COMPARISON OF ASPARTATE TRANSCARBAMOYLASE ACTIVITY BETWEEN *Pseudomonas aeruginosa* WHICH HAS ONE CHROMOSOME AND *Burkholderia cepacia* WHICH HAS THREE CHROMOSOMES

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The pyrimidine biosynthetic pathway is essential and similar in all bacteria. The pathway from *Pseudomonas* is regulated by nucleotides which bind to the upstream region of the *pyrBC'* gene complex. Work in our lab mapped the genes and showed that the *pyrB* and *pyrC'* were part of an overlap complex. The *Pseudomonas aeruginosa* has one circular chromosome. A former *Pseudomonas* now called *Burkholderia cepacia* is similar to *P. aeruginosa* except that it contains three circular chromosomes (CI, CII, CIII) and one large plasmid. The primary chromosome named CI contains the *pyrBC'*. To our knowledge there has been no report of the activity of ATCase in *Pseudomonas* and contrasted with that of *Burkholderia*. Here, we compare the activity of ATCase in *P. aeruginosa* and *B .cepacia*. Cells of both organisms were grown in *Pseudomonas* minimal medium and in Enriched medium. The ATCase was extracted and partially purified from each sample. It is hypothesized that the *B. cepacia* has greater activity for ATCase than do the *Pseudomonas*.

ACKNOWLEDGMENTS

First of all, I would like to thank my mentor and my guide Dr. O'Donovan for having me in his lab, his guidance, his teaching, and his support are so much appreciated and will last with me as long as I live. I would like to thank our lab members for their support, Todd, Hyunju and Sara. I would like to thank Dr. Benjamin and his students for their help and Dr. Allen and his students for sharing their lab to help me with my project. I would like to thank the father of my kids, my dear husband Abdurahman Niazy for his endless support and encouragement all the time and for believing in me. My best friend, Dana thanks for your endless support and believing in me. Last but not least, Mom and Dad thank you for all your prayers and I hope I made you more proud.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Until a short while ago, prokaryotes were known to have one circular chromosome and that was a definitive disparity between prokaryotes and eukaryotes. Now it has been known that some bacteria possess more than one chromosome, linear chromosomes and linear plasmids. Still much remains to be learned about their structure and function. Multiple chromosomes has been found in *Vibrio cholerae, Vibrio parahaemolyticus Leptospira interrogans, Rhodobacter sphaeroides, Brucella melitensis, Rhizobacterium meliloti, Paracoccus denitrificans* and *Burkholderia cepacia. Agrobacterium tumefaciens* has one linear and one circular chromosome. Linear chromosomes can be found in *Streptomyces coelicolor*, *Borella burgdorferi* and *Rhodococcus fascians* (16). In this thesis the chromosomes of *Pseudomonas aeruginosa* and *Burkholderia cepacia* are examined.

The existence of multiple chromosomes in bacteria raises a lot of inquiries about any special functions, dimensions, rearrangements, mode of interactions, DNA synthesis, DNA pathway regulation, stress response, How big is the replicon, and how many more genes does it contain ? In general with multiple chromosomes, the primary replicon is larger and it contains all essential genes and theoretically, secondary chromosomes have the accessory genes. This suggests that purification of the secondary ones would be weaker due to the less usage of them (3). In contrast, another study showed that multiple copies of the same genes can be shown in both chromosomes of *Rhodobacter sphaeroides* such as the three RNA operons (rrnA, rrnB, rrnC) (16).

Chromosomes can originate from different sources, one of which is single chromosome division, another is chromosome doubling, or incorporating a large plasmid carrying its genes.

Multiple chromosomes in bacteria may be different sizes in which it's a challenge to have a synchronic replication. This can cause variation in replication rate and may lead to different gene dosage that will have a deep impact on gene expression and their developmental rates (3).

Replication of multiple chromosomes in bacteria brings into question, the replication of each chromosome is it the same as eukaryotes by which uses one mechanism that will initiate replication in all chromosomes or, different replicons within the same cell may use different factors for replication or, replicons may take advantage of some common and some different replication factors. However, sharing the same factors will lead to competition of utilizing factors and this could be avoided by using different factors or making large quantities of common factors. This might cause difficulty in regulating replication (4).

Pseudomonas aeruginosa is a motile gram negative rod with single circular chromosome. It has a total genome size of 5,900 kbp, (3). It's an aerobic organism but it can also be considered as a facultative anaerobe utilizing nitrate instead of oxygen. It has a single polar flagellum. It can be found in soil and water. It's an opportunistic human pathogen and the most common cause of hospital infection. It can cause different serious infections such as urinary tract infections, respiratory tract infections, bone infections, soft tissue infections and can cause deadly infections in patients with burns and in cystic fibrosis patients where it can colonize the airway epithelia . *P. aeruginosa* can colonize and form biofilms in many different natural and non-natural environments. *P. aeruginosa* secretes different pigments, such as pyoverdine which gives yellow green color (Fig. 4); pyocyanin which gives blue-green color, florescent pigment, and some red-brown color called pyorubin. Virulence of the organism depends on the production of specific cellular extratoxins. Exotoxin A resulting in inhibition of protein synthesis and exotoxin S helps in membrane disruption resulting in cell death. *P .aeruginosa* is resistant to

many antibiotics and disinfectants that can kill other organisms. *P. aeruginosa* infections can be treated with anti-microbial medications. *P. aeruginosa* resistances to medications occur during the course of treatment forming mutations in some chromosomal genes. This is especially a problem in hospitals with immune compromised patients (9,18, 25).

Another organism which causes serious respiratory infection, especially with cystic fibrosis patients, is caused by *Burkholderia cepacia* previously known as *Pseudomonas cepacia* which was first identified as a plant pathogen discovered in rotten onions. It is a Gram negative rod aerobic and non-spore forming, catalase positive, oxidase positive, and it can produce different non-flourescent pigments. It is found in water and soil and can survive for a long time. *B. cepacia* is able to colonize both humans and plants. Nucleotide sequencing has confirmed that *B. cepacia* doesn't belong to *Pseudomonas* and now it has its own genus. The unfamiliar distinguishing character of this organism is its very large DNA (two times the size of *E. coli* and four times that of *Haemophilus influenzae*). Unlike most bacteria *B. cepacia* has the most complex organization of multichromosomes ranging from 2-4 in different isolates and its genome size runs between 5-9 Mb (8). The strain used in this paper is ATCC25416 which has three chromosomes. It has a total genome size of 8,090kbp chromosomes with each containing 3,650kbp(CI) +3,170kbp(CII) + 1,070kbp(CIII) and a 200kbp plasmid (2). Most essential genes are present in the first chromosome CI (3). Another important element in *B. cepacia* is the wide array of insertion sequence (IS), elements which support genomic rearrangement and increase the expression of adjacent genes. *B. cepacia* not only causes infection in cystic fibrosis patients but also in chronic granulomatous patients. *B. cepacia* has an important role in agriculture as a biologic control agent. It has been shown to have a startling potential as biodegradation and biocontrol agents. *B. cepacia* can degrade toxic compounds in pesticides to use as a carbon

source. It can inhibit other plant pathogens and inhibit spore germination. Main crop diseases, such as blight due to *Alternaria solani* or *Alternaria brassicola,* can be controlled by *B. cepacia*. It can also prevent cucumber infection by *Pythium.* In order to prevent such plant diseases, *B. cepacia* can act as an environmentally friendly substitute to toxic fungicides which can't be broken down in nature (8). This use can reduce the use of pesticides and fungicides. Because of its use as an agricultural agent, B. *cepacia* developed as a potential multiantibiotic-resistant human pathogen. This led to a concern regarding the relationship between environmental and clinical isolates and then to the potential threat caused by using *B. cepacia* as a biological control agent(7). Because of the difficulty of accurate *B. cepacia* isolation and the recognition of different *B. cepacia* strains which were identified later as (*B. cepacia* complex) molecular taxonomic techniques developed such as DNA-DNA hybridization which separates *B. cepacia* isolates and that is clinically important (10).

The key function essential for DNA and RNA synthesis is pyrimidine synthesis, which is important for the survival of most organisms (19). *P. aeruginosa* and *B. cepacia* employs the pyrimidine biosynthetic pathway (Fig.1). The genes involved and the enzymes they code for are shown in the illustrated pathway. Glutamine (Gln), adenosine triphosphate (ATP) and bicarbonate are used by carbamoylphosphate synthase (CPSase) to form carbamoylphosphate, glutamate (Glu) or water, and adenosine diphosphate (ADP). Carbamoylphosphate and aspartate (Asp) are catalyzed by aspartate transcarbamolyase (ATCase) to form N-carbamoylaspartate (Fig. 2). Dihydroorotase (DHOase) catalyzes the dehydration, which forms on N-carbamoylaspartate to form dihydroorotate (DHO). Dihydroorotate dehydrogenase (DHODhase) oxidizes DHO (NAD^+-dependent) to make orotate (OA). OA and phosphoribosyl pyrophosphate (PRPP) are used by orotate phosphoribosyl transferase (OPRTase) to make orotidylate (OMP).

Orotidylate decarboxylase catalyzes the decarboxylation of OMP to uridylate (UMP). The phosphorylation of UMP by ATP is catalyzed by UMP kinase (using Mg^{+2} cofactor) to make uridine diphosphate (UDP). Nucleoside diphosphate kinase (Ndk) catalyzes the phosphorylation of UDP by ATP to make uridine triphosphate (UTP). CTP synthetase catalyzes the addition of an amine group to UTP to make CTP. Together UTP and CTP are used in the cell for RNA synthesis or changed to dNTPs and eventually used for DNA synthesis (14).

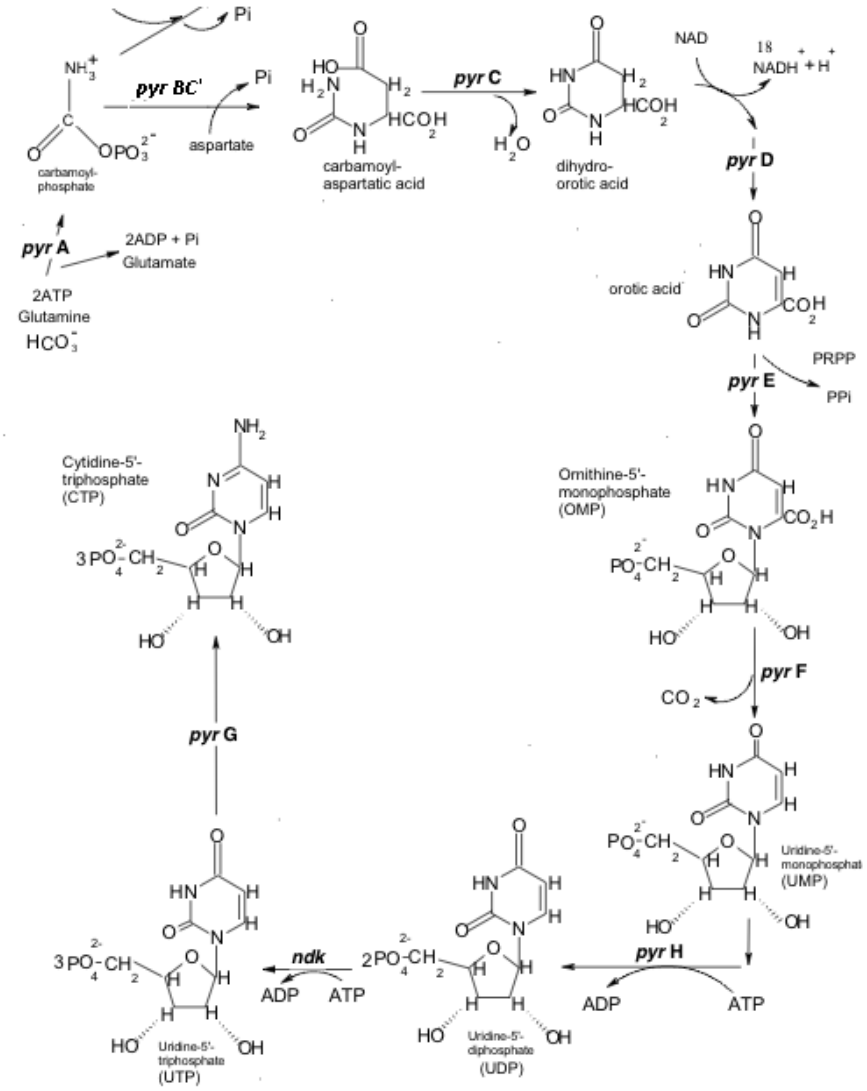

FIG. 1. Pyrimidine biosynthetic pathway.

The pyrimidine biosynthetic pathway including ATCase is found in all organisms, both in eukaryotes and prokaryotes. It's the first committed step in the pyrimidine pathway (Fig. 2). ATCases are divided into three classes according to their molecular weight and regulatory properties. The three classes (21) of bacterial ATCase are classified as, class A, B, and C (Fig.3).

Class A, ATCase is found in *Pseudomonas*. The holoenzyme molecular weight is 470-500 kDa. It exists as a dodecamer, composed of six *pyrB*-encoded 34-kDa polypeptides connected with six *pyr C'*- encoded 45-kDa polypeptides. No *pyr I*- encoded regulatory polypeptide has been found in this class of ATCase. In the 34-kDa polypeptide, the enzyme and the binding site of nucleotide are both located on the same site (1).

Class B, ATCase is found in *Escherichia.coli*. This holoenzyme has a molecular weight of 310 kDa. It exists as a dodecamer, $2B_3.3I_2$ composed of two catalytic trimers (B_3) made of six *pyrB*-encoded 33-kDa identical polypeptides, associated with three regulatory dimers (I_3) made up of six *pyrI* – encoded 17 kDa polypeptides. The *pyrI* encoded regulatory dimers have no ATCase activity. The three dimers carry binding sites for triphosphates. The *pyrB* gene encodes a 153 amino acid long regulatory polypeptide (13). The catalytic polypeptide is composed of two domains, where the carbamoylphosphate binding site is found in the amino terminus domain and the aspartate binding site is found in the carboxy terminus domain. In the regulatory polypeptide, there are also two domains, the effector binding sites for ATP and CTP, which are located in the amino terminus, and a zinc binding site found at carboxy terminus domain (27).

Class C, ATCase is found in *Bacillus subtilis.* This holoenzyme has a molecular weight of 100 kDa and is composed of three identical chains forming catalytic active trimers, each a 23 kDa polypeptide chain, encoded by *pyrB* gene. It show hyperbolic kinetics for substrates,

aspartate and carbamoyl phosphate, and is not controlled by nucleotide effectors such as ATP, CTP, or UTP.

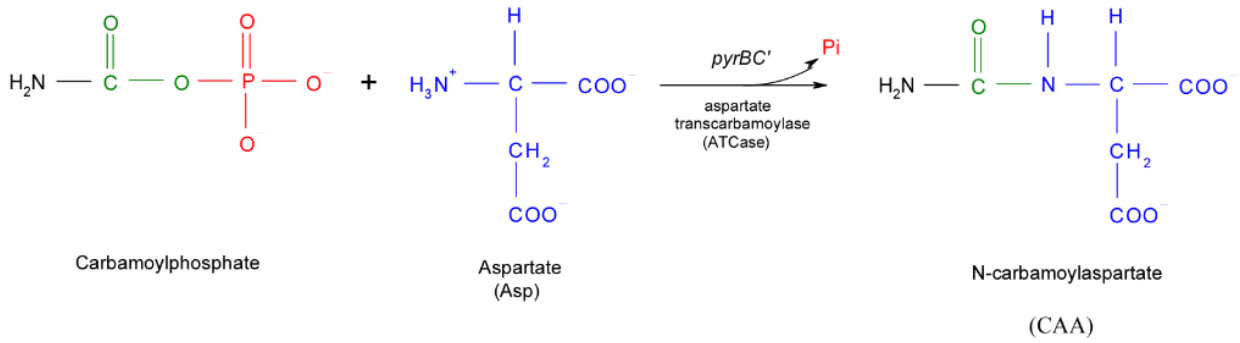

FIG. 2. Bacterial aspartate transcarbamoylase reaction.

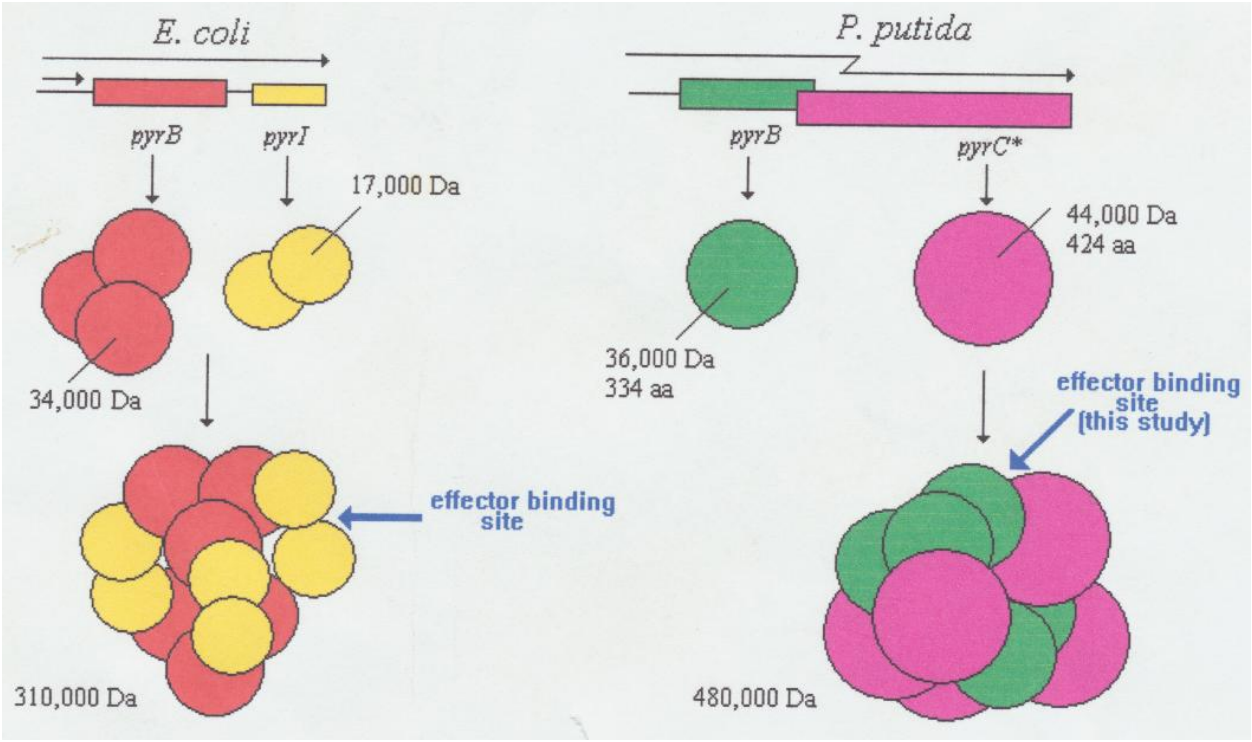

FIG. 3. Bacterial aspartate transcarbamoylase (ATCase) in *Pseudomonas* (Class A) and in *E.coli* (Class B).

CHAPTER II

MATERIALS AND METHOD

Bacterial Strains and Media

The bacterial strains used in this study are listed in Table 1. Sterilization, using autoclave with the settings of 121˚C, and 15psig, for 15 minutes.

Table 1: Bacterial strains.

Strains	*Genotype*	*Source*	*Optimal Temperature*
Pseudomonas aeruginosa (PA01)	Wild type	ATCC	37˚C
Burkholderia cepacia	Wild type	ATCC 25416	30˚C

Pseudomonas Minimal Medium Broth

Pseudomonas minimal medium (PMM) broth for 1L was prepared by the addition of 25ml of 0.5M KH_2PO_4, 25ml of 0.5M K_2HPO_4, 10 ml of 10% $(NH_4)SO_4$, and 10 ml of concentrated base to 930ml ddH_2O. After sterilization and cool down, carbon source was added (0.2% glucose).

Concentrated base consists of (14.6gm of KOH, 20gm nitriloacetic acid, 28.9gm $MgSo_4$ anhydrous, 6.67gm $CaCl_2 \bullet 7H_2O$, 18.5gm $(NH4^+)_6\, Mo_7O_{24} \bullet 7H_2O$, 0.198gm $FeSO_4 \bullet 7H_2O$, and 100 ml Metals 44 in ddH_2O to a final volume of 1L) (20) .

Pseudomonas Minimal Medium Agar

Pseudomonas minimal medium agar for 1L is prepared by adding PMM salts total of 70ml to 430ml ddH2O. In another flask, 16gm of Difco® Bacto® agar is added to 500ml ddH2O. After sterilization of the two portions and cooling to 65˚C, the two portions are mixed together and carbon source is added. PMM agar is then poured into plates and allowed to dry overnight at room temperature. Plates are stored in 4˚C until used.

Enriched Medium LB (Luria-Bertani) Broth

LB enriched medium broth is prepared as indicated on the package. LB miller® or LB Lennox® has been used. For LB Miller® 25gm was dissolved in 1L ddH2O and for LB Lennox® 20gm was dissolved in 1L ddH2O. Then, it has been sterilized by autoclave and cooled before use.

Enriched Medium LB (Luria-Bertani) Agar

LB enriched medium agar is prepared as indicated on the package. LB miller® or LB Lennox® has been used. For LB Miller® 25gm was dissolved in 1L ddH2O and for LB Lennox® 20gm was dissolved in 1L ddH2O. 16gm of Difco® Bacto® agar was added. Then it has been sterilized by autoclave. After cooling down to 65˚C, LB agar was poured into plates and allowed to dry overnight at room temperature. Plates are stored in 4˚C until used.

Dialysis Tubing Preparation

Protocol was adapted from reference (22). After cutting the tubing into smaller pieces, they were boiled for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA pH 8. Then, they were rinsed by ddH2O and then boiled in 1mM EDTA for 10 minutes. After cooling, tubings are stored in 10% ethanol at 4˚C. Before use they were rinsed with ddH2O.

Preparation of Cell Extracts

Bacterial culture of PA01 and *B.cepacia* were started by inoculating 5 ml PMM broth and 5 ml LB broth. After 24 hours incubation at 37ºC for *P.aeruginosa* and 30ºC for *B.cepacia*, cells were transferred into 50 ml PMM broth and 50 ml LB both in 250ml flasks. Flasks were placed at 37˚C in a shaker. OD600 was checked every hour until it reached exponential phase (OD600= 0.5-0.8) of growth. Cell cultures were transferred into 45ml conical tubes. Cells were harvested by centrifugation (25 minutes at 1300xg at 4˚C). Then, cells were washed with PMM salts without the carbon source and centrifuged again (25 minutes at 1300xg at 4˚C). Then, cells were resuspended in 2 ml aspartate transcarbamoylase (ATCase) buffer (2mM β-mercaptoethanol (BME), 20µM ZnSO4, 50mM tris-HCl (pH 8.0) and 20% glycerol). The suspension was subjected to ultrasonic disruption by Branson cell disruptor 200 three times at 0˚C for 1 minute followed by rest at 0˚C for 1 minute. The conical tubes were secured in ice to control the temperature. The disrupted cell suspension was distributed into 1.5ml microcentrifuge tubes and centrifuged 4 minutes at 9,000xg at 4˚C (slow spin). Then, 300µl of the supernatant which contains considerable portion of cell membrane components was transferred into fresh tube and stored at 4˚C. The rest of the suspension was centrifuged 10 minutes at 12,000xg at 4˚C (regular spin). Both slow and regular spins were then transferred into previously prepared dialysis tubing and dialyzed for 20 hours at 4˚C in 2L plastic beaker with stirring. Dialysis buffer consisted of the same ingredients as ATCase breaking buffer except for glycerol (2mM β-mercaptoethanol (BME), 20µM ZnSO4, 50mM tris-HCl [pH 8.0]). The recommended dialysis buffer volume is 150 times the prepared cell extract volume. After dialysis, cell extract was transferred into a fresh tube and stored at 4˚C until assayed. Cell

extract volume before dialysis was 2 ml and after dialysis it went up to 3-4 ml. Assay should be accomplished within 24 hours of cell extract preparation.

Aspartate Transcarbamoylase Assay

ATCase activity was measured for *P.aeruginosa*, *B.cepacia*. ATCase activity was calculated by quantifying the amount of carbamoylaspartate produced in 20 minutes at 30ºC. This was done by using the protocol in (5) with modification using colorimetric procedure (21).

The procedure was carried out in triplicate both from cell extracts produced from PMM and LB broths. It was performed on a microtiter plate (96 wells). There were four controls. Each sample was done in four ascending concentration 10µl, 20µl, 40µl, and 80µl. The assay mixture was total of 100µl adjusted with the addition of ddH2O as needed. Each well contained the following: 4µl tribuffer(50mM 2-(N-morpholino)ethanesulfonic acid (MES), 100µl diethanolamine and 51mM N-ethylmorpholine, pH 9.5), 5µl 20X aspartate (200mM aspartate), and the sample. Microtiter plate was pre incubated at 30ºC for five minutes. Reaction was triggered by the addition of 10µl of freshly prepared 100mM carbamyolephosphate to each well. Microtiter plate was incubated 30 minutes in 30ºC. After that, the reaction was stopped by the addition of 100µl stop solution freshly prepared (21). The microtiter plate was covered with sealing tape to avoid evaporation. Then, the microtiter plate was placed on heating block set on 60ºC for 2 hours. Plate was exposed to direct light for color to develop. After incubation, microtiter plate was allowed to cool in the dark for 10 minutes. Microtiter plate was read at A450 using Bio-tek synergy microplate reader.

Stop buffer was a mixture of two parts of 5mg/ml antipyrine in 50% sulfuric acid (v/v) and one part of 8 gm/ml of 2,3-butanedione monoxime in 5% acetic acid (v/v).

CHAPTER III

RESULTS AND DISCUSSION

Results

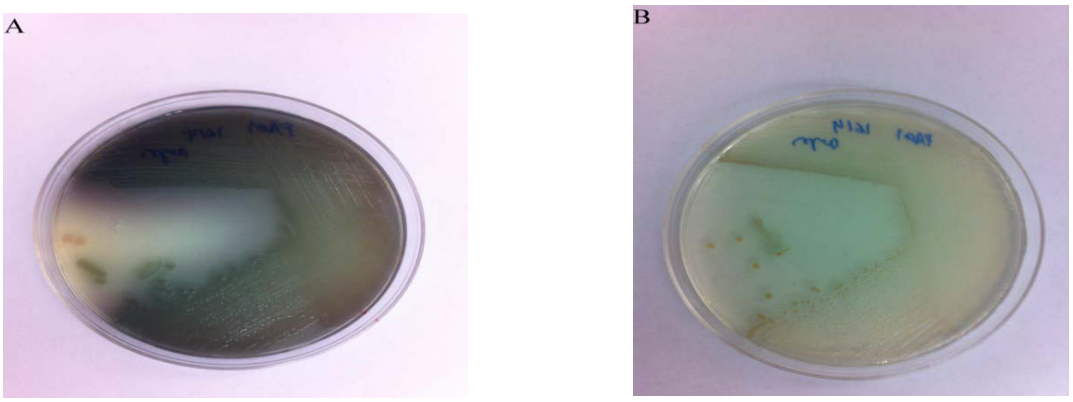

FIG. 4. (A) *P. aeruginosa* growth on enriched medium. (B) *P. aeruginosa* growth on minimal medium.

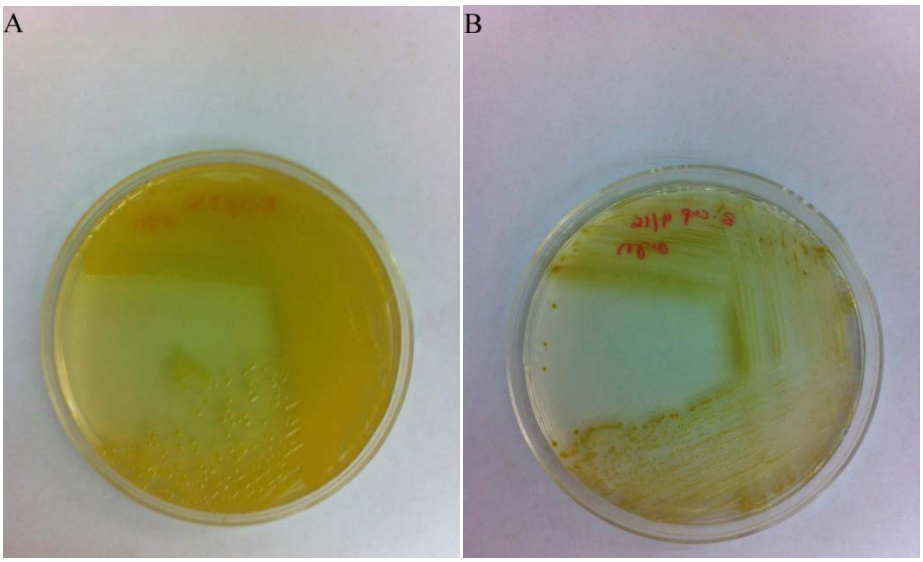

FIG. 5. (A) *Burkholderia cepacia* growth on enriched medium. (B) *B. cepacia* growth on minimal media.

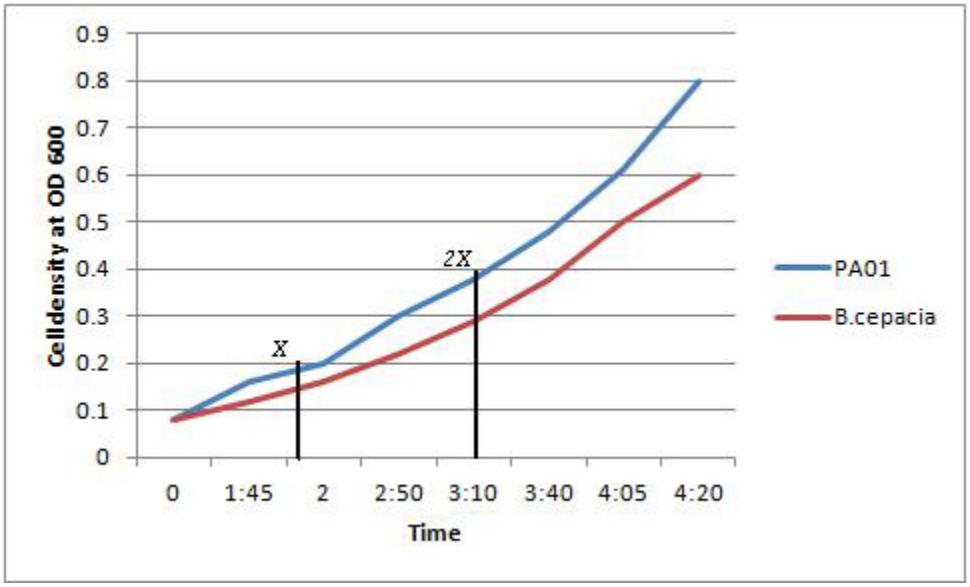

FIG. 6. Growth curve of *P. aeruginosa* and *B. cepacia*. Doubling time indicated by black line (X, 2X).

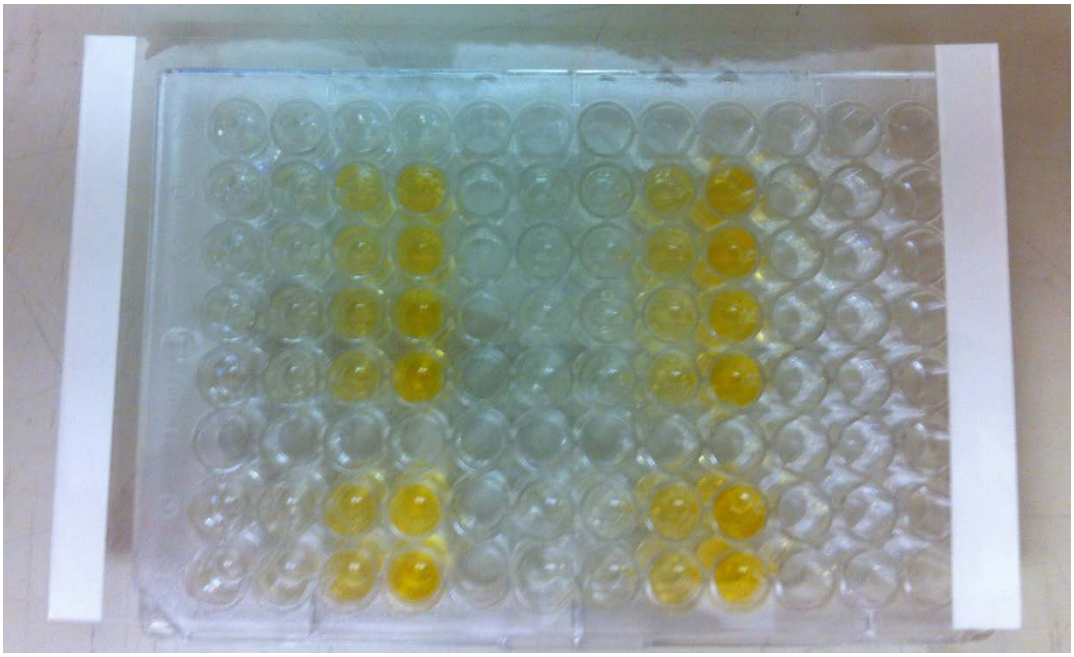

FIG. 7. Microtiter plate showing a developed color which is an indication of ATCase activity.

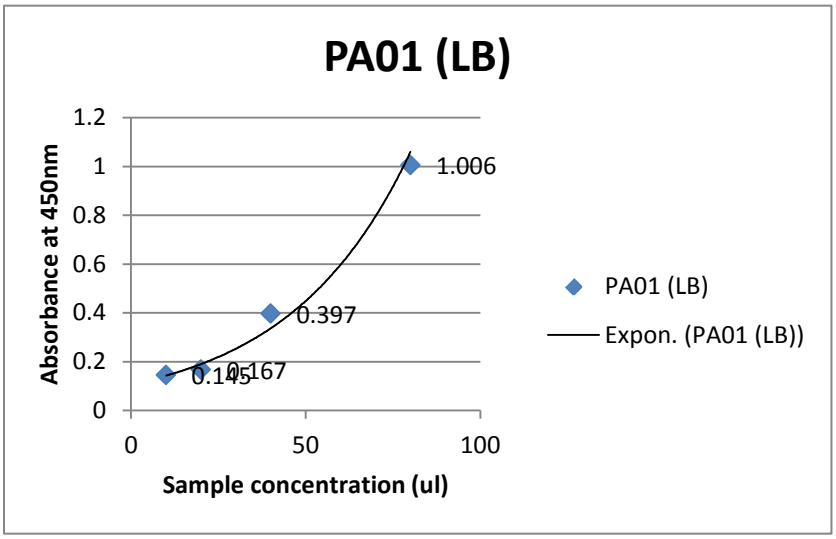

FIG. 8. ATCase activity from *P. aeruginosa* at various concentrations of sample grown on enriched medium.

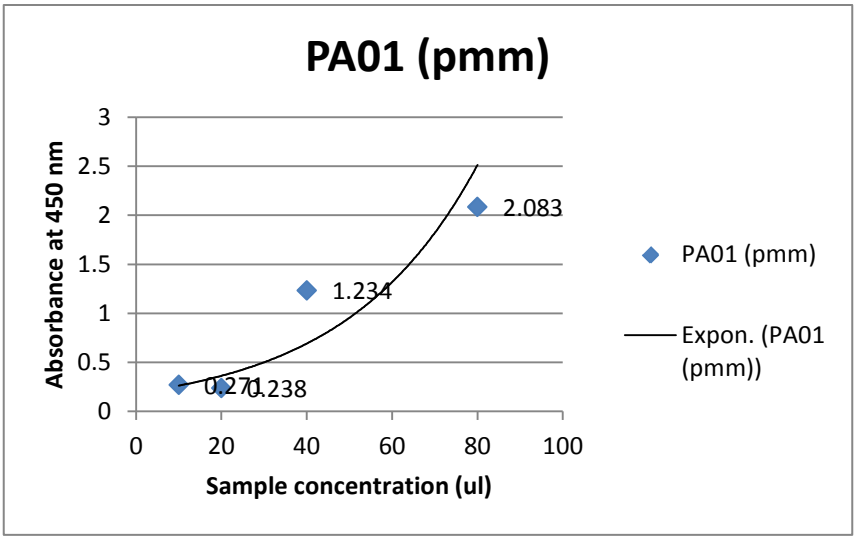

FIG. 9. ATCase activity from *P. aeruginosa* at various concentrations of sample grown on minimal medium.

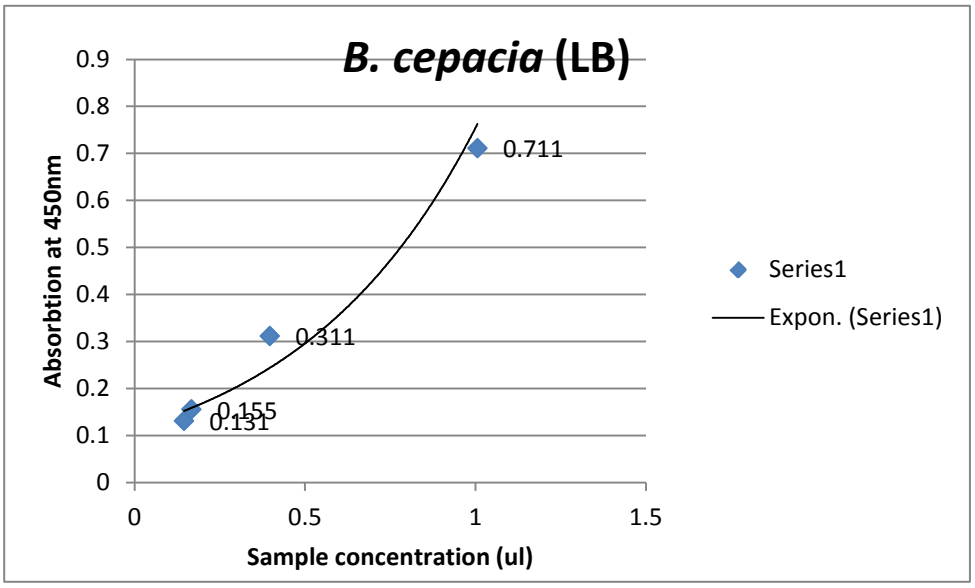

FIG. 10. ATCase activity from *B. cepacia* at various concentrations of sample grown on enriched medium.

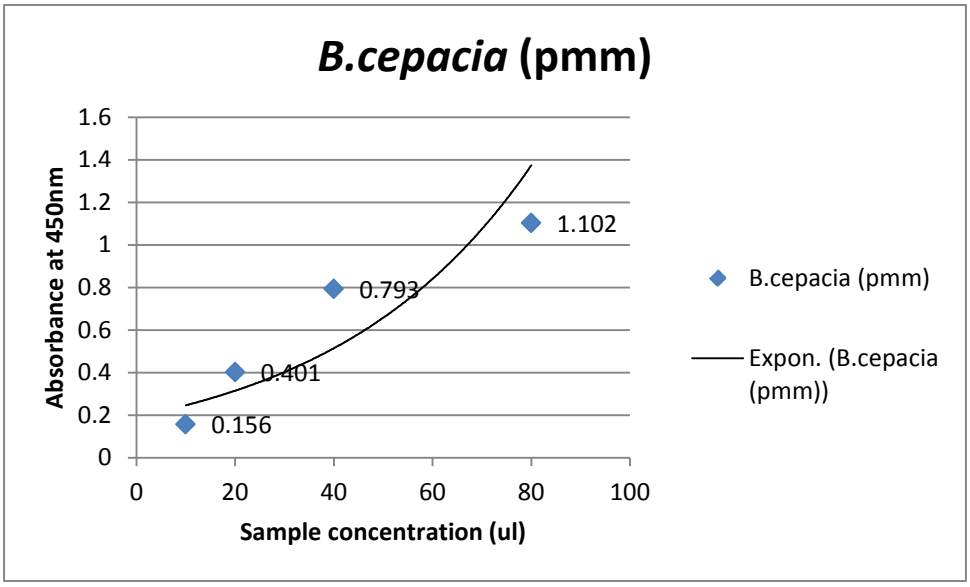

FIG. 11. ATCase activity from *B. cepacia* at various concentrations of sample grown on minimal medium.

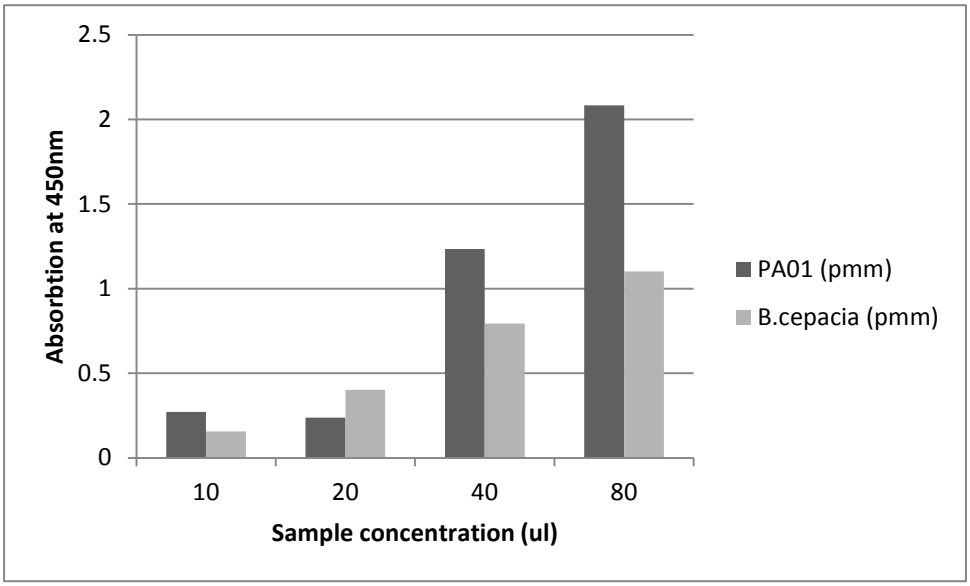

FIG. 12. ATCase activity comparison between *B. cepacia* and *P. aeruginosa* at various concentrations of sample grown on minimal medium.

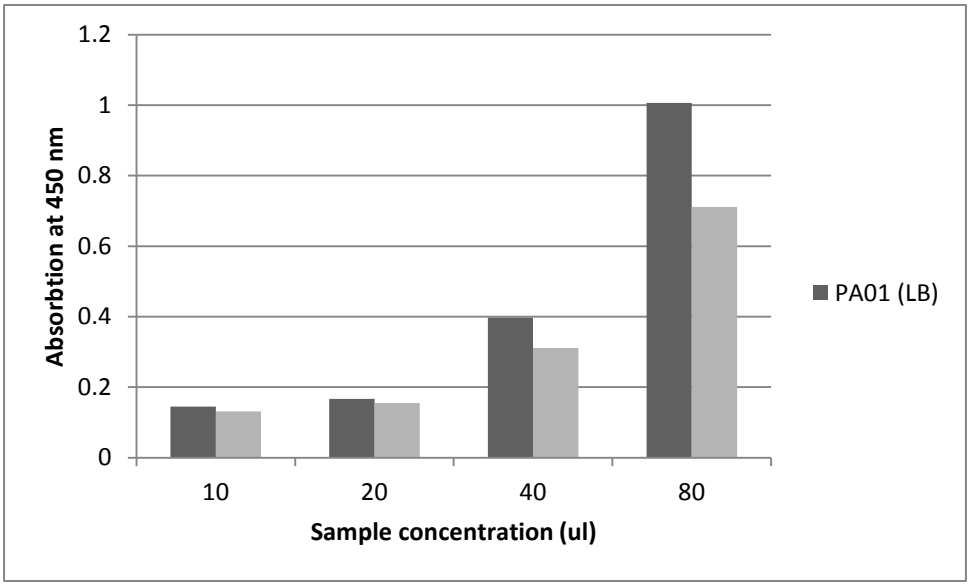

FIG. 13. ATCase activity comparison between *B. cepacia* and *P. aeruginosa* at various concentrations of sample grown on enriched medium.

Discussion

Pseudomonas aeruginosa (PA01) and *B. cepacia* were grown in *Pseudomonas* minimal medium (pmm) and Luria-Bertani (LB) as shown in (Fig.4) and (Fig.5) with PA01 at 37˚C and *B. cepacia* at 30˚C according to their optimal temperature. Difference in bacterial growth between PA01 and *B. cepacia* was noticed. *B. cepacia* growth was slower than PA01 in order to reach optical density at 600nm (OD_{600}) (Fig. 6). Results showed even when it is growing in an enriched medium *B. cepacia* still took a longer time compared to PA01. This raises a question is *B. cepacia* a slow grower? Does it need more time replicating three chromosomes compared to one as in PA01? And by that, is it considered to be faster by replicating two more chromosomes?

Aspartate transcarbamoylase catalyzes the first committed step in the pyrimidine biosynthetic pathway. We hypothesized that an organism with more than one chromosome, such as *B. cepacia* which has three chromosomes should show more ATCase activity, when compared with PA01, which has one chromosome.

The ATCase activity for PA01 and *B. cepacia* was assayed using cell extract prepared from harvesting cells grown in *Pseudomonas* minimal medium (pmm) broth and Luria-Bertani (LB) broth. After reaching OD_{600}, cells were harvested by centrifugation (25 minutes at 1300xg at 4˚C) and then washed with pmm salts and centrifuged as before, in order to remove any media, toxins and inhibitors remaining. Then, cells were broken by the addition of ATCase breaking buffer and then broken by sonication. Each sample suspension was about 2ml. Dialysis is a method of purification to achieve contaminant removal. It's a separation technique to eliminate small unwanted molecules in the solution. The membrane retains the substance wanted by a factor equal to the ratio between the buffer and the sample. The ratio between sample and buffer was 1ml against 150ml buffer which means the unwanted substances was

decreased by 150 fold. Samples were placed in previously prepared dialysis tubing made from high retention seamless cellulose tubing with a molecular weight cutoff of 12,000 which means that any molecules below that limit can liberally pass throughout the membrane while the molecules above the limit cannot pass through and remain inside the tubing. After 20 hours of dialysis at 4˚C with stirring, volume inside the tubing increased by 1-1.5ml. Samples were assayed within 24 hours.

The ATCase activity for PA01 and *B. cepacia* was calculated by measuring the amount of carbamoyal aspartate produced in 20 minutes at 30˚C. It was performed on a microtiter plate. Each sample was run in triplets. Each well contained the following: 4µl tribuffer (50mM 2-(N-morpholino)ethanesulfonic acid (MES), 100µl diethanolamine and 51mM N-ethylmorpholine, pH 9.5) to control the pH. 5µl 20X aspartate (200mM aspartate) as a substrate and the sample to be measured. The microtiter plate was pre incubated at 30ºC for five minutes. Reaction was triggered by the addition of the second substrate, 10µl of freshly prepared 100mM carbamoylphosphate to each well. The microtiter plate was incubated 30 minutes at 30˚C. After that, the reaction was stopped by the addition of 100µl stop solution freshly prepared [18]. The microtiter plate was covered with sealing tape to avoid evaporation. Then, the microtiter plate was placed on heating block set on 60ºC for 2 hours. Plate was exposed to direct light for color to develop. After incubation, microtiter plate was allowed to cool in the dark for 10 minutes. Plate showed a color development which indicates ATCase enzymatic activity (Fig.7). Microtiter plate was read at A_{450} using Bio-tek synergy microplate reader.

The velocity enzyme plot of PA01 at various sample concentrations showed an increase in enzymatic activity from cell extract prepared from cells grown in enriched medium (LB) (Fig.8). The more sample that was added, the more enzyme activity shown. Velocity enzyme

plot of *B. cepacia* also showed increase in enzymatic activity from cell extract prepared from cells grown in enrich medium (LB) (Fig.10).

When comparing both enzyme activity plots of PA01 and *B. cepacia* gathered from cell extract prepared from cells grown in enriched medium (LB) it shows that they have similar activity at similar concentrations. In some cases PA01 showed slightly more activity (Fig.13).

The enzyme activity plot of PA01 at various sample concentrations showed more increase in enzymatic activity from cell extract prepared from cells grown in minimum medium (pmm) (Fig.8). The more sample was added the more enzyme activity was shown. Velocity enzyme plot of *B. cepacia* also showed more increase in enzymatic activity from cell extract prepared from cells grown in enriched medium (pmm) (Fig.11).

When comparing both enzyme activity of PA01 and *B. cepacia* gathered from cell extract prepared from cells grown in enrich medium (pmm) it shows that they have similar activity at similar concentration (Fig.12). These results disapprove our hypothesis and raise questions such as, are only one chromosome turned on in *B. cepacia*? Is only one chromosome carrying the essential genes such as the gene that codes for the ATCase and does it work for all the other chromosomes? Is the enzyme partially inactivated by other substances? Why does *B. cepacia* which has three chromosomes produce similar ATCase activity as in *P. aeruginosa* which has one chromosome?

From research reported in this thesis and that performed by other scientist elsewhere, I wish to make the following points:

- The dogma that bacteria contain only a single circular chromosome is no longer true.
- Plasmids with essential genes are not plasmids but mini chromosomes.

- Whereas *Pseudomonas* and *Escherichia coli* each are known to contain one circular chromosome, many bacteria including *B. cepacia* and *V. cholera* contain multiple chromosomes. In fact *Agrobacterium tumefaciens* has one circular and one linear chromosome.
- In bacterial genomes with more than one chromosome such as *Burkholderia* with three circular chromosomes, the primary chromosome is the largest replisome with the most essential genes (3).
- In the case of *Burkholderia* the ATCase is found on the primary chromosome (12).

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