

CHAPTER 4

CONSTRUCTION OF SINGLE-GENE KNOCKOUT MUTANT STRAINS OF

Burkholderia xenovorans LB400 AND *Burkholderia cenocepacia* J2315

4.0 Introduction

Sigma-54 (encoded by the *rpoN* gene) that involves in many ancillary activities require an additional factor known as Enhanced Binding Protein (EBP) to activate the promoter and subsequently initiates the transcription process by *rpoN* gene. In order to determine the involvement of *rpoN* gene in degradation of xenobiotic compounds and other related activity, *rpoN* gene have to be inactivated. This chapter described the protocol used to knock out and deactivate the *rpoN* gene functions in selected *Burkholderia* species. The establishment of *rpoN* mutants was conducted using pKNOCK suicide vector via homologous recombination. In addition, several related genes such as the *ntrC* gene which involve in nitrogen assimilation have also been knocked out to determine its implication towards nitrogen utilisation in *Burkholderia* species.

In order to inactivate the function of the *rpoN* genes, the information regarding the sequence are needed. To begin with, the *rpoN* gene sequences were obtained from GenBank and subsequently the sequence analysis was conducted.

4.1 Background study and sequence analysis of *rpoN* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315.

The sequences of the *Burkholderia xenovorans* LB400 and the *Burkholderia cenocepacia* J2315 *rpoN* genes were obtained respectively from the GenBank.

In order to identify putative *rpoN* genes in all *Burkholderia* species, whose genomes have been completely sequenced, BLAST search analyses of their translated genomes were performed. Most bacterial species only have a single copy of the *rpoN* gene in their genome but some, especially soil and rhizospheric bacteria including several of the *Burkholderia* species examined, possess two homologs of the *rpoN* genes with potentially different roles in gene regulation (Kullik *et al.* 1991, Poggio *et al.* 2006). For instance *B. cenocepacia* AU1054, *B. cepacia* AMMD, *B. vietnamensis* G4 and *B. phytofirmans* PsJN have two copies of the *rpoN* genes while *B. pseudomallei*, *B. mallei* and *B. thailandensis* E264 only have one copy of *rpoN* gene. The investigation with *Bradyrhizobium japonicum*, which possesses two homologs of the *rpoN* genes of different sizes show that these copies are highly similar and they are functionally interchangeable even though the larger homolog is thought to fulfil the dominant role as an alternative sigma factor (Kullik *et al.* 1991) and *Burkholderia* species such as *B. cepacia* AMMD appear to show the similar characteristic.

The *rpoN1* gene (locus tag: Bxe_A4122) of *Burkholderia xenovorans* LB400 located at chromosome 1 spanning 1518bp and encoding a protein of 505 amino acids while *rpoN2* gene (locus tag: Bxe_B1172) of *Burkholderia xenovorans* LB400 located at chromosome 2 with size

of 1494bp and encoding a protein of 497 amino acids. With the size different of only 24bp, *rpoN1* and *rpoN2* genes of wildtype *Burkholderia xenovorans* LB400 might have their own function where each sigma factor is activated only by its cognate activator protein (Poggio *et al* 2006). Furthermore, pairwise sequence comparison of *rpoN1* and *rpoN2* genes of *B. xenovorans* showed a degree of nucleotide sequences identity and similarity of approximately 58.2% which does not seem to be the results of recent events of duplication and might not functionally interchangeable. Recently it was suggested that some residues in the RpoN box interact with the -24 promoter sequence (Burrows *et al.*, 2003; Doucleff *et al.*, 2005). The difference found in amino acid of the RpoN Box in *rpoN1* and *rpoN2* genes of *Burkholderia* species might be the location where the interaction with -24 promoters sequence occurred.

A putative *rpoN* gene for *Burkholderia cenocepacia* J2315 was found located at its chromosome 1 spanning 1506bp and encoding a protein of 501 amino acids (FJ02783) which was 92.8% homologous with *B. cepacia* AMMD *rpoN2* gene. From the sequence analysis, the *B. cenocepacia* J2315 *rpoN* gene is monocistronic and also identical in sequence to the *B. cenocepacia* K56-2 *rpoN* gene (Saldias *et al.* 2008).

4.1.1 BLAST analysis

Sequence analysis of *rpoN* gene of *Burkholderia* species was commenced with BLAST analysis for nucleotide sequences of *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 individually against GenBank database to obtain the closely related sequence for *rpoN1* and *rpoN2* genes, respectively (Figure 4.1). BLAST or Basic Local Alignment Search Tools able to find the similar sequences with the given reference sequence in GenBank using local alignment.

Based on E-value, the BLAST of *rpoN1* gene shows 19 closely related *rpoN* genes which include *rpoN* gene from *Burkholderia cenocepacia* J2315 while *rpoN2* gene of *Burkholderia xenovorans* LB400 shows 6 closely related *rpoN* sequences.

Sequences producing significant alignments:						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000270.1	Burkholderia xenovorans LB400 chromosome 1, complete sequence	2804	2804	100%	0.0	100%
CP001052.1	Burkholderia phytofirmans PsJN chromosome 1, complete sequence	2276	2276	99%	0.0	94%
CP002519.1	Burkholderia sp. CCGE1001 chromosome 1, complete sequence	1943	1943	99%	0.0	90%
CP002217.1	Burkholderia sp. CCGE1003 chromosome 1, complete sequence	1905	1905	99%	0.0	89%
CP002013.1	Burkholderia sp. CCGE1002 chromosome 1, complete sequence	1855	1855	99%	0.0	89%
CP001043.1	Burkholderia phymatum STM815 chromosome 1, complete sequence	1500	1500	99%	0.0	85%
CP000086.1	Burkholderia thailandensis E264 chromosome I, complete sequence	1391	1391	99%	0.0	84%
CP001503.2	Burkholderia glumae BGR1 chromosome 1, complete sequence	1384	1384	99%	0.0	84%
CP000958.1	Burkholderia cenocepacia MCO-3 chromosome 1, complete sequence	1334	1334	99%	0.0	83%
AP009385.1	Burkholderia multivorans ATCC 17616 DNA, complete genome, chromo	1328	1328	99%	0.0	83%
CP000458.1	Burkholderia cenocepacia HI2424 chromosome 1, complete sequence	1297	1297	99%	0.0	83%
CP000378.1	Burkholderia cenocepacia AU 1054 chromosome 1, complete sequence	1297	1297	99%	0.0	83%
CP002599.1	Burkholderia gladioli BSR3 chromosome 1, complete sequence	1295	1295	99%	0.0	83%
F025783.1	Burkholderia cenocepacia strain J2315 sigma factor RpoN (rpoN) gene	1295	1295	99%	0.0	83%
CP003087.1	Burkholderia sp. YI23 chromosome 1, complete sequence	1279	1279	99%	0.0	82%
CP000614.1	Burkholderia vietnamiensis G4 chromosome 1, complete sequence	1262	1262	99%	0.0	82%
CP000151.1	Burkholderia sp. 383 chromosome 1, complete sequence	1245	1245	99%	0.0	82%
CP000440.1	Burkholderia ambifaria AMMD chromosome 1, complete sequence	1195	1195	99%	0.0	81%
CP001025.1	Burkholderia ambifaria MC40-6 chromosome 1, complete sequence	1168	1168	99%	0.0	81%
FR602359.1	Burkholderia rhizoxinica HK1-454, complete genome	1130	1130	100%	0.0	81%
FP885897.1	Ralstonia solanacearum CFBP2957 chromosome complete genome	538	538	67%	5e-149	77%
CU914168.1	Ralstonia solanacearum strain IPO1609 Genome Draft	534	534	73%	6e-148	76%
CU861906.1	Ralstonia solanacearum strain MolK2 Genome Draft	534	534	73%	6e-148	76%
CP002812.1	Ralstonia solanacearum Po82, complete genome	529	529	66%	3e-146	77%
FR854088.1	Ralstonia syzygii R24, genomic contig 00003-1628	518	518	74%	6e-143	76%

Sequences producing significant alignments:						
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP000271.1	Burkholderia xenovorans LB400 chromosome 2, complete sequence	2760	2760	100%	0.0	100%
CP001053.1	Burkholderia phytofirmans PsJN chromosome 2, complete sequence	1831	1831	99%	0.0	89%
CP002520.1	Burkholderia sp. CCGE1001 chromosome 2, complete sequence	1724	1724	100%	0.0	88%
CP002218.1	Burkholderia sp. CCGE1003 chromosome 2, complete sequence	1624	1624	100%	0.0	86%
CP002014.1	Burkholderia sp. CCGE1002 chromosome 2, complete sequence	1509	1509	100%	0.0	85%
CP001044.1	Burkholderia phymatum STM815 chromosome 2, complete sequence	1157	1157	76%	0.0	85%
CP003087.1	Burkholderia sp. YI23 chromosome 1, complete sequence	915	915	71%	0.0	83%
AP009387.1	Burkholderia multivorans ATCC 17616 genomic DNA, complete genome	516	516	70%	2e-142	77%
CP000870.1	Burkholderia multivorans ATCC 17616 chromosome 3, complete sequen	516	516	70%	2e-142	77%
CP000260.1	Burkholderia cenocepacia MCO-3 chromosome 3, complete sequence	483	483	57%	2e-132	78%
CP000150.1	Burkholderia sp. 383 chromosome 3, complete sequence	483	483	70%	2e-132	76%
CP000460.1	Burkholderia cenocepacia HI2424 chromosome 3, complete sequence	477	477	70%	1e-130	76%
CP000378.1	Burkholderia cenocepacia AU 1054 chromosome 1, complete sequence	477	477	70%	1e-130	76%
CP000616.1	Burkholderia vietnamiensis G4 chromosome 3, complete sequence	466	466	57%	2e-127	77%
CP002599.1	Burkholderia gladioli BSR3 chromosome 1, complete sequence	453	453	53%	2e-123	78%
CP000442.1	Burkholderia ambifaria AMMD chromosome 3, complete sequence	442	442	56%	4e-120	77%
FP885907.1	Ralstonia solanacearum str. CFBP2957 plasmid RCFBPv3_mp, complete	405	405	38%	5e-109	80%
CP002820.1	Ralstonia solanacearum Po82 megaplasmid, complete sequence	383	383	38%	2e-102	79%
AL646053.1	Ralstonia solanacearum GMI1000 megaplasmid complete sequence	379	379	36%	3e-101	80%
FR854083.1	blood disease bacterium R229, genomic contig 00007-1627	377	377	38%	1e-100	79%
FP885891.2	Ralstonia solanacearum PS107 megaplasmid mpPS107, complete sequen	377	377	38%	1e-100	79%
FR854092.1	Ralstonia syzygii R24, genomic contig 00003-1629	372	372	38%	5e-99	79%
CU914166.1	Ralstonia solanacearum strain IPO1609 Genome Draft	368	368	34%	6e-98	80%
FP885896.1	Ralstonia solanacearum str. CMR15 plasmid CMR15_mp, complete gen	366	366	38%	2e-97	79%

Figure 4.1: BLAST analysis for *rpoN1* gene (above) and *rpoN2* gene (below) of *Burkholderia xenovorans* LB400

4.1.2 Multiple sequence alignment (MSA)

Multiple sequence alignment conducted for the amino acid sequences of *rpoN* genes from closely related *Burkholderia* species, exhibited the highly conserved region within the sequences. This highly conserved sequence might be sequence/ signature sequence motif that have specific function in that particular gene. It was observed that *rpoN1* and *rpoN2* genes have a region called as RpoN box with 10 highly conserved amino acids (ARRTVAKYRE). However from the analysis, the RpoN boxes for *rpoN2* genes have a different amino acid at the end of the sequence (ARRTVAKYRH) (Figure 4.2). From the previous research by Poggio and co workers (2006), it was suggested that certain amino acid in RpoN box are able to recognise the -24 sequence of the specific promoter for DNA binding and initiate the transcription process. The difference in *rpoN1* and *rpoN2* genes might indicates the different function played by both genes.

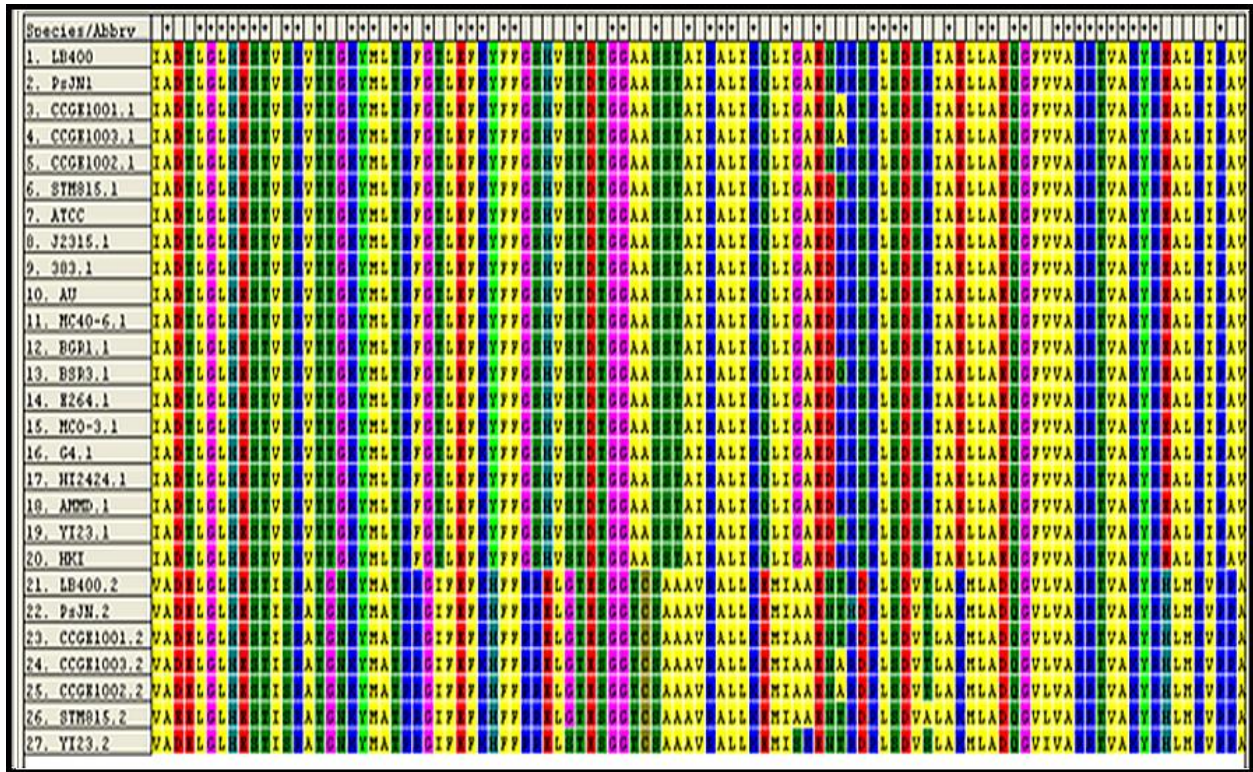


Figure 4.2: Multiple sequence alignment of the amino acids sequences of *rpoN1* and *rpoN2* genes of closely related *Burkholderia* species using CLUSTALX2.

4.1.3 Distance matrix of *rpoN1* and *rpoN2* genes of *Burkholderia* species

The distance matrix between *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 and *rpoN* gene of *Burkholderia cenocepacia* J2315 were also calculated using their nucleotide sequences. The results show that *rpoN1* gene of *Burkholderia xenovorans* LB400 is closely related with *rpoN* of *Burkholderia cenocepacia* J2315 with distance matrix of 0.08. By contrast, the distance matrix between *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 is high with value of 0.64 (Figure 4.3).


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[ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 ]
[ 1]
[ 2] 0.00
[ 3] 0.01 0.01
[ 4] 0.02 0.02 0.02
[ 5] 0.05 0.05 0.05 0.05
[ 6] 0.08 0.08 0.08 0.07 0.09
[ 7] 0.08 0.08 0.08 0.08 0.09 0.01
[ 8] 0.08 0.08 0.07 0.08 0.09 0.09 0.09
[ 9] 0.08 0.08 0.08 0.08 0.09 0.11 0.11 0.09
[10] 0.13 0.13 0.13 0.13 0.14 0.15 0.15 0.14 0.12
[11] 0.15 0.15 0.14 0.14 0.15 0.17 0.17 0.16 0.15 0.13
[12] 0.17 0.17 0.17 0.16 0.17 0.18 0.18 0.18 0.17 0.15 0.11
[13] 0.20 0.20 0.20 0.20 0.21 0.22 0.22 0.20 0.19 0.19 0.20 0.21
[14] 0.20 0.20 0.20 0.20 0.21 0.22 0.22 0.20 0.19 0.19 0.20 0.21 0.00
[15] 0.20 0.20 0.20 0.20 0.21 0.22 0.22 0.21 0.19 0.19 0.19 0.21 0.06 0.06
[16] 0.20 0.20 0.20 0.20 0.22 0.22 0.22 0.21 0.20 0.20 0.20 0.22 0.11 0.11 0.11
[17] 0.20 0.20 0.20 0.20 0.22 0.22 0.22 0.20 0.20 0.20 0.19 0.21 0.11 0.11 0.11 0.13
[18] 0.19 0.19 0.20 0.19 0.20 0.21 0.22 0.20 0.20 0.20 0.19 0.22 0.15 0.15 0.17 0.17 0.17
[19] 0.23 0.23 0.23 0.23 0.24 0.25 0.25 0.22 0.21 0.22 0.23 0.24 0.21 0.21 0.20 0.22 0.21 0.20
[20] 0.23 0.23 0.24 0.24 0.25 0.26 0.25 0.25 0.25 0.23 0.25 0.27 0.24 0.24 0.23 0.25 0.23 0.25 0.28
[21] 0.64 0.64 0.64 0.64 0.66 0.66 0.67 0.63 0.63 0.65 0.65 0.66 0.67 0.67 0.69 0.68 0.68 0.69 0.69 0.70
[22] 0.65 0.65 0.65 0.66 0.67 0.66 0.67 0.63 0.65 0.64 0.64 0.65 0.65 0.65 0.67 0.66 0.65 0.66 0.69 0.68 0.11
[23] 0.64 0.64 0.64 0.63 0.66 0.65 0.66 0.63 0.63 0.65 0.62 0.63 0.65 0.65 0.64 0.63 0.66 0.66 0.68 0.68 0.13 0.14
[24] 0.63 0.63 0.63 0.63 0.64 0.64 0.65 0.61 0.62 0.63 0.61 0.64 0.65 0.65 0.65 0.65 0.65 0.67 0.68 0.68 0.14 0.14 0.09
[25] 0.61 0.61 0.61 0.62 0.63 0.63 0.63 0.62 0.62 0.61 0.61 0.64 0.65 0.65 0.65 0.66 0.66 0.67 0.68 0.69 0.15 0.15 0.17 0.18
[26] 0.65 0.65 0.65 0.65 0.66 0.64 0.65 0.64 0.65 0.65 0.66 0.65 0.70 0.70 0.71 0.69 0.69 0.69 0.75 0.72 0.22 0.25 0.23 0.23 0.25
[27] 0.64 0.64 0.64 0.64 0.64 0.65 0.66 0.62 0.63 0.66 0.66 0.66 0.69 0.69 0.69 0.68 0.69 0.70 0.71 0.70 0.27 0.29 0.28 0.30 0.29 0.28

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Fig. 4.3: Distance matrix of *rpoN* genes of *Burkholderia* species using MEGA5.05.

- | | |
|---|---|
| 1) <i>rpoN1 B. xenovorans</i> LB400 | 2) <i>rpoN1 B. phytofirmans</i> PsJN |
| 3) <i>rpoN1 Burkholderia</i> sp CCGE1001 | 4) <i>rpoN1 Burkholderia</i> sp CCGE1003 |
| 5) <i>rpoN1 Burkholderia</i> sp CCGE1002 | 6) <i>rpoN1 B. phymatum</i> STM815 |
| 7) <i>rpoN1 B. multivorans</i> ATCC17616 | 8) <i>rpoN1 B. cenocepacia</i> J2315 |
| 9) <i>rpoN1 Burkholderia</i> sp. 383 | 10) <i>rpoN1 B. cenocepacia</i> AU1054 |
| 11) <i>rpoN1 B. ambifaria</i> MC40-6 | 12) <i>rpoN1 B. glumae</i> BGR1 |
| 13) <i>rpoN1 B. gladioli</i> BSR3 | 14) <i>rpoN1 B. thailandensis</i> E264 |
| 15) <i>rpoN1 B. cenocepacia</i> MCO-3 | 16) <i>rpoN1 B. vietnamensis</i> G4 |
| 17) <i>rpoN1 B. cenocepacia</i> HI2424 | 18) <i>rpoN1 B. ambifaria</i> AMMD |
| 19) <i>rpoN1 Burkholderia</i> sp Y123 | 20) <i>rpoN1 B. rhizoxinica</i> HKI 454 |
| 21) <i>rpoN2 B. xenovorans</i> LB400 | 22) <i>rpoN2 B. phytofirmans</i> PsJN |
| 23) <i>rpoN2 Burkholderia</i> sp CCGE1001 | 24) <i>rpoN2 Burkholderia</i> sp CCGE1003 |
| 25) <i>rpoN2 Burkholderia</i> sp CCGE1002 | 26) <i>rpoN2 B. phymatum</i> STM815 |
| 27) <i>rpoN2 Burkholderia</i> sp Y123 | |

4.1.4 Phylogenetic tree construction

In order to determine the difference between *rpoN1* and *rpoN2* genes, phylogenetic tree was constructed based on the nucleotide sequences using Neighbor-Joining method with Kimura-2 parameter. Bootstrap of 1000 repetition shows the confidence level for clustering of the genes (Figure 4.4)

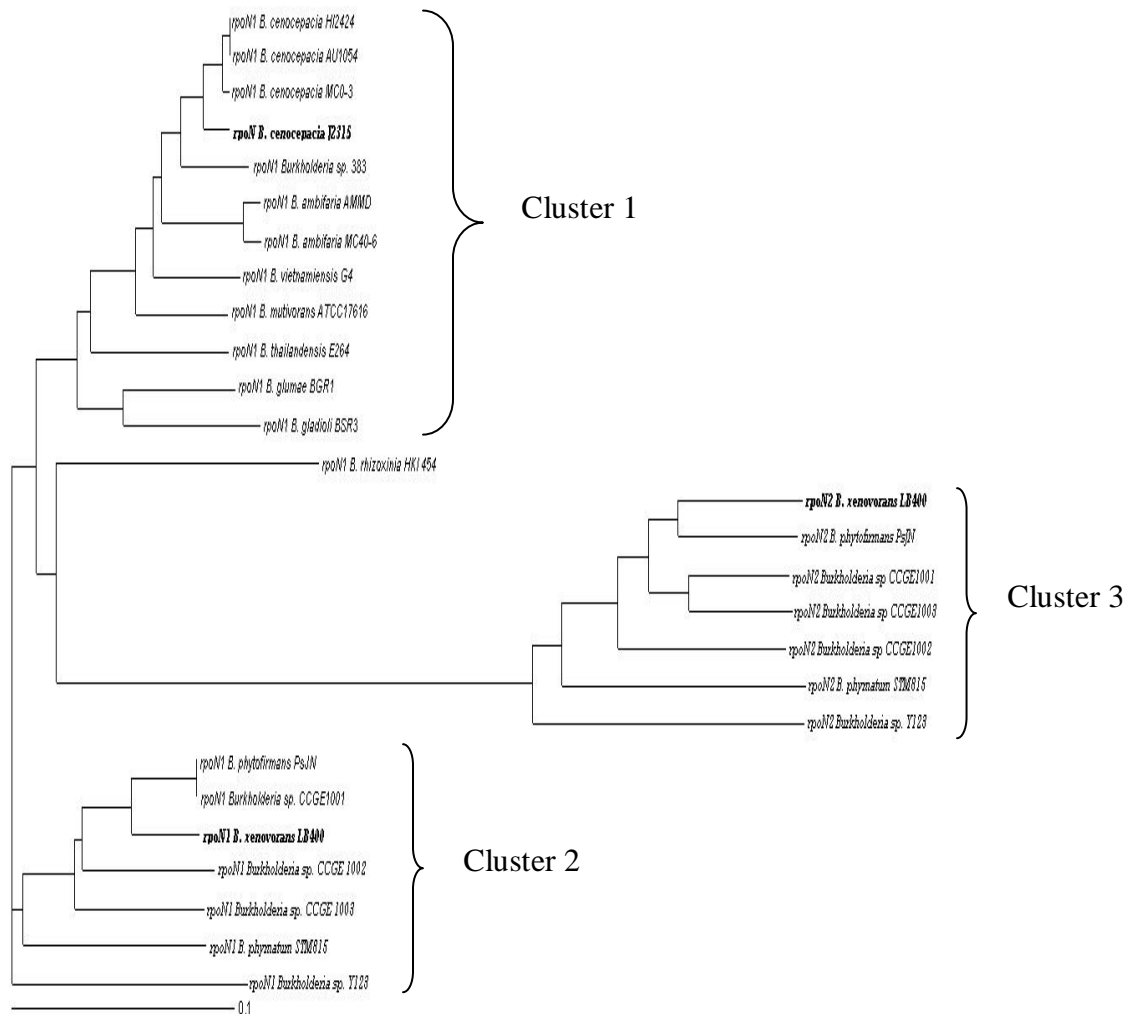


Figure 4.4: Phylogenetic tree of *rpoN* genes of *Burkholderia* species.

The phylogenetic tree clearly illustrated three major clusters generated from previous multiple sequence alignment. The *rpoN* genes that clustered in cluster 1 are mostly from members of *Burkholderia cepacia* complex (Bcc), a group of pathogenic bacteria especially towards cystic fibrosis patients. This includes *Burkholderia cenocepacia* J2315 who has a highest implication towards CF patients compared to other Bcc members. In the second cluster, the *rpoN* genes are originated from chromosome 1 of the bacteria which mostly isolated from soil and rhizospheric environment including *Burkholderia xenovorans* LB400 who has an ability to degrade PCBs and biphenyl compounds. However the *rpoN2* gene of *Burkholderia xenovorans* LB400 was not clustered together with *rpoN 1* gene but forms a different cluster together with other *rpoN* genes from chromosome 2 of soil and rhizospheric bacteria. This result demonstrated that *rpoN* genes from pathogenic bacteria are not closely related to *rpoN* genes from soil and rhizospheric bacteria. This result also showed that *rpoN1* and *rpoN2* genes are not closely related. This was confirmed by pairwise analysis of the nucleotide sequences where the *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 have sequence identity and similarity of only 58.2% which indicates that these two *rpoN* genes might have different function and able to initiate transcriptions process independently (Figure 4.5).

```
Alignment Submission Details Submit Another Job
Alignment
View Alignment File
#####
# Program: needle
# Rdate: Wed 23 May 2012 12:55:34
# Commandline: needle
#
# -auto
# -stdout
#
# -asequence caboss_needle-I20120523-125512-0860-94044279-pg.a.sequence
# -bsequence caboss_needle-I20120523-125512-0860-94044279-pg.b.sequence
# -datafile EDNAFULL
#
# -gapopen 10.0
# -gapextend 0.5
# -endopen 10.0
# -endextend 0.5
# -aformat3 pair
# -snucleotide1
# -snucleotide2
# Align_format: pair
# Report_file: stdout
#####
#-----
#
# Aligned_sequences: 2
# 1: PpoN1
# 2: PpoN2
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1711
# Identity: 995/1711 (58.2%)
# Similarity: 995/1711 (58.2%)
# Gaps: 410/1711 (24.0%)
# Score: 2406.5
#
#
```

Figure 4.5: Pairwise sequence analysis of *rpoN1* and *rpoN2* gene for *Burkholderia xenovorans* LB400.

Since the similarity of *rpoN1* and *rpoN2* genes are very low, this study would like to investigate the effects of inactivation of each gene towards the ability of *Burkholderia xenovorans* LB400 in degrading dibenzofuran. In order to do that, *rpoN1* and *rpoN2* genes were amplified and knocked out using the pKNOCK vector system.

4.2 Identification and amplification of *rpoN* and *ntrC* genes from *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315

The primer sets were designed to amplify the full length and partial gene sequences of the *rpoN* genes (Section 2.6.2). Using the RpLBF/R, Rp2LBF/R and JRpoNFS/R primer pairs, the complete *rpoN1* and *rpoN2* genes of *Burkholderia xenovorans* LB400 (located at chromosome 1 and chromosome 2, respectively) and *Burkholderia cenocepacia* J2315 representing fragments *ca.* 1500 bp in size were successfully amplified while the RpLBIF/R, Rp2LBIF/R and JRpoNF2/R2 primer pairs gave partial fragments *ca.* 600bp in size (Figures 4.5 and 4.6)

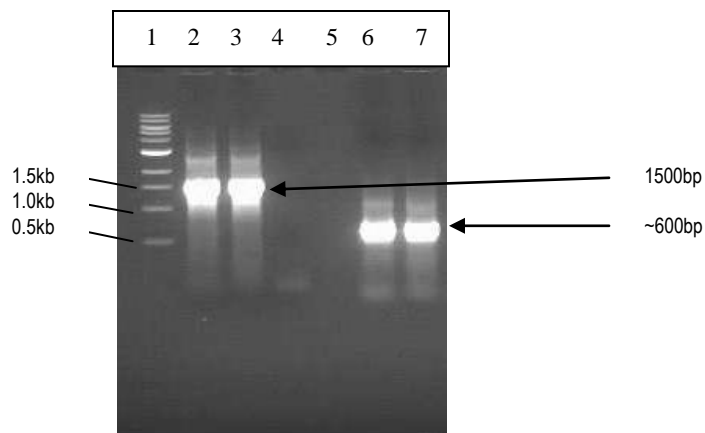


Figure 4.5: EtBr-stained 1% agarose gel electrophoresis of the *rpoN* gene amplicons generated by PCR from *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315.

- 1: 1kb DNA size marker
- 2: The amplified *rpoN* gene of *Burkholderia xenovorans* LB400
- 3: The amplified *rpoN* gene of *Burkholderia cenocepacia* J2315
- 6: The amplified partial *rpoN* gene of *Burkholderia xenovorans* LB400
- 7: The amplified partial *rpoN* gene of *Burkholderia cenocepacia* J2315

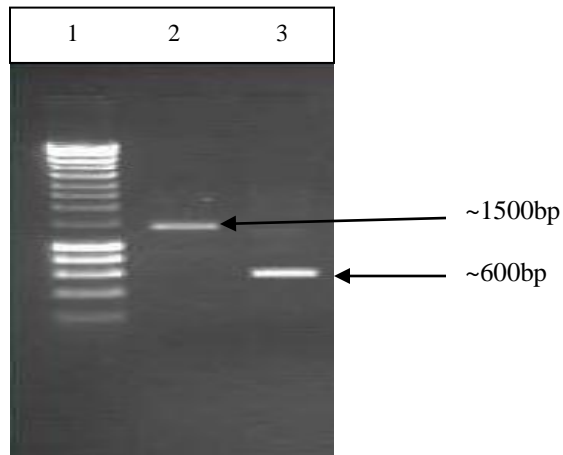


Figure 4.6: EtBr-stained 1% agarose gel electrophoresis of the complete and partial *rpoN2* gene of *Burkholderia xenovorans* LB400.

1. Hyperladder 1 DNA size marker
2. The amplified *rpoN2* gene of *Burkholderia xenovorans* LB400
3. The amplified partial *rpoN2* gene of *Burkholderia xenovorans* LB400

The full length versions of the *ntrC* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 were successfully amplified using the *NtrC*-U1F/*NtrC*-R1 and NtLB178F/NtLB1514R primer pairs generating amplicons *ca.* 1600 bp and *ca.* 1500 bp, respectively. The partial versions of the *ntrC* genes from *Burkholderia cenocepacia* and *Burkholderia xenovorans* were also amplified using *KpnI*-*NtrC*-F1/ *XbaI*-*NtrC*-R2 and NtLB591F /NtLB1134R primer pairs to generate amplicons *ca.* 888 bp (Figure 4.7A) and *ca.* 600 bp in size (Figure 4.7B), respectively.

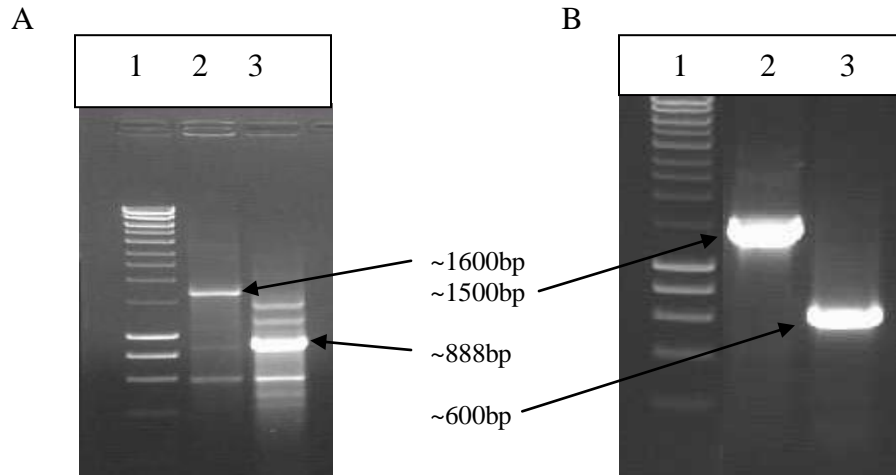


Figure 4.7: EtBr-stained 1% agarose gel electrophoresis of the complete and partial *ntrC* genes of *Burkholderia cenocepacia* J2315 (A) and *Burkholderia xenovorans* LB400 (B).

- A
- 1: Hyperladder I DNA size marker
 - 2: The amplified *ntrC* gene of *Burkholderia cenocepacia* J2315
 - 3: The amplified partial *ntrC* gene of *Burkholderia cenocepacia* J2315
- B
- 1: Hyperladder I DNA size marker
 - 2: The amplified *ntrC* gene of *Burkholderia xenovorans* LB400
 - 3: The amplified partial *ntrC* gene *Burkholderia xenovorans* LB400

4.3 Cloning of the *rpoN* and *ntrC* genes into pGEM T Easy

The *rpoN* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 were separately cloned into the pGEM T Easy cloning vector and the resultant recombinant plasmids were digested with *EcoRI* yielding two DNA fragments representing 3 kbp of the cloning vector and *ca.* 1500 bp of the DNA insert (Figures 4.8 and 4.9).

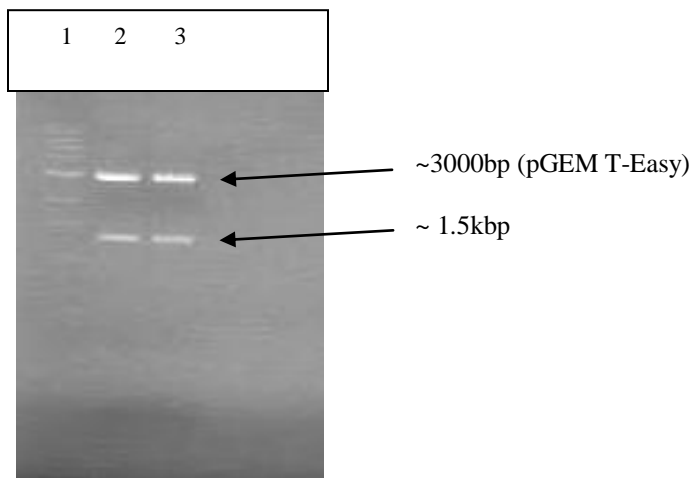


Figure 4.8: EtBr-stained 1% agarose gel electrophoresis of *EcoRI*-digested plasmids carrying the *rpoN* gene.

1: Hyperladder I DNA size marker

2: *EcoRI*-digested plasmid carrying the *rpoN* gene of *Burkholderia cenocepacia* J2315

3: *EcoRI*-digested plasmid carrying the *rpoN1* gene of *Burkholderia xenovorans* LB400.

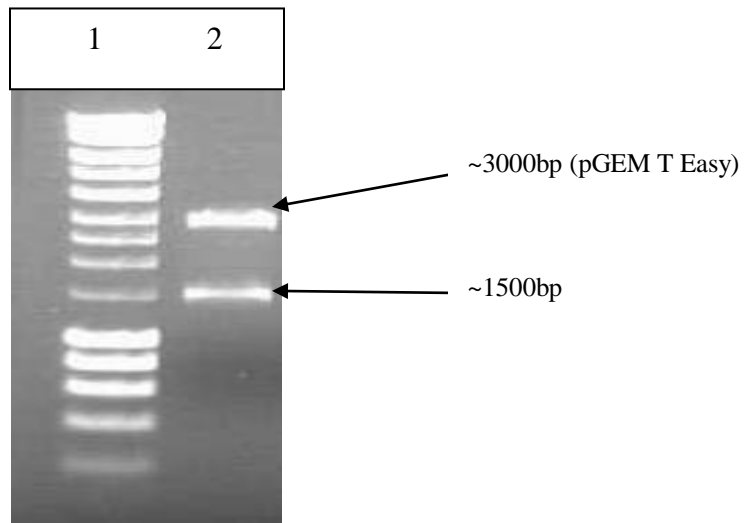


Figure 4.9: EtBr-stained 1% agarose gel electrophoresis of *Eco*RI-digested plasmid carrying the *rpoN2* of *Burkholderia xenovorans* LB400

1: Hyperladder I DNA size marker

2: *Eco*RI-digested plasmid carrying the *rpoN2* gene of *Burkholderia xenovorans* LB400.

The *nrC* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 were also successfully cloned into pGEM T Easy cloning vector. The *Eco*RI digestion of recombinant plasmid showed two DNA fragments representing 3 kbp of the cloning vector, *ca.* 1500 bp (*Burkholderia xenovorans* LB400) and 1600 bp (*Burkholderia cenocepacia* J2315) of the DNA inserts (Figures 4.10A and 4.10B respectively).

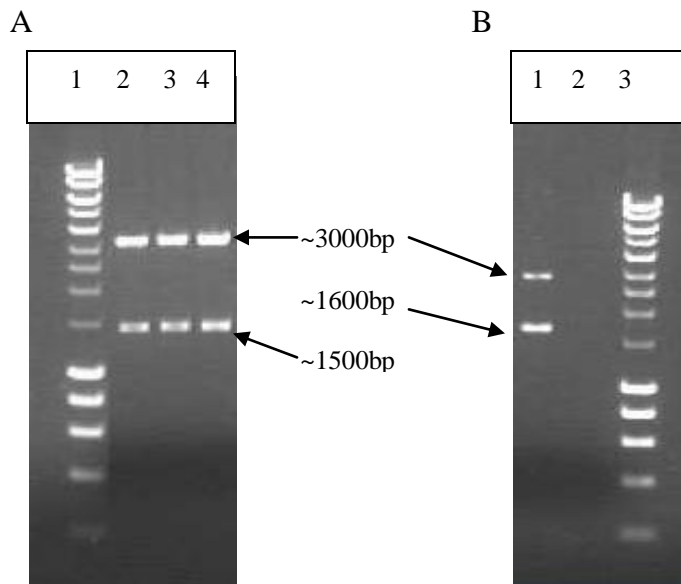


Figure 4.10: EtBr-stained 1% agarose gel electrophoresis of *Eco*RI-digested replicate plasmids carrying the *nrC* genes of *Burkholderia xenovorans* LB400 (A) and *Burkholderia cenocepacia* J2315 (B).

A 1: Hyper ladder I DNA size marker

2-4: *Eco*RI-digested plasmids carrying *nrC* gene of *Burkholderia xenovorans* LB400

B 1: *Eco*RI-digested plasmid carrying *nrC* gene of the *B. cenocepacia* J2315 *nrC* gene.

3: Hyper ladder I DNA marker

4.4 Cloning of partial copies of the *rpoN* and *ntrC* genes into pGEM T Easy

The partial sequences of the *rpoN1* and *rpoN2* genes of the *Burkholderia xenovorans* LB400 and *rpoN* gene of *Burkholderia cenocepacia* J2315 were separately cloned into pGEM T Easy and the resultant recombinant plasmids were designated as pGRPL (pGEM::*rpoNS*_{LB400}), pGRP2L (pGEM::*rpoN2S*_{LB400}) and pGRPJ (pGEM::*rpoNS*_{J2315}). Following *EcoRI* digestion of these plasmids, two DNA fragments representing *ca.* 3000 bp of the cloning vector and *ca.* 600 bp of DNA insert were produced. Similarly successful insertion of the partial *ntrC* gene from *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 in pGNtC (pGEM::*ntrCS*_{J2315}) and pGNtL (pGEM::*ntrCS*_{LB400}) has been confirmed following digestion of the recombinant plasmid with *EcoRI* to produce bands corresponding to 3 kbp of the cloning vector and 888 bp DNA insert in pGNtC and 600bp DNA insert in pGNtL. Figure 4.11 shows *EcoRI* digestion of partial *rpoN* and *ntrC* insertion from *Burkholderia cenocepacia* J2315 in pGEM T Easy while Figures 4.12A and 4.12B show the *EcoRI*-digestion of partial *rpoN1* gene (pGRpL) and *rpoN2* gene (pGRp2L) of *Burkholderia xenovorans* LB400, respectively.

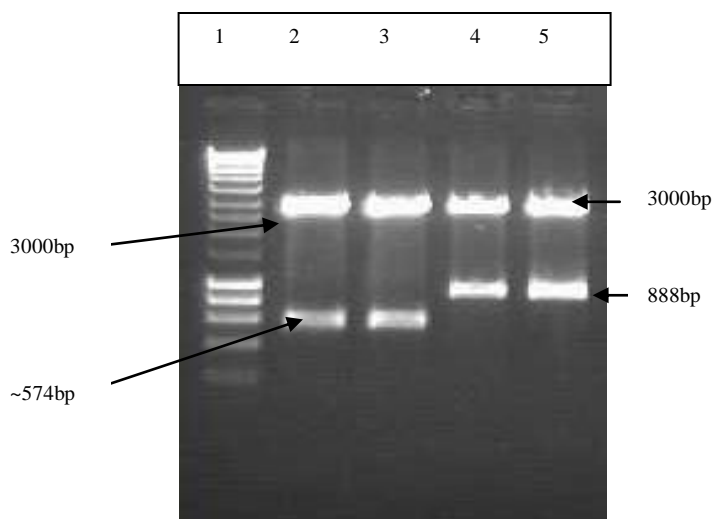


Figure 4.11: EtBr-stained 1% agarose gel electrophoresis of *Eco*RI-digested replicate plasmids carrying a partial copy of the *Burkholderia cenocepacia* J2315 *rpoN* gene (pGRPJ) and a partial copy of the *Burkholderia cenocepacia* J2315 *ntrC* gene (pGNtC).

1: Hyperladder I DNA size marker

2: pGRPJ (pGEM::*rpoNS*_{J2315})

3: pGRPJ (pGEM::*rpoNS*_{J2315})

4: pGNtrC (pGEM::*ntrCS*_{J2315})

5: pGNtrC (pGEM::*ntrCS*_{J2315})

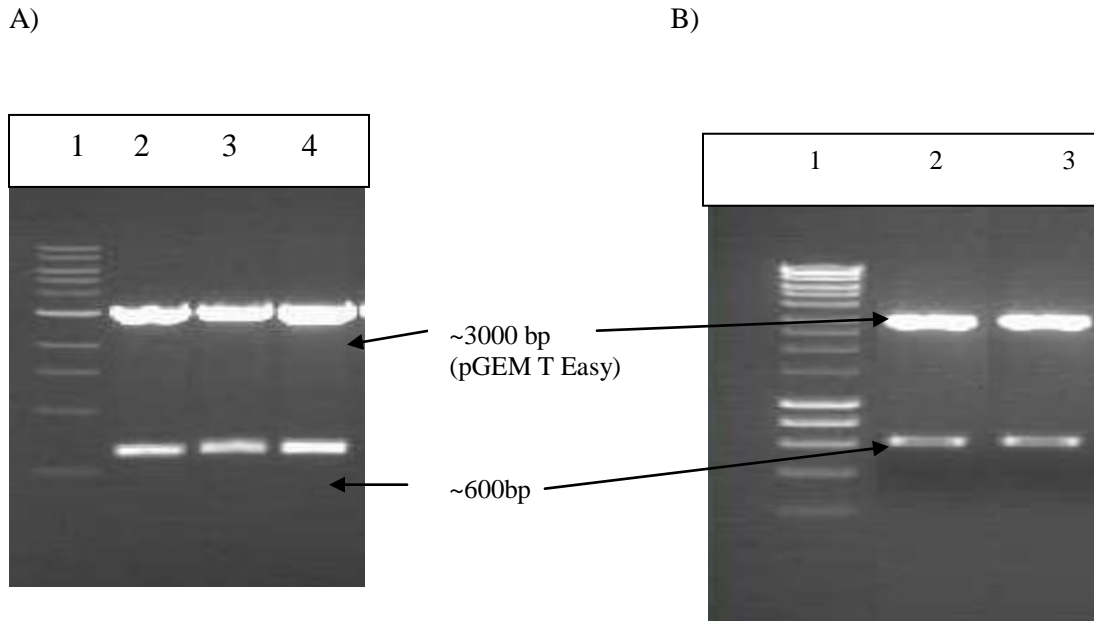


Figure 4.12: EtBr-stained 1% agarose gel electrophoresis of *EcoRI*-digested replicate plasmids carrying a partial copy of the (panel A) *Burkholderia xenovorans* LB400 *rpoN1* gene (pGRpL) and (panel B) *Burkholderia xenovorans* LB400 *rpoN2* gene (pGRp2L).

A)

- 1: 1kb DNA size marker
- 2: pGRpL (pGEM::*rpoNS*_{LB400})
- 3: pGRpL (pGEM::*rpoNS*_{LB400})
- 4: pGRpL (pGEM::*rpoNS*_{LB400})

B)

- 1: Hyperladder 1 DNA size Marker
- 2: pGRp2L (pGEM::*rpoN2S*_{LB400})
- 3: pGRp2L (pGEM::*rpoN2S*_{LB400})

The partial *ntrC* gene of the *Burkholderia xenovorans* LB400 was also successfully cloned into pGEM T Easy. Figure 4.13 shows *Eco*RI digestion of replicate recombinant plasmids designated as pGNtL (pGEM::*ntrCS*_{LB400}) showing 3 kbp of the cloning vector and *ca.* 600 bp DNA insert.

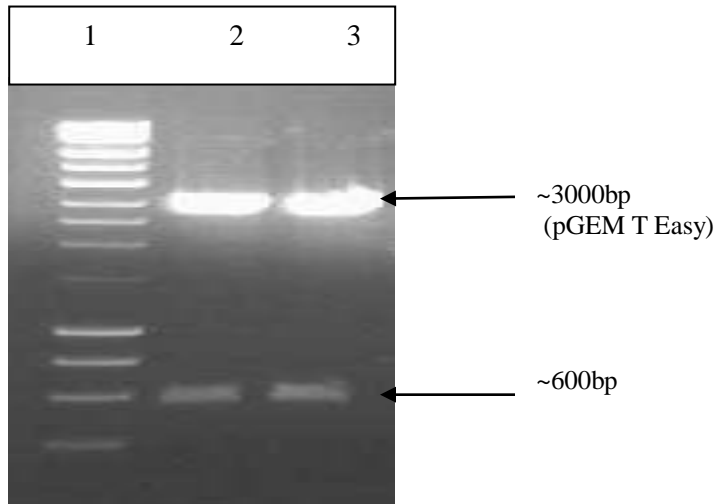


Figure 4.13: EtBr-stained 1% agarose gel electrophoresis of *Eco*RI-digested replicate plasmids carrying a partial copy of *ntrC* gene of *B. xenovorans* LB400.

1: Hyperladder 1 DNA marker

2: pGNtL (pGEM::*ntrCS*_{LB400})

3: pGNtL (pGEM::*ntrCS*_{LB400})

4.5 Cloning of partial copies of the *rpoN* and *ntrC* genes into the suicide vector pKNOCK

Following restriction of the pGRPL, pGRPJ, pGNtL and pGNtC with *EcoRI*, the insert fragments corresponding to the partial gene sequences of the *rpoN* genes and *ntrC* genes of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 were excised from the gels and ligated into linearised pKNOCK-cm which carry chloramphenicol resistant cassette at the *EcoRI* site prior to transformation into competent *E. coli* BW19851 cells. The resulting constructs were designated as pKRpJ (pKNOCKcm::*rpoNS*_{J2315}), pKRpL (pKNOCKcm::*rpoNS*_{LB400}), pKNtJ (pKNOCKcm::*ntrCS*_{J2315}) and pKNtLB (pKNOCKcm::*ntrCS*_{LB400}). Following *EcoRI* digestion of these recombinant plasmids, DNA fragment with sizes of *ca.* 600 bp for the partial *rpoN* genes and *ca.* 888 bp of the *ntrC* gene plus 1,800 bp of the pKNOCK-cm plasmid in each digestion were produced (Figure 4.14). For pKNtLB, DNA fragments of *ca.* 600 bp of the *ntrC* gene and 1800 bp of pKNOCK-cm plasmid were obtained (Figure 4.15).

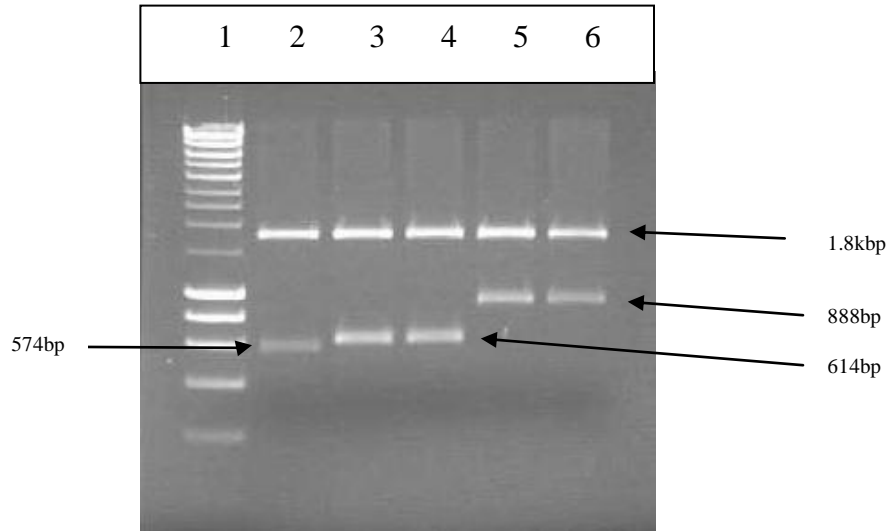


Figure 4.14: EtBr-stained 1% agarose gel electrophoresis of *EcoRI*-digested pKNOCK-cm replicate recombinants carrying partial copies of *Burkholderia cenocepacia* J2315 *rpoN* (pKRPJ; 1.8 kbp + 564 bp), *Burkholderia xenovorans* LB400 *rpoN* gene (pKRPL1 and 2; 1.8 kbp + 614 bp) and *Burkholderia cenocepacia ntrC* genes (pKNTJ1 and 2; 1.8 kbp + 888 bp).

- | | |
|--|--|
| 1: Hyperladder I DNA size marker | 4: pKRPL (pKNOCKcm:: <i>rpoNS</i> _{LB400}) |
| 2: pKRPJ (pKNOCKcm:: <i>rpoNS</i> _{J2315}) | 5: pKNTJ (pKNOCKcm:: <i>ntrCS</i> _{J2315}) |
| 3: pKRPL (pKNOCKcm:: <i>rpoNS</i> _{LB400}) | 6: pKNTJ (pKNOCKcm:: <i>ntrCS</i> _{J2315}) |

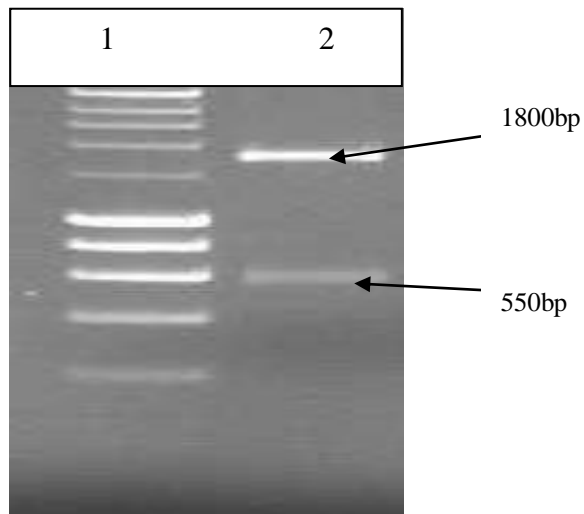


Figure 4.15: EtBr-stained 1% agarose gel electrophoresis of *Eco*RI-digested pKNOCK-cm replicate recombinant carrying partial copies of the *Burkholderia xenovorans* LB400 *ntrC* gene (pKNtLB; 1.8 kbp + 550 bp)

1: Hyperladder I DNA size marker

2: pKNtLB (pKNOCKcm::*ntrCS*_{LB400})

The insert fragment of partial *rpoN2* gene from chromosome 2 of *Burkholderia xenovorans* LB400 in pGRp2L was also purified and ligated into linearised pKNOCK-Tc which carry tetracycline-resistance cassette at the *EcoRI* site prior to transformation into competent *E. coli* BW19851 cells. The *EcoRI* digestion of the resulting construct, designated as pKRp2L, yielded DNA fragment of *ca.* 600 bp for the partial *rpoN2* gene and *ca.* 2,200 bp of the pKNOCK-tc vector in each digestion (Figure 4.16).

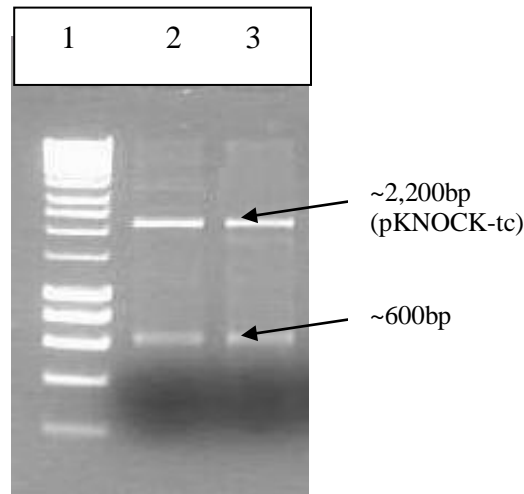


Figure 4.16: EtBr-stained 1% agarose gel electrophoresis of *EcoRI*-digested pKNOCK-tc replicate recombinants carrying partial copies of the *Burkholderia xenovorans* LB400 *rpoN2* gene (pKRp2L; 2.2 kbp + 600 bp).

1: Hyperladder I DNA size marker

2: pKRp2L (pKNOCKtc::*rpoNS*_{LB400})

4.6 Screening for putative *rpoN* and *ntrC* mutants

Biparental matings between the wild type *Burkholderia* strains and *E. coli* BW19851 carrying the pKNOCK-cm or pKNOCK-tc target gene constructs described above were performed and the progeny screened for mutated genes by PCR amplification. Putative knockout mutants were scored when PCR amplification using primers JRpoNFS/R failed to amplify amplicons of the expected size. Only one potential *rpoN* gene knock-out in putative mutants of *B. cenocepacia* J2315 was obtained when PCR failed to amplify amplicon of the expected size in lane 3 (*rpoN* mutant candidate 1 of *Burkholderia cenocepacia* J2315) (Figure 4.17). In order to determine if the absence of expected DNA fragment was due to the mutation of the *rpoN* gene, the amplification of mutated *rpoN* gene was complemented with the amplification of the active *ntrC* gene of *Burkholderia cenocepacia* J2315 using *ntrC* primers (NtrC-UF1/R1). The expected *ntrC* gene fragment was obtained in lane 7 as shown by the the wild type *Burkholderia cenocepacia* J2315 in lane 6 (Figure 4.17).

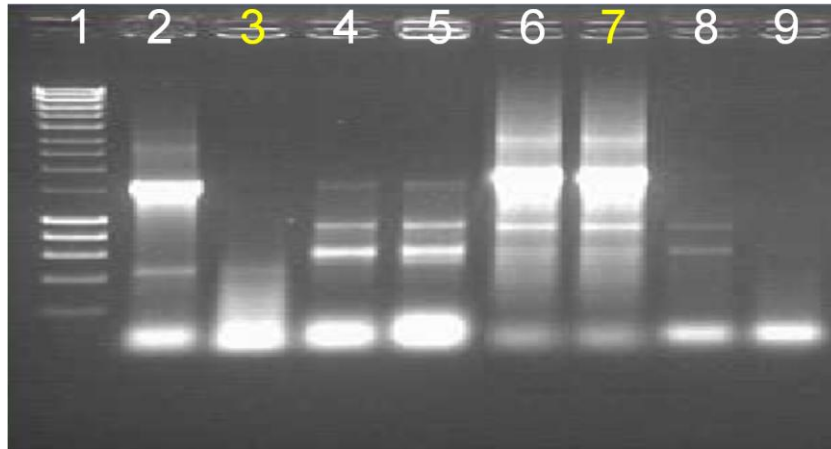


Figure 4.17: Colony PCR amplification assay of putative pKNOCK-cm *Burkholderia cenocepacia* J2315 *rpoN* mutants. Primer pairs used to generate the amplicons are shown in brackets.

1= Hyperladder I DNA size marker

2= Wildtype *Burkholderia cenocepacia* J2315 (JRpoNFS/R)

3= *rpoN* mutant candidate 1 of *Burkholderia cenocepacia* J2315 (JRpoNFS/R)

4= *rpoN* mutant candidate 2 of *Burkholderia cenocepacia* J2315 (JRpoNFS/R)

5= *rpoN* mutant candidate 3 of *Burkholderia cenocepacia* J2315 (JRpoNFS/R)

6= Wildtype *Burkholderia cenocepacia* J2315 (*NtrC*-UF1/R1)

7= *rpoN* mutant candidate 1 of *Burkholderia cenocepacia* J2315 (*NtrC*-UF1/R1)

8= *rpoN* mutant candidate 2 of *Burkholderia cenocepacia* J2315 (*NtrC*-UF1/R1)

9= *rpoN* mutant candidate 3 of *Burkholderia cenocepacia* J2315 (*NtrC*-UF1/R1)

Putative *ntrC* knockout mutants of *Burkholderia cenocepacia* J2315 was also scored when PCR failed to amplify amplicons of the expected size normally found with the wildtype *Burkholderia cenocepacia* J2315 as shown in lane 1 (Figure 4.18). Even though both *ntrC* mutant candidate 1 (lane 2) and 2 (lane 3) failed to amplify the desired fragment size, the *ntrC* mutant candidate 2 (lane 7) gave the expected amplicon as that of the wildtype *Burkholderia cenocepacia* J2315 as shown in lane 5 when amplified using *rpoN* primers which will be considered as potential mutant (Figure 4.18).

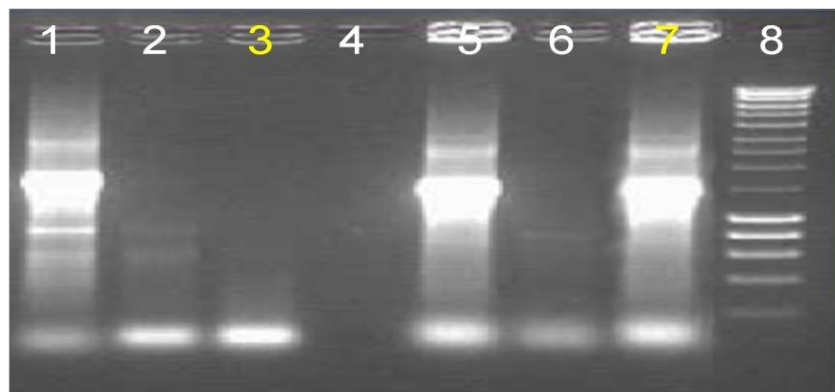


Figure 4.18: Colony PCR amplification assay of putative pKNOCK-cm *Burkholderia cenocepacia* J2315 *ntrC* mutants. Primer pairs used to generate the amplicons are shown in brackets.

1= Wildtype *Burkholderia cenocepacia* J2315 (*NtrC-UF1/R1*)

2= *ntrC* mutant candidate 1 of *Burkholderia cenocepacia* J2315 (*NtrC-UF1/R1*)

3= *ntrC* mutant candidate 2 of *Burkholderia cenocepacia* J2315 (*NtrC-UF1/R1*)

4= -NA-

5= Wildtype *Burkholderia cenocepacia* J2315 (*JRpoNFS/R*)

6= *ntrC* mutant candidate 1 of *Burkholderia cenocepacia* J2315 (*JRpoNFS/R*)

7= *ntrC* mutant candidate 2 of *Burkholderia cenocepacia* J2315 (*JRpoNFS/R*)

8= Hyperladder I DNA size marker

The *rpoN1* mutant candidate of *Burkholderia xenovorans* LB400 were screened by amplification using RpLBF/R primers. Putative *rpoN1* knockout of *Burkholderia xenovorans* LB400 mutant was scored when PCR failed to amplify the expected *rpoN1* gene as shown by wildtype *Burkholderia xenovorans* LB400 in lane 2 (Figure 4.19). The *ntrC* gene carried by wildtype *Burkholderia xenovorans* LB400 and *rpoN1* mutant candidates was also amplified to complement the negative identification. Even though *rpoN1* mutant candidates 1, 2 and 3 of *Burkholderia xenovorans* LB400 (lanes 3, 4 and 5, respectively) showed the absence of normal *rpoN1* gene fragment as found in wildtype *Burkholderia xenovorans* LB400, only *rpoN1* mutant candidates 1 and 2 (lanes 7 and 8, respectively) gave positive amplicons when amplified with *ntrC* primers (Figure 4.19). The *rpoN1* mutant candidate 3 of *Burkholderia xenovorans* LB400 in lane 9 does not show the expected *ntrC* gene thus indicated that it might not have been derived from *Burkholderia xenovorans* LB400.

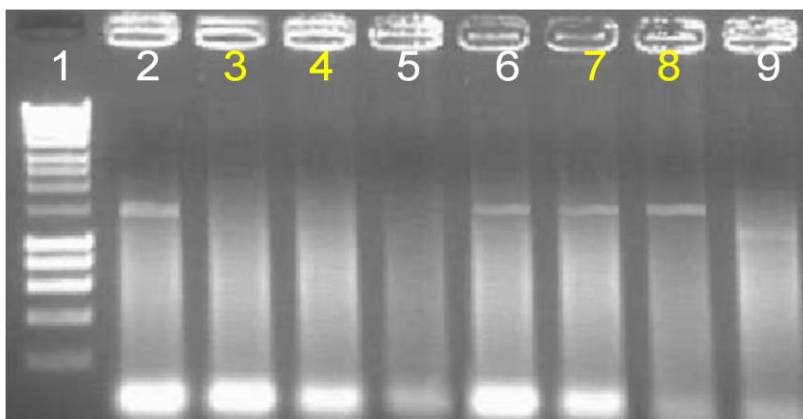


Figure 4.19: Colony PCR amplification assay of putative pKNOCK-cm *Burkholderia xenovorans* LB400 *rpoN* mutants. Primer pairs used to generate the amplicons are shown in brackets.

1= Hyperladder I DNA size marker

2= Wildtype *Burkholderia xenovorans* LB400 (RpLBF/R)

3= *rpoN1* mutant candidate 1 of *Burkholderia xenovorans* LB400 (RpLBF/R)

4= *rpoN1* mutant candidate 2 of *Burkholderia xenovorans* LB400 (RpLBF/R)

5= *rpoN1* mutant candidate 3 of *Burkholderia xenovorans* LB400 (RpLBF/R)

6= Wildtype *Burkholderia xenovorans* LB400 (NtLBF/R)

7= *rpoN1* mutant candidate 1 of *Burkholderia xenovorans* LB400 (NtLBF/R)

8= *rpoN1* mutant candidate 2 of *Burkholderia xenovorans* LB400 (NtLBF/R)

9= *rpoN1* mutant candidate 3 of *Burkholderia xenovorans* LB400 (NtLBF/R)

Figure 4.20 shows that only one potential *ntrC* mutant candidate of *Burkholderia xenovorans* LB400 was obtained. The *ntrC* mutant candidate 2 of *Burkholderia xenovorans* LB400 not only shows the absence of expected amplicon of *ntrC* gene (lane 4) which as found in wildtype *Burkholderia xenovorans* LB400 but also shows the presence of expected *rpoNI* gene of *Burkholderia xenovorans* LB400 (lane 8). The *ntrC* mutant candidates 1 (lane 7) and 3 (lane 9) did not show the presence of *rpoNI* gene thus indicated that it might not have been derived from *Burkholderia xenovorans* LB400.

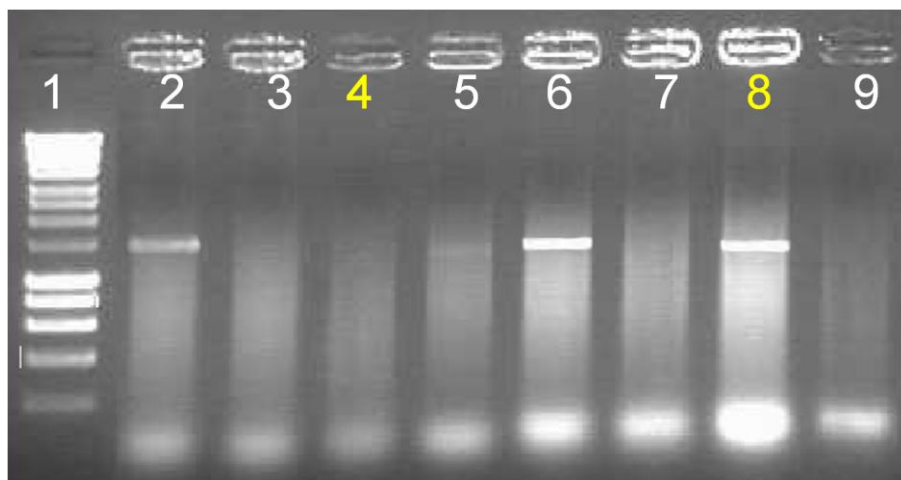


Figure 4.20: Colony PCR amplification assay of putative pKNOCK-cm *Burkholderia xenovorans* LB400 *ntrC* mutants. Primer pairs used to generate the amplicons are shown in brackets.

1= Hyperladder I DNA size marker

2= Wildtype *Burkholderia xenovorans* LB400 (NtLBF/R)

3= *ntrC* mutant candidate 1 of *Burkholderia xenovorans* LB400 (NtLBF/R)

4= *ntrC* mutant candidate 2 of *Burkholderia xenovorans* LB400 (NtLBF/R)

5= *ntrC* mutant candidate 3 of *Burkholderia xenovorans* LB400 (NtLBF/R)

6= Wildtype *Burkholderia xenovorans* LB400 (RpLBF/R)

7= *ntrC* mutant candidate 1 of *Burkholderia xenovorans* LB400 (RpLBF/R)

8= *ntrC* mutant candidate 2 of *Burkholderia xenovorans* LB400 (RpLBF/R)

9= *ntrC* mutant candidate 3 of *Burkholderia xenovorans* LB400 (RpLBF/R)

The *rpoN2* mutant candidates of *Burkholderia xenovorans* LB400 were screened using Rp2LBIF/R and RpLBF/R primer pairs which amplify the *rpoN2* gene and *rpoN1* gene of *Burkholderia xenovorans* LB400, respectively. Figure 4.21 shows that *rpoN2* mutant candidates 1, 3 and 5 of *Burkholderia xenovorans* LB400 (lanes 3, 5 and 7, respectively) gave negative identification when PCR failed to amplify the expected *rpoN2* gene as shown by wildtype *Burkholderia xenovorans* LB400 in lane 2. However, only the *rpoN2* mutant candidate 1 of *Burkholderia xenovorans* LB400 in lane 9 was considered as potential mutant since it yielded the expected *rpoN1* gene as shown by wildtype *Burkholderia xenovorans* LB400 in lane 8.

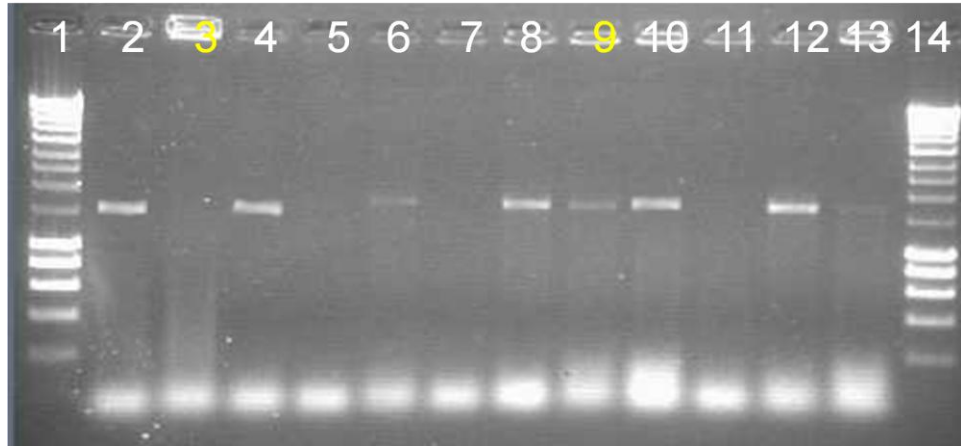


Figure 4.21: Colony PCR amplification assay of putative pKNOCK-tc *Burkholderia xenovorans* LB400 *rpoN2* mutants. Primer pairs used to generate the amplicons are shown in brackets.

1= Hyperladder I DNA size marker

2= Wildtype *Burkholderia xenovorans* LB400 (Rp2LBF/R)

3= *rpoN2* mutant candidate 1 of *Burkholderia xenovorans* LB400 (Rp2LBF/R)

4= *rpoN2* mutant candidate 2 of *Burkholderia xenovorans* LB400 (Rp2LBF/R)

5= *rpoN2* mutant candidate 3 of *Burkholderia xenovorans* LB400 (Rp2LBF/R)

6= *rpoN2* mutant candidate 4 of *Burkholderia xenovorans* LB400 (Rp2LBF/R)

7= *rpoN2* mutant candidate 5 of *Burkholderia xenovorans* LB400 (Rp2LBF/R)

8= Wildtype *Burkholderia xenovorans* LB400 (RpLBF/R)

9= *rpoN2* mutant candidate 1 of *Burkholderia xenovorans* LB400 (RpLBF/R)

10= *rpoN2* mutant candidate 2 of *Burkholderia xenovorans* LB400 (RpLBF/R)

11= *rpoN2* mutant candidate 3 of *Burkholderia xenovorans* LB400 (RpLBF/R)

12= *rpoN2* mutant candidate 4 of *Burkholderia xenovorans* LB400 (RpLBF/R)

13= *rpoN2* mutant candidate 5 of *Burkholderia xenovorans* LB400 (RpLBF/R)

14= Hyperladder I DNA size marker

4.7 Confirmation of the presence of *rpoN* and *ntrC* mutants by PCR amplification

Positive PCR identification using combination of primers that amplify the gene of interest with pKNOCK internal primers will further confirmed the insertion of pKNOCK construct into the chromosome of *Burkholderia* mutants. The putative *rpoN* mutant of *Burkholderia cenocepacia* J2315 were amplified using a combination of *rpoN* primers and pKNOCK internal primers which sequences were based on the sequences of the chloramphenicol cassette (CmF/CmR) and the oriV/oriT region of the pKNOCK-cm vector (OriF/OriR) (Section 3.6.13). Two amplicons obtained using combination primers of JRpoNFS/OriR (lane 5) and JRpoNFS/cmR (lane 9) (Figure 4.22) were separately excised and purified prior to ligation into pGEM T Easy and subsequently transformed into *E.coli* JM109. Plasmid extracted from resultant transformants was sequenced to confirm the presence of the mutants.

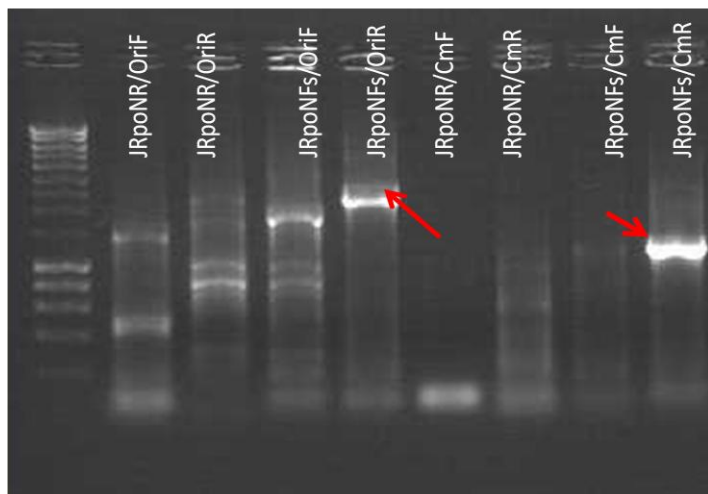


Figure 4.22: Colony PCR amplification of *Burkholderia cenocepacia* J2315 *rpoN* mutant using a combination of RpoN primers and pKNOCK-cm internal primers. Arrows indicate the potential amplicons for sequencing analysis.

Examination of the sequence of the recombinant plasmid containing the mutated fragment of *Burkholderia cenocepacia* J2315 *rpoN* gene confirmed that the mutation had been successfully inserted into the bacterial chromosome with the partial *rpoN* gene was observed at the 5' end (indicated as red line) while chloramphenicol cassette was located at 3' end (indicated as yellow line). In between the *rpoN* gene and chloramphenicol cassette, the vector backbone was indicated with blue line (Figure 4.23)

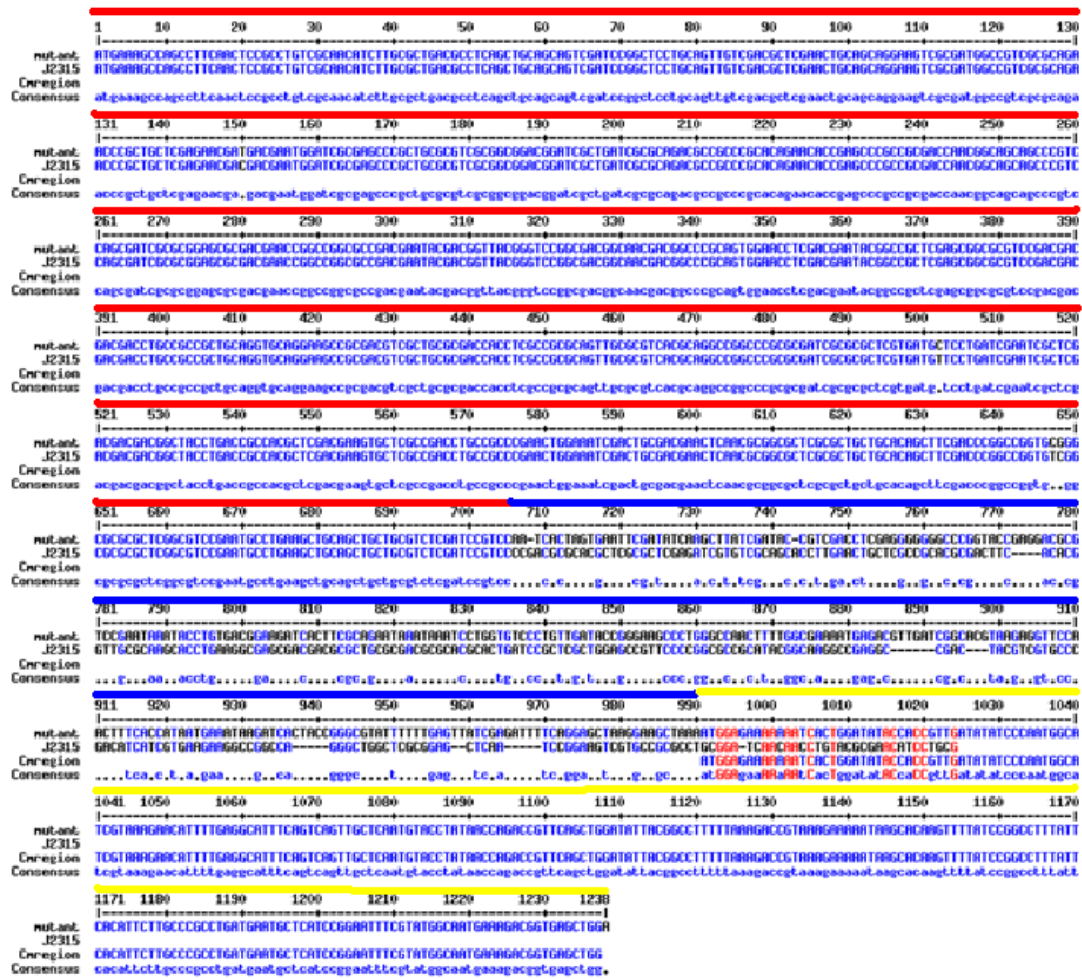


Figure 4.23: Alignment of DNA sequences of *rpoN* gene of *Burkholderia cenocepacia* J2315, chloramphenicol cassette from pKNOCK-cm vector and the *Burkholderia cenocepacia* J2315 *rpoN* mutant.

Putative *ntrC* mutants of J2315 were amplified using a combination of *ntrC* primers (NtrCUF/NtrCR1) and pKNOCK internal primers (CmF/CmR and OriF/OriR). Four amplicons were obtained using the primer pairs of NtrCUF1/OriF (lane 2), NtrCUF1/OriR (lane 3), NtrCR1/OriR (lane 4) and NtrCR1/CmF (lane 8) as shown in Figure 4.24. Plasmid extracted from resultant transformants was sequenced to confirm the presence of the mutants.

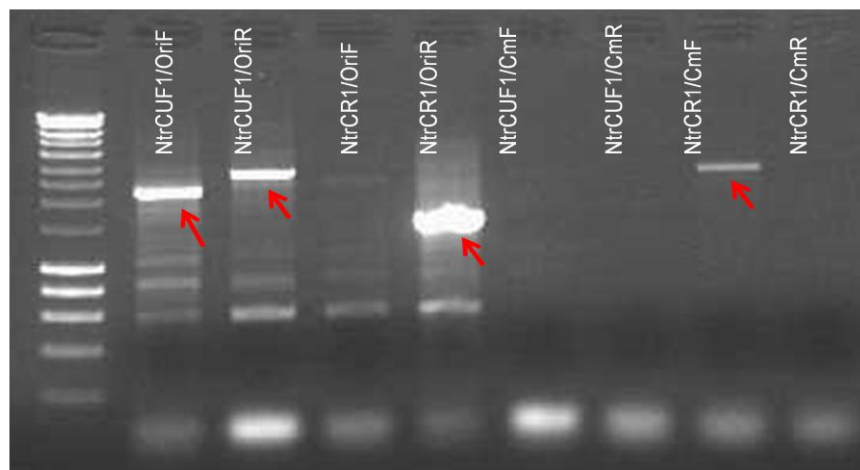


Figure 4.24: Colony PCR amplification of *B. cepacia* J2315 *ntrC* mutant using a combination of *ntrC* primers and pKNOCK-cm internal primers. Arrows indicate the potential amplicons for sequencing analysis.

Both putative *rpoN1* and *rpoN2* mutants of *Burkholderia xenovorans* LB400 were also amplified using RpLBF/R and Rp2LBF/R, respectively with combination of pKNOCK internal primers. Four amplicons were obtained for the *rpoN1* mutants of *Burkholderia xenovorans* LB400 when amplified using RpLBF/OriF (lane 2), RpLBF/CmR (lane 7), RpLBR/CmR (lane 8) and RpLBR/CmR (lane 9) as shown in Figure 4.25. Plasmid extracted from resultant transformants was sequenced to confirm the presence of the mutants.

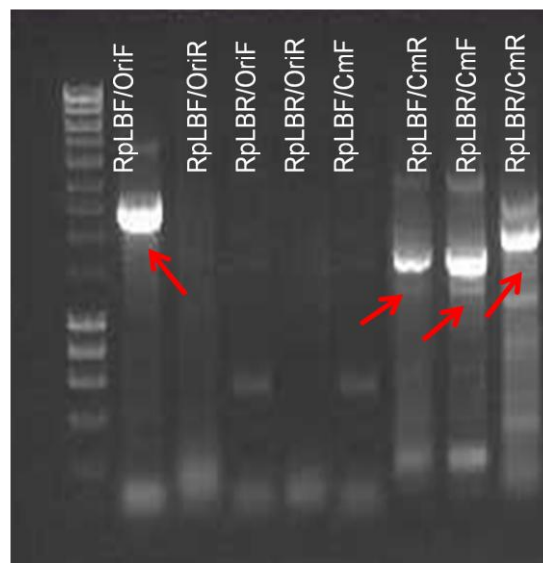


Fig. 4.25: Colony PCR amplification of *B. xenovorans* LB400 *rpoN* mutant using a combination of *rpoN* primers and pKNOCK-cm internal primers. Arrows indicate the potential amplicons for sequencing analysis.

Only combination of *rpoN2* primers with internal pKNOCK primers based on oriT/oriV region were used to amplify *rpoN2* mutant of *Burkholderia xenovorans* LB400. Two amplicons were obtained using primer pairs of Rp2LBR/OriF (lane 3) and Rp2LBR/OriR (lane 4) as shown in Figure 4.26. Plasmid extracted from resultant transformants was sequenced to confirm the presence of the mutants.

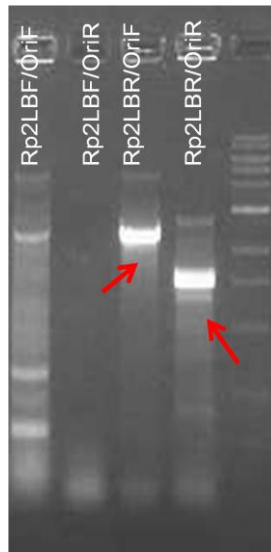


Figure 4.26: Colony PCR amplification of *B. xenovorans* LB400 *rpoN2* mutant using a combination of *rpoN2* primers and pKNOCK-tc internal primers. Arrows indicate the potential amplicons for sequencing analysis.

The putative *ntrC* mutants of *Burkholderia xenovorans* LB400 were also amplified using combination of *ntrC* primers and pKNOCK-cm internal primers CmF/CmR and OriF/OriR. Three amplicons were obtained from the amplification using primer pairs of NtLBF/OriR (lane 3), NtLBR/OriF (lane 4) and NtLBF/CmF (lane 6) as shown in Figure 4.27. Plasmid extracted from resultant transformants was sequenced to confirm the presence of the mutants.

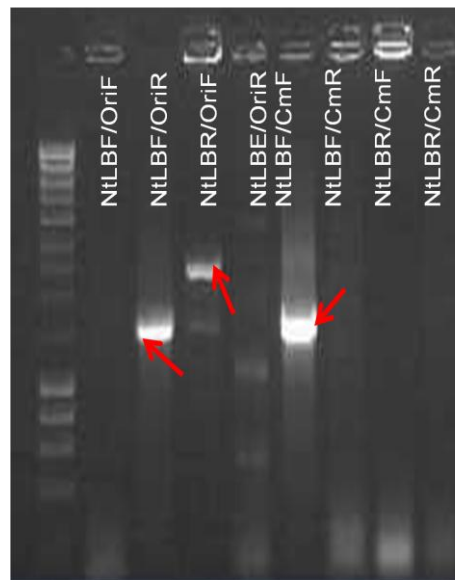


Fig. 4.27: Colony PCR amplification of *B. xenovorans* LB400 *ntrC* mutant using a combination of *ntrC* primers and internal primers based on the sequence of the pKNOCK-cm vector. Arrows indicate the potential amplicons for sequencing analysis.

4. 8 Discussion

Investigations on the role of the *rpoN* gene in *Burkholderia* species require the development of loss-of-function mutants. However, genetic studies of this nature with the *Burkholderia* genus have been hampered by their inherent resistance to most antibiotics commonly used for genetic selection and limited availability of cloning vectors. Recent advances have been made to develop mutation systems for *Burkholderia cenocepacia* (Saldias *et al.* 2008) and a similar system is described here with the successful development of a system to knock out the *rpoN* genes in *Burkholderia* based on the pKNOCK plasmid. The plasmid pKNOCK is a suicide vector which utilises the R6K γ -origin of replication to provide and encode the π protein in *trans* through growth in *E. coli* (Kolter *et al.* 1978).

Confirmation of the successful generation of *rpoN* and *ntrC* gene mutants of both *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 is based on negative PCR amplification of the full length gene amplicons as compared to the wild types. The absence of expected size of amplicon was due to the insertion of pKNOCK vector which carried the partial fragment of respective gene. Amplification using primers that were designed to amplify the full length of the gene will only results in bigger amplicons with additional size from pKNOCK vector and partial insertion of the particular gene. However, this bigger DNA amplicon which includes the target gene, pKNOCK vector and partial insertion of the gene was difficult to be amplified. To complement this assay, the amplification of other genes which possessed by both mutant and wild type were added. This amplification step was able to confirm that the mutants were not different from their wild type except for the mutation in the target gene. The amplification of full

length gene amplicons has been used as initial screening step and will be further analysed. Several potential mutants of *rpoN* and *ntrC* genes of *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 have been identified using this method. However this assay does not preclude other interpretations and with this in mind, attempts have been made to amplify the region between the inserted DNA and the suicide vector. To amplify this region, primers within the pKNOCK region, *oriT/oriV* (Alexeyev and Shokolenko, 1995) and the chloramphenicol cassette (Alexeyev *et al.*, 1995) have also been designed for combined use with forward and reverse primers of the genes both upstream and downstream of the inserted DNA. From this combination of primers, two DNA fragments were amplified from *rpoN* mutant of *Burkholderia cenocepacia* J2315 while four DNA fragments were obtained from potential *ntrC* mutant of *Burkholderia cenocepacia* J2315. The *rpoN* mutant of *Burkholderia xenovorans* LB400 showed four DNA fragments whereas only three fragments were obtained from *ntrC* mutant of *Burkholderia xenovorans* LB400. Since the *rpoN2* mutant of *Burkholderia xenovorans* LB400 was constructed using pKNOCK-tc, only combinations with OriF/OriR from *oriT/oriV* region of pKNOCK-tc were used. Two fragments were obtained from these combinations. All these fragments were ligated and transformed into pGEM T easy vector prior to DNA sequencing. The resultant amplicon from *rpoN* mutant of J2315 showed an identical sequence to the *Burkholderia cenocepacia* J2315 *rpoN* gene of the wild type and the chloramphenicol cassette of pKNOCK-cm at 5'-end and 3'-end, respectively with pKNOCK vector backbone in between them. This DNA alignment confirmed the insertional mutagenesis was successfully achieved and verifies that the strategy can be used for screening of other mutants. The *rpoNI* mutant of *Burkholderia xenovorans* LB400 and two other *ntrC* mutants of both *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 also showed the same pattern of

sequence with partial sequence identical to its original *rpoN* gene sequence and the remaining sequence resemble the vectors backbone and antibiotic resistance cassette of pKNOCK-cm. Since the *rpoN2* mutant of *Burkholderia xenovorans* LB400 was using *oriT/oriV* sequence in its primer combination, the amplicon consist of partial *rpoN2* original sequence and *oriT/oriV* was obtained. The amplification of partial *rpoN* gene associated with partial pKNOCK sequence indicates the successful mutant construction.

4.9 Summary

The knock-out system for *rpoN* and *ntrC* genes for both *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 was successfully developed using a series of pKNOCK suicide vectors via homologous recombination. The mutant detection using PCR amplification with combination of primers for *rpoN* and *ntrC* genes and primers designed based on internal sequence of pKNOCK vector able to confirm the construction of mutants when complemented with DNA sequencing.