AlkB1 as a key enzyme for alkane degradation in *Pseudomonas aeruginosa* PAO1 strain

R.R.P.Y.K. Rathnayaka[1#], Erandika H. Hemamali[1,2] Cristina Bez[3], Iris Bertani[3], Vittorio Venturi[3] and Inoka C. Perera[1]

[1]University of Colombo, Colombo, Sri Lanka
[2]Currently at Saint Louis University, St. Louis, Missouri, United States of America
[3]International Center for Genetic Engineering and Biotechnology, Trieste, Italy
#Corresponding author: yasarakithmini@gmail.com

1 Introduction

Alkanes are a common type of contaminant present in the environment. They are the main constituent in crude oil (Wu et al., 2018). Alkanes can make up as much as 90% of crude oil, which is a significant portion (Muriel-Millán et al, 2019). Alkanes are chemically inert and apolar (Labinger and Bercaw, 2002). Therefore, many microbes present difficulties in metabolizing alkanes because of their limited solubility in water, the propensity to concentrate in cell membranes, and the energy requirement for molecular activation (Fernando, 2009). However, some aerobic and anaerobic microorganisms can use diverse alkanes as their carbon and energy source.

Use of biological organisms to remove contaminants present in the environment is referred as bioremediation (Medic et al, 2020). Microorganisms such as bacteria and fungi are widely used in bioremediation processes to remove different types of pollutants like hydrocarbons, pesticides, and heavy metals. Bioremediation is becoming more popular due to its efficiency, relatively low cost and environmental friendliness compared to other treatment techniques (Wang et al, 2017).

Pseudomonas aeruginosa is an n-alkane-degrading bacteria and it can degrade long-chain alkanes efficiently (Belhaj et al., 2002). It is a common bacterium that inhabits both soil and water environments and can infect plants and animals as an opportunistic pathogen (Marin et al, 2003). *P. aeruginosa* undergoes terminal oxidation of n-alkanes. Alkane 1-monooxygenase enzyme catalyzes this oxidation reaction with the aid of accessory proteins rubredoxin and rubredoxin reductase. Two of the alkane monooxygenases designated AlkB1 and AlkB2, are encoded by *P. aeruginosa*. Alkanes from C12 to C22 are oxidized by AlkB2, while n alkanes from C16 to C24 are oxidized by AlkB1 (Marin et al., 2003). The *alkB1* and *alkB2* genes may not express themselves simultaneously in the cell, indicating that distinct transcription factors are responsible for controlling each gene's expression. The objective of this study is to construct *P. aeruginosa* PAO1 knockout *alkB1* mutant and compare the growth of *P. aeruginosa* PAO1 and its knockout *alkB1* mutant, when exposing to different lengthy n-alkanes.

2 Methodology

2.1 Construction of *P. aeruginosa* PAO1 knockout *alkB1* mutant

Upstream and downstream regions of *alkB1* gene were PCR amplified by using alkB1 fr5 kpn, alkB1 fr5 xho, alkB1 fr6 BglII, and alkB1 fr6 Xba primers. PCR fragments were then cloned into pGEM-T plasmid and transformed into *Escherichia coli* DH5〈 chemical competent cells and positive transformants were isolated by blue/white colony selection followed by colony PCR. Plasmids (pGEM-T_alkB1 fr5 and pGEM-T_alkB1 fr6) were isolated using EuroClone plasmid extraction kit. Extracted plasmids were sequenced to confirm the restriction sites. All fragments were digested with respective enzymes and gel purified. Next, tetracycline resistance gene (Tc) was PCR amplified from pGEM_Tc plasmid, digested with XhoI and, gel purified. Subsequently, fr 5, fr 6, and Tc were cloned into pBluescript SK +/- plasmid in the order of Fr5_Tc_Fr6. Positive clones were confirmed by colony PCRs and restriction digestion. The positive plasmid was named pBluescript_AlkB1. The Fr5_Tc_Fr6 fragment was isolated from pBluescript_AlkB1 and cloned into pEX suicide plasmid (pEX_AlkB1) and transformed into *E. coli* donor strain. The pEX_AlkB1 was subsequently transferred to *P. aeruginosa* PAO1 via conjugation (Hmelo et al, 2015). The knockout mutants were isolated through tetracycline selection, followed by sucrose counter selection at room temperature. Sucrose resistant colonies were further confirmed by colony PCR.

2.2 Growth of *Pseudomonas aeruginosa* PAO1 and its *alkB1* mutant on glucose

An overnight culture of *Pseudomonas aeruginosa* PAO1 was prepared in M9 minimal medium supplement with glucose as the source of carbon. *P. aeruginosa* cells were harvested by centrifugation (4000g, 12 minutes) from the overnight culture and any remaining glucose was removed by washing the cells in M9 medium without glucose (Hemamali et al,

2022). The M9 media (10 ml) supplemented with 0.2 % (w/v) glucose was inoculated with 20 µl of *P. aeruginosa* cells diluted to an OD 0.01 and incubated in a shaking incubator under aerobic conditions at 37 ºC and OD_{600} was measured using NanoDrop One^c (Thermo scientific) hourly for 24 hours. Same steps were repeated for *⊗alkB1* mutant of PAO1 and finally growth curves were plotted as OD_{600} vs time.

2.3 Growth of *Pseudomonas aeruginosa* PAO1 and its ⊗*alkB1* mutant on different lengthy n-alkanes

An overnight culture of *Pseudomonas aeruginosa* PAO1 was prepared in M9 minimal medium supplement with glucose as the source of carbon. *P. aeruginosa* cells were harvested by centrifugation (4000g, 12 minutes) from the overnight culture and any remaining glucose was removed by washing the cells in M9 medium without glucose. The M9 media (10 ml) supplemented with 0.2 % (v/v) or (w/v) n-hexadecane, n-eicosane, n-docosane, and n-octacosane separately, were inoculated with 20 µl of *P. aeruginosa* cells diluted to an OD 0.01 and incubated in a shaking incubator under aerobic conditions at 37 ºC. *P aeruginosa* cells were harvested daily from three flasks for each alkane and the cell pellet was entirely dissolved in 10 ml of M9 media free-from alkanes and the OD_{600} was measured using NanoDrop One^c (Thermo scientific) daily. Same steps were repeated for *⊗alkB1* mutant of PAO1 and finally growth curves were plotted as OD_{600} vs time.

3 Results and Discussion

Growth of PAO1 and its *alkB1* knockout mutant was observed over time in M9 minimal media containing glucose as the carbon source. Both can utilize glucose efficiently and reaches the stationary phase within 24 hours (Fig.1). PAO1 starts exponential phase after 8 hours and reaches stationary phase by 21 hours while *⊗alkB1* mutant starts exponential phase after 9 hours and reaches stationary phase by 23 hours (Fig.1). During stationary phase, cell growth of PAO1 is higher compared to *⊗alkB1* mutant. But even without the *alkB1* gene, the mutant can utilize glucose efficiently.

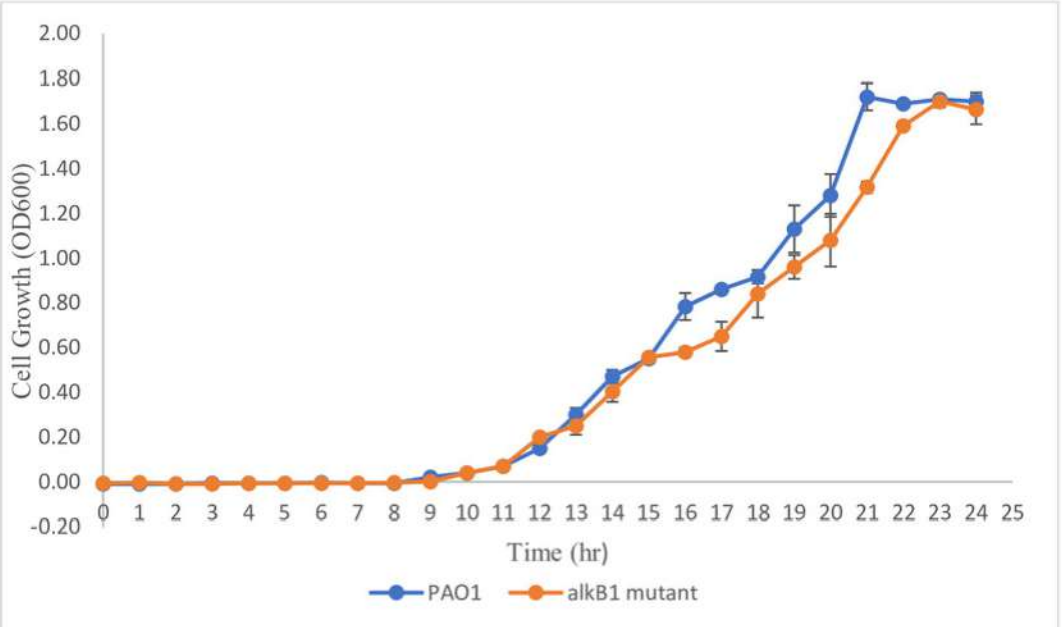

Fig.1: Growth of *Pseudomonas aeruginosa* PAO1 and its *⊗aklB1* mutant on glucose ($C_6H_{12}O_6$)

The utilization of n-alkanes by PAO1 and its *⊗alkB1* mutant was investigated through the observation of their cell growth over time in M9 minimal media that included a range of n-alkanes, from C16 to C28 as the sole carbon source. Cell growth of *⊗alkB1* mutant is lower compared to PAO1 in all the exposed n-alkanes (n-hexadecane, n-eicosane, n-docosane, n-octacosane) which indicate its lower utilization of n alkanes (Fig.2).

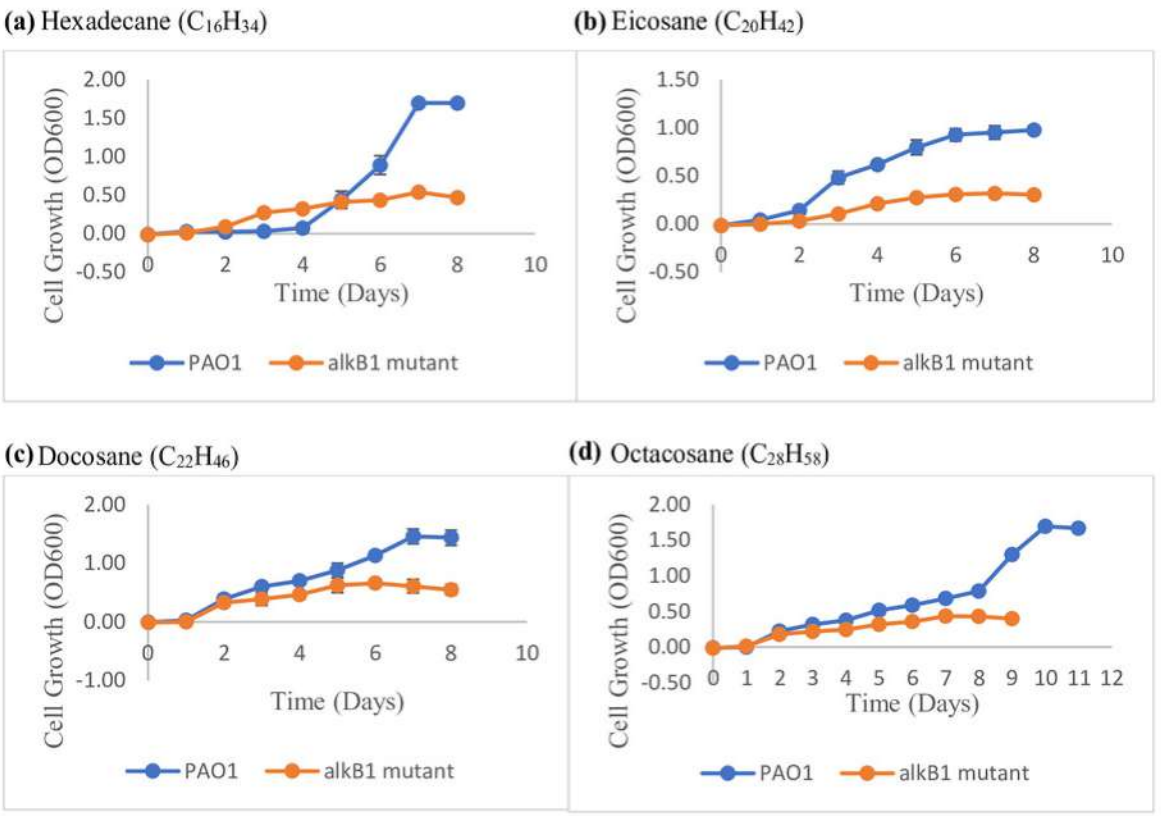

Fig.2: Growth of *Pseudomonas aeruginosa* PAO1 and its *⊗aklB1* mutant on different n alkanes (a) Hexadecane, (b) Eicosane, (c) Docosane, and (d) Octacosane

PAO1 exhibits highest cell growth in n-hexadecane followed by n-octacosane where stationary phase is reached in 7 days and 10 days respectively (Fig.2.a, d). *⊗alkB1* mutant shows highest cell growth in n-docosane followed by n-hexadecane where stationary phase is reached in 6 days and 7 days respectively (Fig.2.c, a). According to the growth curves, *alkB1* gene has played a major role in n-alkane utilization of PAO1. Despite the reduced growth of *⊗alkB1* mutant compared to PAO1, it retains the ability to degrade n alkanes suggesting that other genes like *alkB2* may be involved in the process of n-alkane degradation. Therefore, this study needs to be continued to construct *⊗alkB2* mutant and *⊗alkB1/⊗alkB2* double mutant and their growth curves should be compared with PAO1.

Wang et al (2017), has conducted a functional characterization of AlkB1 and AlkB2 alkane hydroxylases in *Pseudomonas aeruginosa* NY3 strain. They have constructed *P. aeruginosa* NY3 *⊗alkB1* and *⊗alkB2* mutants and *⊗alkB1/⊗alkB2* double mutant and their cell growth has been monitored in Mineral Salt medium (MSM) medium containing n-alkanes ranging from C12 to C24 for 6 days. NY3 has shown a rapid growth rate for all the n-alkanes with OD_{600} value between 0.5-0.7 within 24 hours. The double mutant had lowest growth followed by *⊗alkB2* mutant and then *⊗alkB1* mutant for all the tested n-alkanes. This implies that *alkB1* and *alkB2* are both involved in the degradation of n-alkanes, with *alkB2* having a major role.

Conclusion

In this study, growth of PAO1 and its *⊗alkB1* mutant was compared while exposing them to M9 minimal medium supplemented with glucose and n-alkanes ranging from C16 to C28. Both PAO1 and *⊗alkB1* mutant efficiently utilize glucose indicating that *alkB* genes are activated only when alkanes are available. When PAO1 and its *⊗alkB1* mutant were exposed to four n-alkanes, cell growth of *⊗alkB1* mutant is lower compared to PAO1 in all the exposed n-alkanes, which indicate its lower utilization of n-alkanes. Based on the growth curve analysis, *alkB1* gene appears to play a significant role in degrading n-alkanes. Further studies need to be conducted to construct PAO1 *alkB* knockout mutants and their growth has to be compared with PAO1 in order to understand the dynamics of *alkB* regulation of *P. aeruginosa* PAO1 strain. Understanding the role and regulation of genes involved in pollutant degradation in bacteria will help to determine novel pathways of pollutant degradation and creation of engineered organisms which can be used in bioremediation process in an industrial scale.

Keywords: *Pseudomonas aeruginosa*, alkane degradation, *alkB* gene

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