on the bacteria stage of growth and its local population density detected by *S. pneumoniae* quorum-sensing ability. This is the justification on why the recombinant amplicon was introduced into *S. pneumoniae* culture during growth stage that corresponds to OD660 of 0.07-0.08. The streptococcal growth stage (local population) at OD660 of 0.07-0.08 can be referred as streptococcal 'window of time' for exogenous DNA uptake. During that window, cascade of genes expression that leads to the production of natural competence stimulating peptide (CSP) which ultimately results in the entry of exogenous DNA into the streptococcal genome. This natural competence state can be enhanced by exogenously adding exogenous competence stimulating peptide (CSP-1) to ensure competence state.

In term of transformation efficiency, the flanking sequences (in this work F1/R1 and F3/R3 sequences) play major role as the homologous recombination recognition sequences. In homologous recombination process, the flanking sequences serve as tool for genome-wide site recognition in the double cross-over recombination reaction. In gene deletion construct, they serve as deletion site recognition. Previous recent work on the study of transformation efficiency of *Streptococcus mitis* (*S. mitis*) conducted by Salvadori et al. [11], revealed that the larger flanking sequences can provide higher successful transformation rate rendering the streptococcal transformation process more efficient. In this current work, the sizes of such flanking sequences i.e. F1/R1 and F3/R3 flanking amplicons were 796 bp and 972 bp respectively. In the work mentioned here [11], the flanking sequences of size 1,5000 bp increased the transformation efficiency by 300% compared to 500 bp and size 3,500 bp accomplished 600% transformation rate compared to 1,000 bp flanking sequences. Thus, the size of flanking sequence can be further optimised particularly for *S. pneumoniae* study sample to form foundational work for any transformation work in the future.

Absence of apha-3 gene promoter in the gene deletion construct design might be the factor for no expression of kanamycin resistance protein thus the absence of kanamycin resistant mutant on the selective plates. The design of the recombinant amplicon in this work only comprised of the flanking sequences (F1/R1 and F3/R3) and the gene apha-3 (F2/R2). The worker excluded the promoter in the recombinant amplicon construct design to avoid polar transcriptional effects on native bacterial genes as mentioned in the reference work [12]. Polar transcriptional effects are defined as the disruptive effects of foreign (introduced) promoter on the adjacent or downstream native genes [12]. For the study of gene's functional differences between mutant and its wild type, as the aim of this work i.e. the study of Hyl gene expression in CodY mutant, it was preferable to eliminate any potential disruptive effects. However, given the negative transformation results, the problem of poor expression of apha-3 gene can be addressed in future works by including apha-3 promoter within gene deletion construct design.

Other than transformation efficiency and transformation construct design factor, the absence of kanamycin-resistant mutant can also be justified with the property of the CodY gene itself. After three attempts of transformation with no viable transformant, this work can justifiably conclude that CodY is an essential gene of *S. pneumoniae* study sample. Essential gene is defined as important gene that the absence of such gene is lethal to an organism deficient of it [9]. Thus, it is postulated that CodY is an essential gene for *S. pneumoniae* study sample. From this light of reasoning, this work can assume that if there was successful transformation and gene deletion in the first place, the zero survival of mutants in the selective plates was due to the lethality of CodY gene absence instead of due to the factors inherent to the transformation process. Study on the essentiality of CodY gene in *S. pneumoniae* of various strains have been done by Caymaris et al. [6]. Caymaris' team attempted to inactivate CodY gene via minitransposon insertion mutagenesis and resulted in failure to inactivate CodY in all of samples attempted. After genomic-wide analysis of *S. pneumoniae* strain D39, they also reported that CodY cannot be inactivated without the presence of ectopic (in abnormal place) copy of complementing CodY gene. Thus, drawing conclusion from the previous work and this current work, it is postulated that CodY's essentiality may be universal across variety of *S. pneumoniae* strains, including this study sample strain.

4. CONCLUSION

This study supports the use of PCR as a significant approach in the study of bacterial genetics. The genomic DNA of *S. pneumoniae* was successfully extracted and purified in large amount. The plasmid DNA (pSF-OXB14) which contain kanamycin resistance gene (apha-3) was also successfully propagated, extracted and purified. Both of DNA samples can be used as DNA study templates for future studies particularly relating to *S. pneumoniae* and its transformation. All PCR amplification protocols with all three designed primer sets gave the expected positive results. This work also proved that gene deletion construct (recombinant amplicon) can constructed effectively by totally employing PCR methods. PCR is indeed versatile tool with effective analytic, manipulative and constructive functions.

The seemingly negative results of no viable CodY mutants which construction was the primary objective of this work, may further prove the essentiality of CodY gene in *S. pneumoniae* study sample. This work thus postulates that CodY gene is essential for the study sample and thus other strategies are needed to construct CodY mutants and study its regulatory relationship with enzyme hyaluronate lyase. More independent transformation attempts with various optimisations i.e. flanking sequences size optimisation, media conditions optimisation and deletion construct design optimisation should be implemented in future works to ultimately construct *S. pneumoniae* CodY deficient mutant.

In overall conclusion, further study is needed to confirm which factors discussed above conclusively affect the transformation process of *S. pneumoniae* study sample involving CodY gene and whether CodY is indeed its essential gene as postulated.

APPENDIX

Supplementary Table 1: Details of primers used in this study

Primer Set	Primer	Sequence (5'-3')	Primer Length (bp)	GC (%)	T_m (°C)	Amplicon Length (bp)
CodY						
F1/R1	Forward Primer F1	CCA CTA CAG TTG ACA AAG AGC CTA TTT TCG CTG ATT CTC CAC TAC	45	44.4	65.1	796
	Reverse Primer R1	TGG AGC ACC TGC TGT TAG TTT TGA ATG ATT GAA CAA GAT GGA TTG	45	40	65	
F2/R2	Forward Primer F2	ATG ATT GAA CAA GAT GGA TTG CAC GCA	27	40.7	59.5	795
	Reverse Primer R2	TCA GAA GAA CTC GTC AAG AAG GCG ATA	27	44.4	59.2	
F3/R3	Forward Primer F3	TCG CCT TCT TGA CGA GTT CTT CTG ATT TCT ATT GAC AAG TTG CCT	45	42.2	66	972
	Reverse Primer R3	GTG GAT GAA TTG ACT CGT GGT TTG AAA ATT CGT GGC TTC CGT GC	44	47.7	67.4	
CodY-MBD						
F1/R1	Forward Primer F1	CCA CTA CAG TTG ACA AAG AGC CTA TTT TCG CTG ATT CTC CAC TAC	45	44.4	65.1	796
	Reverse Primer R1	TGG AGC ACC TGC TGT TAG TTT TGA ATG ATT GAA CAA GAT GGA TTG	45	40	65	
F2/R2	Forward Primer F2	ATG ATT GAA CAA GAT GGA TTG CAC GCA	27	40.7	59.5	795
	Reverse Primer R2	TCA GAA GAA CTC GTC AAG AAG GCG ATA	27	44.4	59.2	
F3/R3	Forward Primer F3	TCG CCT TCT TGA CGA GTT CTT CTG A + ATG + GCGTTGTAAGGGAGCTCAT C	48	50	68.8	620
	Reverse Primer R3	GCG AGC ATC CTT ACC AAA TTT CTC AAA GTT GGC ACG AAT GGT TTC	45	44.4	66.4	
CodY-DBD						
F1/R1	Forward Primer F1	TCT ACG CTC TCT CAA ACG AAT ATC AGG TCA GTC C	34	47.1	62.6	762
	Reverse Primer R1	TAT CAT TAC GCC AAA TAT GAA TGA TTG AAC AAG ATG GAT TGC A	44	34.1	62.3	
F2/R2	Forward Primer F2	ATG ATT GAA CAA GAT GGA TTG CAC GCA	27	40.7	59.5	794
	Reverse Primer R2	TCA GAA GAA CTC GTC AAG AAG GCG ATA	27	44.4	59.2	
F3/R3	Forward Primer F3	TAT CGC CTT CTT GAC GAG TTC TTC TGA ATG TTT TCA CCT CGA ATT	45	40.0	65.1	944
	Reverse Primer R3	AGC GCG TGG TTT GGA TAT TTC AGG TGT	27	48.1	62.5	

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