CHAPTER 5

PHYSIOLOGICAL AND METABOLIC CHARACTERISTICS OF THE SINGLE-GENE KNOCKOUT MUTANT STRAINS

5.0 Introduction

The *rpoN* gene is known for its involvement in nitrogen assimilation in which the mechanisms also involves nitrogen response *ntrB* and *ntrC*. By knocking out both *rpoN* and *ntrC* genes separately, the effects of loss-of-function of these genes towards nitrogen utilisation can be determined. This is not only served as indicator that inactivation of *rpoN* or *ntrC* genes were successful but also the function of *rpoN* gene in *Burkholderia* species.

Another physiological characteristic that has an important role in bioremediation efficiency of the bacteria is the ability to form biofilm. Microbes are usually found in close association with surface where they stick together with the slime they secreted (Wimpenny *et al.* 2000; Costerton *et al.* 1999). Due to their high microbial biomass and ability to immobilize compounds, biofilm systems are suitable for the treatment of recalcitrant compounds. Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival (especially during unfavourable condition) as they are protected within the matrix (Decho, 2000). Saldias *et al.* (1998) demonstrated that RpoN play an important role in formation of biofilm in *Burkholderia cenocepacia* K56-2.

Other than biofilm formation, motility is also an important factor not only in pathogenecity but also in bioremediation process. Motility involves the development of flagella or pilin that contributed in mobilisation of the cells. It was demonstrated by Saldias *et al*. (1998) that RpoN might play an important role in flagella development in *Burkholderia cenocepacia* K56-2. The inactivation of *rpoN* genes might abolish or reduce the motility of the bacteria thus the chemotactic ability towards target compounds will be reduced. In this study, the motility of the wildtype *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 and their mutants were determined using the motility agar plate technique.

5.1 Nitrogen utilisation by *Burkholderia cenocepacia* J2315, *Burkholderia xenovorans* LB400 and their single-gene knockout mutants

Comparison of nitrogen utilisation is very important as both *rpoN* gene and *ntrC* genes are involved in the regulation of nitrogen metabolism. Six different nitrogen sources were used to study the effect of mutation of the *rpoN* and *ntrC* genes on nitrogen utilisation particularly with regard to the time taken by the mutants to overcome the lag phase of growth and then multiply themselves as compared to the wildtype. Ammonium (NH4-), nitrate (NO3-), histidine (His), alanine (Ala), asparagines (Asn) and glutamine (Gln) were used as the sole nitrogen source for growth of the NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) of *Burkholderia cenocepacia* J2315 and wildtype *Burkholderia cenocepacia* J2315 [Figure 5.1(A to F)]. ANOVA analysis of nitrogen utilisation assay comparing the NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) with wildtype *Burkholderia cenocepacia* J2315 showed that there was no significant difference in utilisation of

alanine for both NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) compare to wildtype *Burkholderia cenocepacia* J2315 with p-values of 0.304 and 0.301, respectively. However both NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) show significant differences in utilisation of ammonium (p-values of 0.008 and 0.003), nitrate (p-values of 6.0×10^{-6} and 1.5×10^{-5}), histidine (p-values of 3.0×10^{-7} and 3.0×10^{-7}), asparagines (p-values of 0.0018 and 0.0002) and glutamate (p-values of 0.0019 and 0.003), respectively compared to wildtype *Burkholderia cenocepacia* J2315.

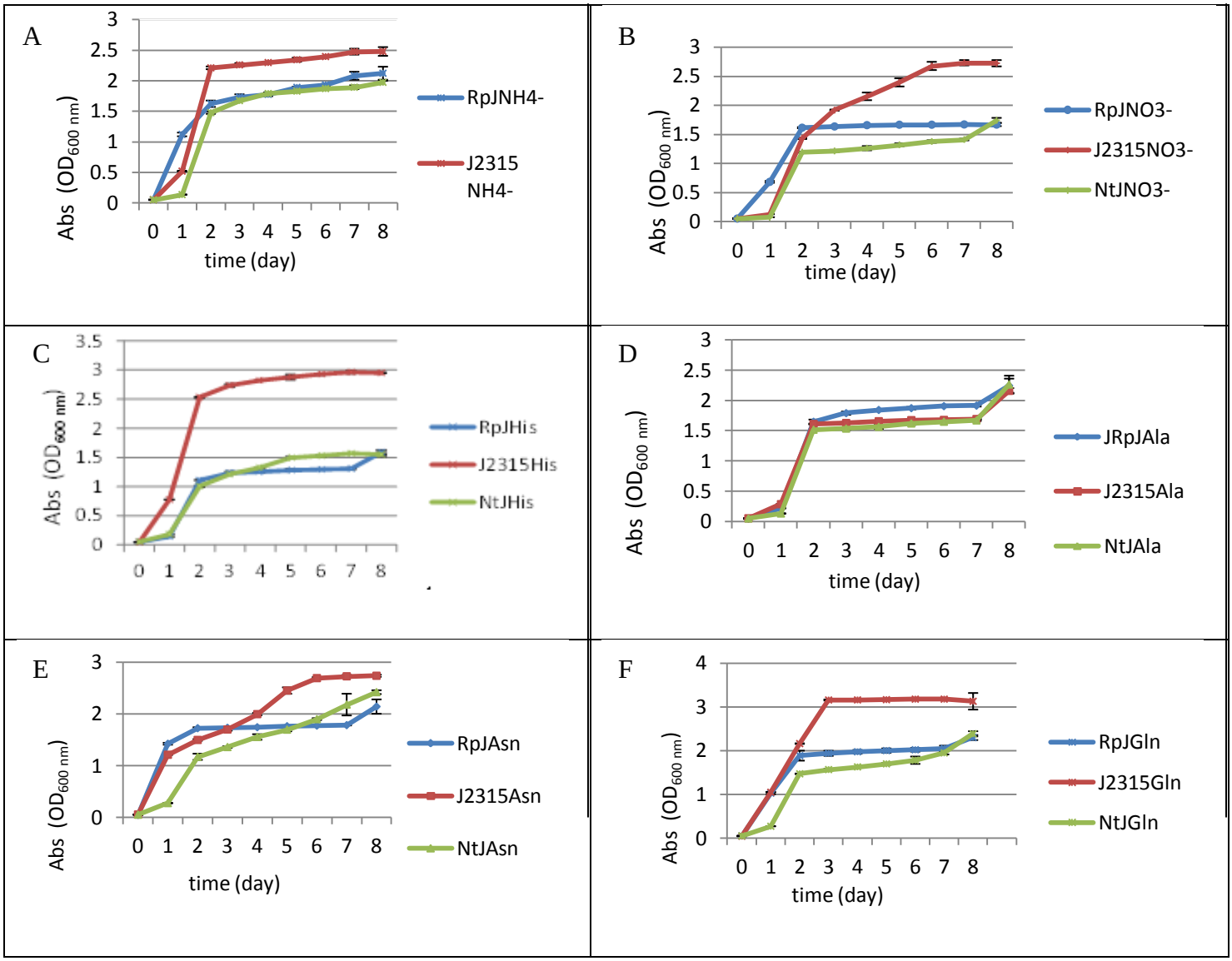

Figure 5.1: Growth curves comparison of NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) to wildtype *Burkholderia cenocepacia* J2315 in M9 minimal media supplemented with various nitrogen sources. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

A: Utilisation of ammonium by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).
B: Utilisation of nitrate by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).
C: Utilisation of histidine by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).
D: Utilisation of alanine by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).
E: Utilisation of asparagine by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).
F: Utilisation of glutamine by wildtype *Burkholderia cenocepacia* J2315 (J2315), NRPJ (*rpoN* mutant) (RpJ) and NNTJ (*ntrC* mutant) (NtJ).

Nitrogen utilisation assay was also conducted for NRPLB (*rpoN1*mutant), NRP2LB (*rpoN2* mutant), NNTLB (*ntrC* mutant) and wildtype *Burkholderia xenovorans* LB400 [Figure 5.2(A to F)]. The effect of mutation of *rpoN1, rpoN2* and *ntrC* genes in *B. xenovorans* LB400 was found to be different from that of *Burkholderia cenocepacia* J2315. The NNTLB (*ntrC* mutant) showed significant differences from wildtype *Burkholderia xenovorans* LB400 in ammonium utilisation with p-value of 3.54×10^{-5}. The NRPLB (*rpoN1*mutant) show higher growth (μ=0.33) compared to wildtype *Burkholderia xenovorans* LB400 (μ= 0.06) while NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant) were impaired. The growth of wildtype *Burkholderia xenovorans* LB400 and its mutants were impaired when supplied with nitrate as sole nitrogen source.

While the total growth of the NRPLB (*rpoN1*mutant) and NRP2LB (*rpoN2* mutant) were affected when using histidine and asparagine as sole nitrogen sources, only utilisation of asparagine in NRPLB (*rpoN1*mutant) and NRP2LB (*rpoN2* mutant) was found impaired while the utilisation of histidine is reduced in the NRP2LB (*rpoN2* mutant) compared to wildtype *Burkholderia xenovorans* LB400. However there was no significant difference that was observed in utilisation of histidine (p-value of 0.437) and asparagine (p-value of 0.872) by NNTLB (*ntrC* mutant). The NRP2LB (*rpoN2* mutant) showed no significant difference in total growth compared to wild type when supplemented with alanine while it was impaired in the NRPLB (*rpoN1*mutant) and NNTLB (*ntrC* mutant).

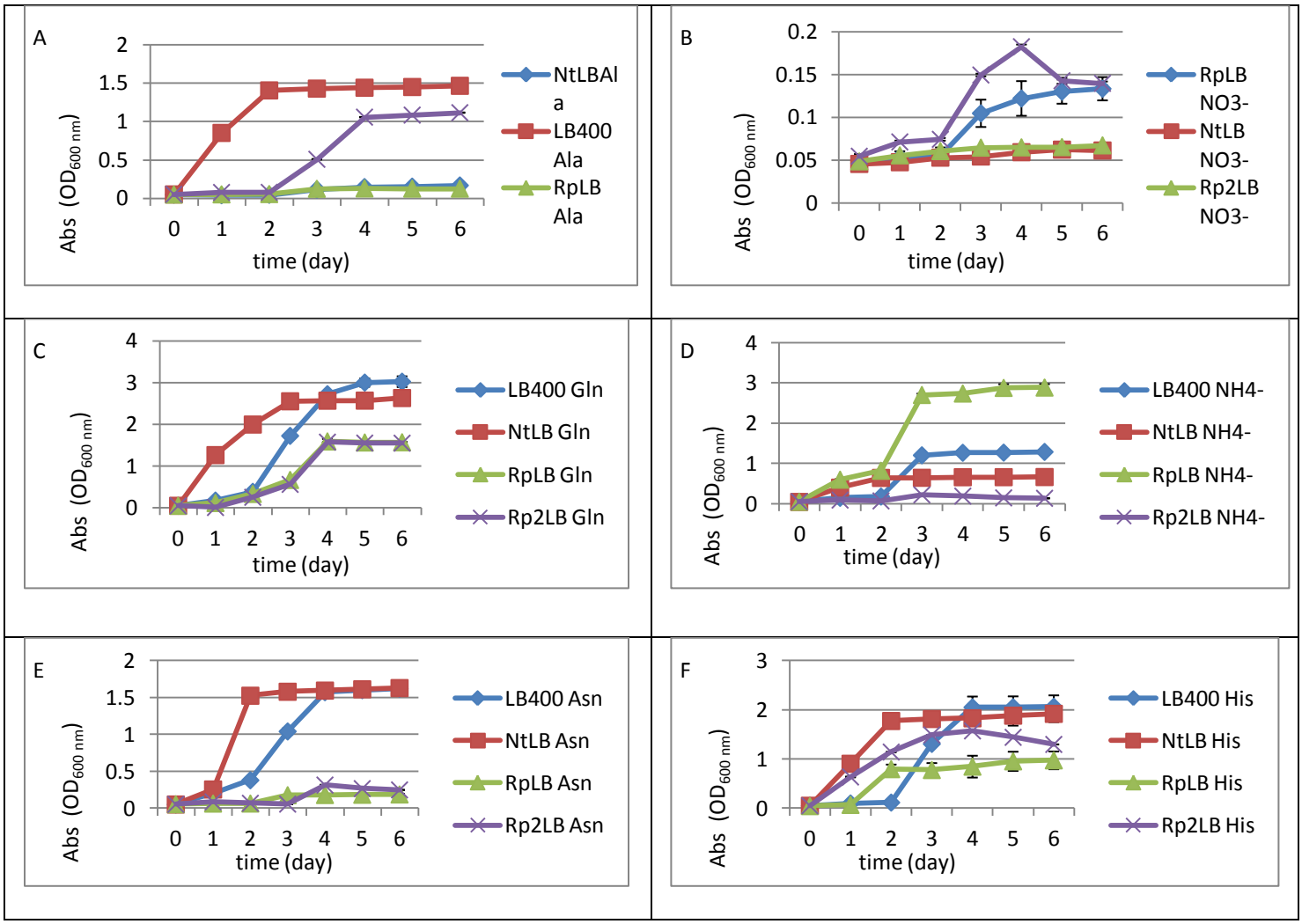

Figure 5.2: Growth curves comparison of the NRPLB (*rpoN1*mutant), NRP2LB (*rpoN2* mutant), NNTLB (*ntrC* mutant) and wildtype *Burkholderia xenovorans* LB400 in M9 minimal media supplemented with various nitrogen sources. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

A: Utilisation of ammonium by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).
B: Utilisation of nitrate by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).
C: Utilisation of histidine by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).
D: Utilisation of alanine by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).
E: Utilisation of asparagine by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).
F: Utilisation of glutamine by wildtype *Burkholderia xenovorans* LB400 (LB400), NRPLB (*rpoN1* mutant) (RpLB), NRP2LB (*rpoN2* mutant) (Rp2LB) and NNTLB (*ntrC* mutant) (NtLB).

The growth rate during the exponential phase was calculated based on the equations: $\mu = \ln O.D/t_d$. The growth rate for NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) were reduced in utilisation of ammonia by 40% and 52.5%, histidine 89% and 99% and glutamine 18% and 51%, respectively (Table 5.1). Although the growth in alanine did not show any significant difference, but when supplied with nitrate and asparagine as sole nitrogen source, the growth rate of NRPJ (*rpoN* mutant) was higher compared to wildtype *Burkholderia cenocepacia* J2315 while in NNTJ (*ntrC* mutant) the growth rate was reduced. This result showed that *rpoN* and *ntrC* genes involve in nitrogen utilisation by *Burkholderia cenocepacia* J2315. Although the inactivation *of rpoN* and *ntrC* genes of *Burkholderia cenocepacia* J2315 did not totally abolish the ability of nitrogen utilisation, the growth rates observed were reduced significantly.

However the results of *Burkholderia xenovorans* LB400 show different patterns from *Burkholderia cenocepacia* J2315. Inactivation of *rpoN1* gene of *Burkholderia xenovorans* LB400 reduced the growth of the bacteria in alanine, asparagine, histidine and glutamine respectively. However, the growth rate of NRPLB (*rpoN1*mutant) was increased in ammonium when compared to wildtype *Burkholderia xenovorans* LB400 which is contrast with *rpoN2* gene inactivation where the growth rate of the NRP2LB (*rpoN2* mutant) was reduced significantly compared to wildtype *Burkholderia xenovorans* LB400. This indicates that *rpoN2* gene might play an important role in ammonium utilisation for *Burkholderia xenovorans* LB400. For alanine, asparagine, histidine and glutamine, the NRP2LB (*rpoN2* mutant) growth rates were reduced which suggested that inactivation of the *rpoN2* gene affects the assimilation of nitrogen sources even though not totally abolished. However the results with nitrate showed that while

utilisation by the parental isolate was poor, as compared to some of the other nitrogen sources, the NRPLB and NRP2LB mutants failed to grow on nitrate and this observation was confirmed by repeating the experiments in solid cultures even with extended incubation of up to 10 days. This result suggests that the loss of the *rpoN* gene might affect the expression of key enzymes in nitrate utilisation by *Burkholderia xenovorans* LB400. The time taken for NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were delayed ranging from 1-2 days compared to wildtype *Burkholderia xenovorans* LB400.

Table 5.1: Growth rate (μ h^{-1}) of the bacteria grown in different nitrogen sources.

	Growth rate (μ) h^{-1}						
	J2315 wildtype	NRPJ (*rpoN* mutant)	NNTJ (*ntrC* mutant)	LB400 wildtype	NRPLB (*rpoN1* mutant	NRP2LB (*rpoN2* mutant)	NNTLB (*ntrC* mutant)
Ammonia	0.40	0.24	0.19	0.06	0.33	Na	Na
Nitrate	0.18	0.24	0.09	Na	Na	Na	Na
Histidine	0.46	0.05	0.005	0.18	Na	0.11	0.15
Alanine	0.26	0.25	0.21	0.08	Na	0.01	Na
Asparagine	0.20	0.27	0.08	0.11	Na	Na	0.12
Glutamine	0.39	0.32	0.19	0.25	0.17	0.11	0.24

5.2 Biofilm formation assay

The ability of the NRPLB (*rpoN1*mutant), NRP2LB (*rpoN2* mutant), NNTLB (*ntrC* mutant), NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) as well as the wildtype strains of *Burkholderia xenovorans* LB400 and *Burkholderia cenocepacia* J2315 to produce biofilm were examined. The NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) of *Burkholderia cenocepacia* J2315 showed reduced biofilm formation by approximately 46% and 42% respectively (Figure 5.3). Statistical analysis was performed to determine the relationship between wildtype *Burkholderia cenocepacia* J2315 and NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant). Both NRPJ (*rpoN* mutant) *and* NNTJ (*ntrC* mutant) show significant differences to wildtype *Burkholderia cenocepacia* J2315 with p-values of 0.003 and 0.006 respectively which is lower than $\alpha = 0.05$. However, no significant difference was obtained when comparing the NRPLB (*rpoN1*mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant), with wildtype *Burkholderia xenovorans* LB400 (Figure 5.4).

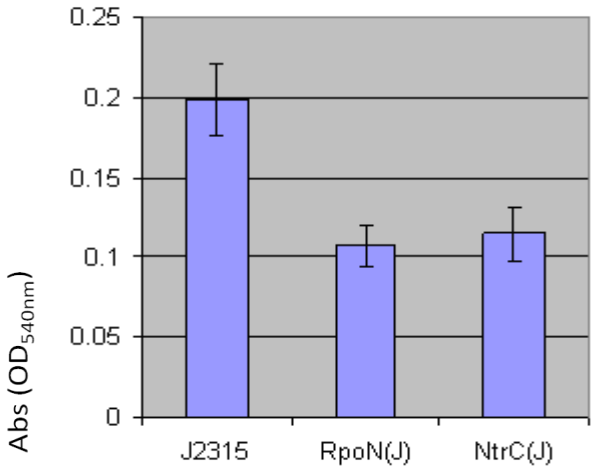

Figure 5.3: Quantitative comparison of biofilm formation by *Burkholderia cenocepacia* J2315, NRPJ (*rpoN* mutant) [*RpoN* (J)] and NNTJ (*ntrC* mutant) [*NtrC*(J)]. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

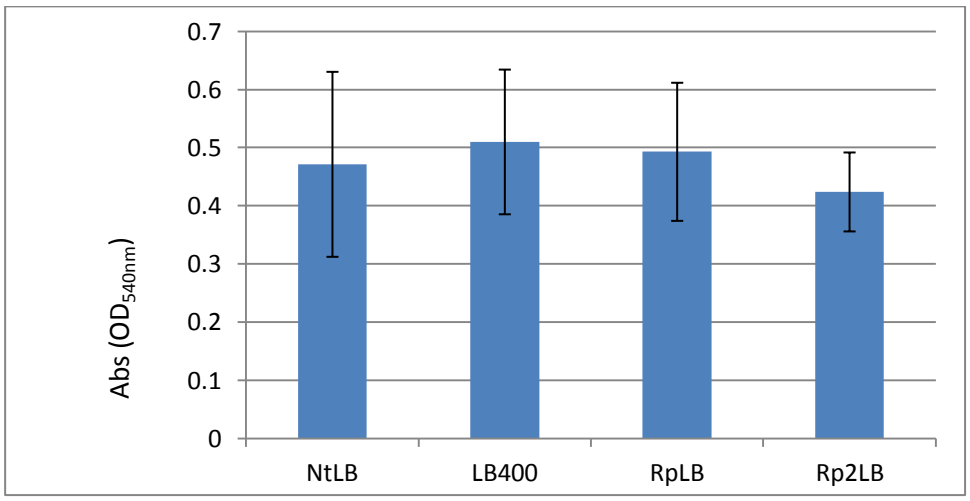

Figure 5.4: Quantitative comparison of biofilm formation by *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant). Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

5.3 Motility assay

Motility assays on NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) were performed for 48h where diameter measurements of the colony were taken every 24h. Figures 5.5A, 5.5B and 5.5C show the colony formed by wildtype *Burkholderia cenocepacia* J2315, NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) on motility agar plates, respectively.

A) Wildtype *Burkholderia cenocepacia* J2315

B) NRPJ (*rpoN* mutant)

C) NNTJ (*ntrC* mutant)

Figure 5.5: Motility assay on motility agar plates, incubated at 37°C for 48h.

The average colony diameters measured following 24h incubation for wildtype *Burkholderia cenocepacia* J2315, NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) were 2.8 cm, 2.4 cm and 2.5 cm, respectively after 48h incubation, the diameter increased to 4.0 cm, 3.7 cm and 3.9 cm, respectively (Figure 5.6). ANOVA analysis showed that there was no significant difference between NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) from wildtype *Burkholderia cenocepacia* J2315 with p-values of 0.514 and 0.821, respectively of which were greater than the significance level ($\alpha = 0.05$).

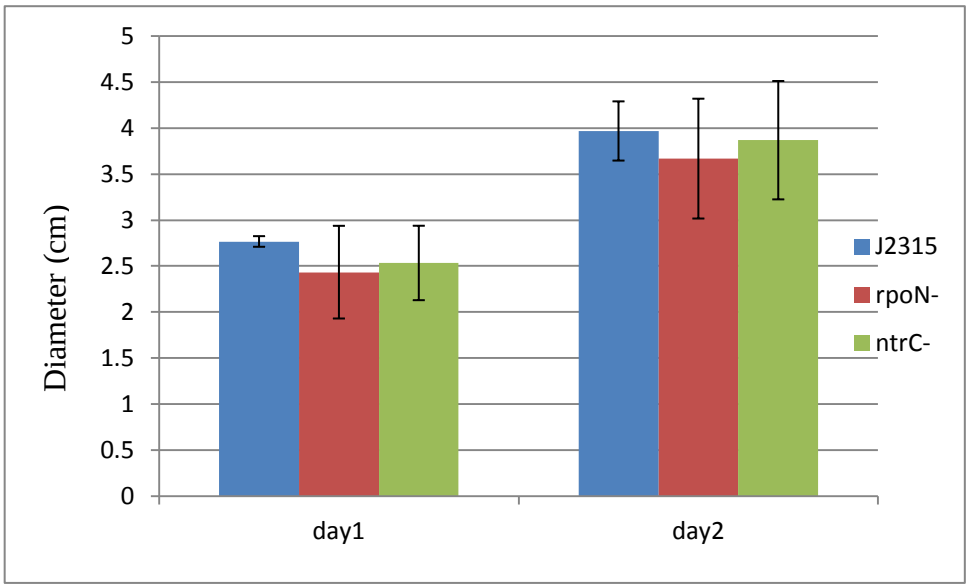

Figure 5.6: Average colony sizes of wildtype *Burkholderia cenocepacia* J2315, NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) measured after 24h and 48h of incubation on motility agar plates. Three independent experiments were performed in triplicate. Error bars represent the standard error of the mean.

The motility assay was also performed on wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant) to observe the effect of loss of *rpoN* and *ntrC* function towards motility. The average colony diameters measured after 24h incubation for wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant) were 3.75 cm, 2.6 cm, 1.9 cm and 3.7 cm, respectively. After 48h incubation, the diameters for wildtype *Burkholderia xenovorans* LB400 and NNTLB (*ntrC* mutant) were doubled (7.7 cm and 8 cm, respectively) while NRPLB (*rpoN1* mutant) showed slight growth in 48h incubation (3.7 cm). However, the NRP2LB (*rpoN2* mutant) diameter increased 3-fold to 6.2 cm in 48 hours incubation (Figure 5.7). ANOVA analysis revealed that NRPLB

(*rpoN1* mutant) showed significant difference from wildtype *Burkholderia xenovorans* LB400 with p-value lower than $\alpha = 0.05$ whereas no significant difference were obtained between NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant).

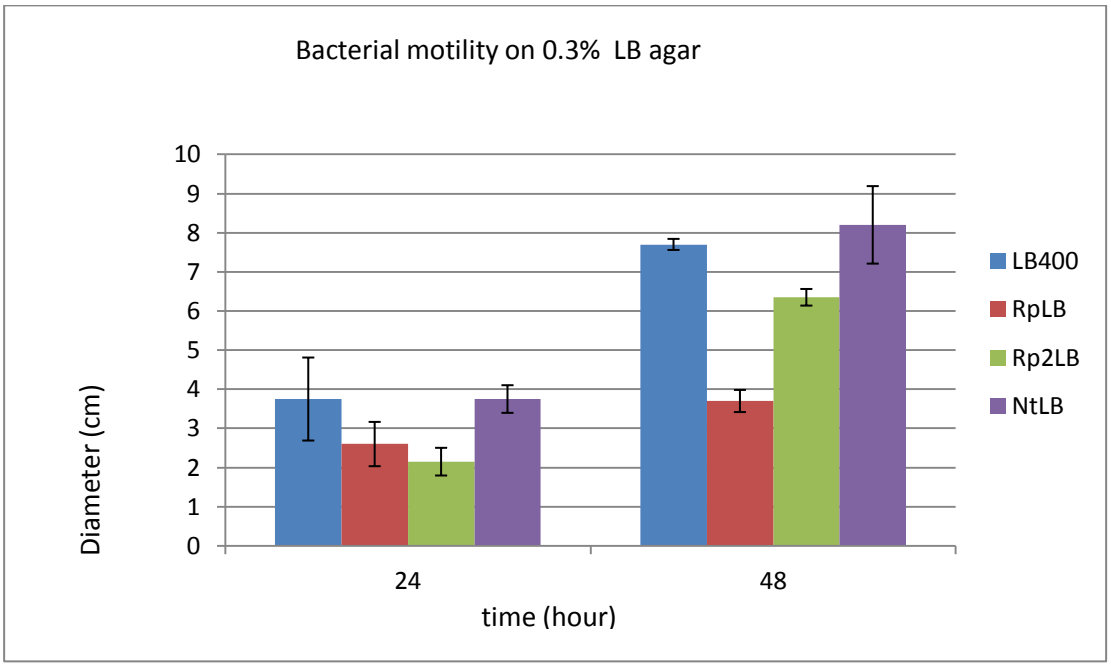

Figure 5.7: Average colony sizes of wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant) measured after 24h and 48h of incubation on motility agar plates. Three independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

LB400 : Wildtype of *Burkholderia xenovorans* LB400
RpLB : NRPLB (*rpoN1* mutant)
Rp2LB: NRP2LB (*rpoN2* mutant)
NtLB : NNTLB (*ntrC* mutant)

5.4 Discussion

The *rpoN* gene has been demonstrated to involve in several physiological characteristics of the bacterial such as nitrogen utilisation, biofilm formation and motility. In order to confirm the construction of the single knockout mutants, further analyses were conducted to examine the metabolic and physiological responses of the mutants in terms of their nitrogen utilisation, biofilm formation and motility.

RpoN and NtrC were well known involved in metabolism of nitrogenous compounds. Previous work by Jones *et al*. (2007), Zhao *et al*. (2005) and Hendrikson *et al*. (2001) showed significant changes in nitrogen utilisation in a *Pseudomonas* sp. with *rpoN* loss-of-function mutants. Investigations of the nitrogen utilisation profile of the NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) as compared to the wildtype *Burkholderia cenocepacia* J2315 revealed that ammonia, nitrate, histidine, asparagines and glutamine utilisation were impaired while alanine utilisation was unaltered. These results suggest that loss of *rpoN* and *ntrC* function might affect the expression of key enzymes involved in nitrogen metabolism.

However the nitrogen utilisation profiles in NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant) were found different from NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant). The nitrate utilisation was impaired in NNTLB (*ntrC* mutant) and NRP2LB (*rpoN2* mutant) while reduction in utilisation was observed in NRPLB (*rpoN1* mutant). However, the growth of wildtype *Burkholderia xenovorans* LB400 was also low indicating the nitrate (inorganic source of nitrogen) is not readily utilised by *Burkholderia*

xenovorans LB400. The glutamine utilisation was reduced in both NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) which indicated the loss of *rpoN* function might alter the ability to utilise glutamine in *Burkholderia xenovorans* LB400. Although growth in both histidine and asparagine were affected when *Burkholderia xenovorans* loss the function of *rpoN* gene, only the growth in asparagine was found to be impaired for both NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) whereas for histidine utilisation, both NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) showed some reduction compared to wildtype *Burkholderia xenovorans* LB400. No significant differences between NNTLB (*ntrC* mutant) and wildtype *Burkholderia xenovorans* LB400 were observed in both asparagines and histidine utilisation. The result suggested that only alteration of *rpoN1* and *rpoN2* genes might change the utilisation of histidine and asparagines. Different phenomenon was observed in alanine utilisation where inactivation of *rpoN1, rpoN2 and ntrC* impaired the growth of the NRPLB (*rpoN1* mutant), NRP2LB (*rpoN2* mutant) and NNTLB (*ntrC* mutant). Some of the mutants also exhibited enhanced activity in utilisation of nitrogenous compounds. The loss of *rpoN1* function in NRPLB (*rpoN1* mutant) showed higher growth in utilisation of ammonia but reduced in NNTLB (*ntrC* mutant). Inactivation of *rpoN1* gene in *Burkholderia xenovorans* LB400 might affect other genes involved in ammonia metabolism upstream or downstream of the gene subsequently increased the growth of the cell. The growth of NRP2LB (*rpoN2* mutant) was impaired when supplemented with ammonia suggesting that loss of function of *rpoN2* gene might affect the metabolism of ammonia in LB400.

The physiological response such as biofilm formation was also examined in this study. The ability of NRPJ (*rpoN* mutant) and NNTJ (*ntrC* mutant) of to produce biofilm was

reduced by 46% and 42%, respectively compared to wildtype *Burkholderia cenocepacia* J2315. The result demonstrated that inactivation of *rpoN* and *ntrC* genes did not completely abolish biofilm formation. However the result suggested that *rpoN* gene in J2315 might be involve in biofilm formation. Even though *ntrC* gene is well known for its involvement in nitrogen metabolism rather than biofilm, its inactivation might affects other gene(s) that are involve in biofilm formation and subsequently reduce its ability. The biofilm formation was unaffected with inactivation of *rpoN* and *ntrC* genes in *Burkholderia xenovorans* LB400 which suggested that both genes were not involve in biofilm formation in *Burkholderia xenovorans* LB400.

Since motility is a well characterised property associated with biofim formation (O'Toole *et al*., 2000; Pratt and Kolter, 1998), the motility assay was also conducted. The motility assay was conducted over a period of 48 hours. No significant difference in biofilm formation ability was observed between the NRPJ (*rpoN* mutant) and wildtype *Burkholderia cenocepacia* J2315. This suggested that *rpoN* gene is not required for motility in *Burkholderia cenocepacia* J2315 although clonally related *Burkholderia cenocepacia* K56-2 needs RpoN for motility (Saldias *et al.*, 2008). For *Burkholderia xenovorans* LB400, even though inactivation of *rpoN2* gene did not show any significant difference from wildtype *Burkholderia xenovorans* LB400, inactivation of *rpoN1* gene reduced the motility in *Burkholderia xenovorans* LB400 which indicated the involvement of *rpoN1* gene in motility of *Burkholderia xenovorans* LB400. The motility assay also showed that *ntrC* gene in both *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 might not be concerned with motility as its primary function and is

more related to nitrogen utilisation as inactivation of *ntrC* genes in both strain showed no significant differences from their wildtype counterpart.

5.5 Summary

The mutants were further characterised for their physiological and metabolic changes from the wildtype. In general, the inactivation of *rpoN* and *ntrC* genes of both *Burkholderia cenocepacia* J2315 and *Burkholderia xenovorans* LB400 affects the nitrogen utilisation suggesting the involvement of *rpoN* and *ntrC* genes in nitrogen assimilation process. However, for *rpoN* genes in *Burkholderia xenovorans* LB400, the ability to utilise nitrogen is different between *rpoN1* and *rpoN2* gene. This is further demonstrated with ability to form biofilm and motility which show different output between these two *rpoN* genes of *Burkholderia xenovorans* LB400. These results suggest that *rpoN1* and *rpoN2* genes play a different role in their function as they are not closely related.