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The Absence of GacA/S signal transduction system affects the frequency of base substitution and frameshift mutations in Pseudomonas putida KT2400

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Abstract:

GacA/GacS two-component system can be found in Gram-negative bacteria, including enteric bacteria and Pseudomonas. *gacS* gene encodes for a membrane-bound sensor kinase GacS, whereas a transcriptional response regulator GacA is encoded by *gacA* gene. The aim of this thesis was to investigate whether the inactivation of the GacA/GacS two-component system could affect mutation frequency in *Pseudomonas putida*. Two test systems were employed for measuring mutation frequency: chromosomal Rif^R assay and a plasmidial test system based on lactose degradation. Rif^R phenotype of bacteria is a result of mutations that decrease the affinity of rifampicin binding to the β subunit of RNA polymerase. This makes this enzyme insensitive to rifampicin. The second test system is based on the monitoring mutations in *lacZ* gene encoding for β-galactosidase, which turns the tester strains from Lac^- to Lac^+ phenotype. Usage of both test systems revealed that the inactivation of *the gacA* gene elevates mutation frequency in *P. putida*.

Keywords:

Pseudomonas putida KT2440, GacA/S signal transduction system, test systems, mutation frequency

CERCS:

B230- Microbiology, baceriology, virology, mycology

GacA/S signaalraja puudumine mõjutab raaminihke- ja asendusmutatsioonide tekkesagedust *Pseudomonas putida* KT2440 tüves

Lühikokkuvõte:

GacA/GacS kahekomponendiline süsteem on kirjeldatud erinevates Gram negatiivsetes bakteriliikides, kuhu kuuluvad ka enterobakterid ja erinevad Pseudomonase liigid. Geen *gacS* kodeerib membraan-seoselist sensorkinaasi GacS ning transkriptsiooni regulaator GacA, mis saab signaali GacS-lt, on kodeeritud geeni *gacA* poolt. Käesoleva

bakalaureusetöö eesmärgiks oli välja selgitada, kas GacA/GacS süsteemi inaktiveerimine mõjutab bakteris *Pseudomonas putida* mutatsioonisagedust. Mutatsioonisageduse mõõtmiseks kasutati kahte testsüsteemi: kromosomaalset Rif^R süsteemi ja plasmiidset laktoosi lagundamisel põhinevat Lac^+ süsteemi. Rif^R fenotüübiga bakterites on tekkinud mutatsioonid, mis vähendavad rifampitsiini seondumist RNA polümeraasi β-subühikuga, muutes sel viisil ensüümi rifampitsiini suhtes tundetuks. Teine testsüsteem põhineb β-galaktosidaasi kodeerivas geenis *lacZ* tekkivate mutatsioonide tuvastamisel, mis võimaldavad Lac^- testertüvedel hakata lagundama laktoosi (Lac^+ reversioon). Töö tulemustena selgus, et *gacA* geeni inaktiveerimine bakteris *P. putida* põhjustas mutatsioonisageduse suurenemist mõlemate testsüsteemide rakendamisel.

Võtmesõnad:

mutatsiooniprotsessid, mutatsioonisagedused, GacA/S signalisatsioonirada, *Pseudomonas putida* KT2440, testsüsteemid

CERCS: B230- Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

CAA: Casamino acids, a mixture of amino acids and some small peptides obtained from acid hydrolysis of casein.

A: Adenine

Amp: Ampicilin

C: Cytosine

CFU: colony-forming units

DNA: Deoxyribonucleic Acid

dNTP: deoxyribonucleotide triphosphate.

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

G: Guanine

Glc: glucose

Lactose: Lac

Lac^+: strain has the ability to degrade on lactose

LB medium: Luria-Bertani medium

OH: hydroxyl functional group

PCR: Polymerase chain reaction

Phe: phenol

Pn: Penicillin

P. aeruginosa: Pseudomonas aeruginosa

Rif: Rifampicin

Rif^R: Rifampicin resistance

Sm: Streptomycin

T: Thymine

TAE buffer: buffer solution including a mixture of acetic acid, EDTA and Tris base

UV light: Ultraviolet light

INTRODUCTION

Living organisms on the Earth belong to three domains, Eukarya, Archaea and Bacteria. It is undeniable that bacterial inhabitants in nature need to have various adaptation processes to cope with the constantly changing environment as well as stressful situations. Under stress conditions, which might be resulted from the lack of nutrition, inappropriate temperature and pH, bacterial populations are not able to grow and stay in the stationary phase. During this stationary phase, mutations appear in the bacterial genome, and several systems of bacterial organisms have been discovered to enhance the development of mutations under stressful conditions. The majority of mutations are deleterious and although the mutations are considerable, the mutation rate is relatively low due to the limitation by error-prevention and error-correction mechanism (Drake, 1991). The spontaneous mutation rate in Escherichia *coli* is around 10^{-10} to 10^{-9} (Fowler et al., 1974; Mackay et al., 1994; J. H. Miller, 1996). Bacteria are able to develop rapidly under the stresses even though they have several processes to control and reduce spontaneous mutation rates significantly, which means that some of the mutations are beneficial and allow bacteria to evolve and adapt to the given environment in a better way (Rosenberg, 2001; Rosenberg et al., 2012). This process is also known as stationary phase mutagenesis.

Obviously, mutations are the raw material for bacterial evolution. It is necessary to have a deeper understanding of the formation processes that allows intervention in the adaptation of bacteria-related problems; antibiotic resistance is one of those needed. Researches in the bacterial's mechanisms of mutagenic processes hence acknowledging the development of bacteria antibiotic resistance decreasing the prospective possibilities of multi-resistant bacteria evolvement is crucial. This is because complications causing by the capability of bacteria to progress the antibiotics resistance. Either mutations or a lateral gene transfer from other organisms can help the bacteria acquire resistance. Over the past few decades, there is more and more useful information about mutations in bacteria thanks to the development of methods as well as the availability of the modern DNA sequencing technique. In spite of the fact that most experiments have been conducted in *E. coli* strain, mutational systems have also been researched in other strains, such as Pseudomonas *putida* in our laboratory. *P. putida*, which is widely found in nature such as soil, water, plants and is commonly recognized as the fast-growing bacteria, plays an instrumental role in pathogenesis. There has only been few systems that have been studied sufficiently for the analysis of pseudomonas mutagenic processes such as the test systems based on the utilization of phenol. These systems were developed to monitor mutagenesis both in growing and starving

condition for *P. putida* by isolating mutants which have the ability to consume phenol as the sole carbon source (Kasak et al., 1997; Tegova et al., 2004)

P. putida is also considered as an organism that is unable to degrade lactose (Liu, 2011). In this thesis, I used *P. putida* strain which has been modified to achieve the ability to degrade on lactose (Lac^+) and monitored the frequency of mutation by using two test systems, which are rifampicin (Rif^R) assay and test system based on lactose degradation. The GacA/S system has also been used to monitor and compare the differences when present within the test system.

The sensors kinase, GacS, and GacA, which is a transcriptional regulator, are parts of a two-component system for monitoring secondary metabolite production and many other facets of bacterial physiology (Workentine et al., 2009). This two-factor regulatory system in bacteria is accountable for sensing and responding to external stimuli. Because of the function in virulence, subordinates sensing, production of biological regulatory factors, and the construction of biofilms, this two-component system has been previously researched in *Pseudomonas* sp. especially in *Pseudomonas fluorescens* (Haas and Amp; Defago, 2005) as well as *Pseudomonas aeruginosa* (Parkins et al., 2001; Goodman et al., 2004; Kay et al., 2006). Upon activation, GacA stimulates the synthesis of some small regulating RNA binding on the inhibitory protein RsmA/E, hence diminishing the target gene inhibition (Lapouge et al., 2008)In this thesis, the role of the two-component GacA and GacS system on mutagenic processes in *P. putida* was examined by comparing mutation frequency in *P. putida* wild-type strain KT2440 and its GacA-deficient derivative by employing Rif^R and Lac^+ assays.

1 LITERATURE REVIEW

1.1 Factors influencing the evolution of bacteria

1.1.1 Bacteria and DNA

Bacteria are abundant and might be found on the peaks of the mountains, in the seas, in the acidic hot spring, radioactive waste and around humans in general (Fredrickson et al., 2004). Therefore, bacteria have had plenty of time to evolve and to develop various types of descendants. Bacterial evolution relates to the genetic modifications, so-called mutations, that can result from adaptations in the light of environmental changes or from a host's immune response. Bacteria are able to develop quickly due to their short generation periods and large communities. Mutations can be categorized as consistent with the types of alternatives in the DNA sequence.

Deoxyribonucleic acid (DNA), is a double helix intertwined molecule (two antiparallel chains) of chemical building blocks called nucleotides ("antiparallel" implies that they are of reverse polarity, one strand runs from 3' to 5' and the other from 5' to 3', due to a free 5'-phosphate group on one and 3'-OH group on the other end) that has individual code for the growth, survival, and reproduction of organisms within all cellulars and is passed from mother cell to daughter cell. These building blocks are composed of three different parts: a phosphate group, a sugar group and one of four forms of nitrogen bases (Adenine (A) and Guanine (G), which are purines, Cytosine (C) and Thymine (T), which are pyrimidines). Nucleotides are connected with each other into chains, together with the sugar and phosphate groups alternating in order to generate a DNA strand.

1.1.2 Bacterial genome (bacterial chromosome and plasmids)

While eukaryotes have two or more chromosomes, prokaryotes, such as bacteria, contain a single circular chromosome composed of double-stranded DNA in a loop and does not have a nucleus. The DNA is positioned within the nucleoid of the cell. Bacteria can also have DNA that is not located in chromosomes but rather in plasmids, which are small, rounded, double-stranded DNA molecules. Plasmids naturally exist in bacterial cells, and also in some eukaryotes (Lodish et al., 2000). Frequently, the genes carried in plasmids offer the bacteria genetic benefits, which can, for instance, include antibiotic resistance.

1.1.3. Mutations in bacteria and classification

Bacteria have to evolve and tailor to various conditions, and it is evident that a mutation that is generally defined as an inheritable modification of a living organism's genetic material (either RNA or DNA) is the fundamental source of all variation. There are three types of mutations; specifically, these are base substitutions (so-called point mutations), insertions and deletions.

Base substitution is the mutation that takes place while one single nucleotide exchange from one to any other leading to the alternate from one base pair to another inside the DNA sequence. There are missense mutations, nonsense mutations and silent mutations. Such substitutions leading to changes in amino acid sequences are referred to as missense mutations. When a codon is modified to premature stop codon, it is known as nonsense mutation. When silent mutation happen, there is no change in amino acid caused by base substitution. Obviously, most of the point mutations do not substitute the amino acid sequence because of the abundance of genetic code. Genetic code is a universal group of rules utilized by living organisms (such as humans, bacteria, animals,...) to translate nucleotides in codons of mRNA into proteins. There are two types of point mutations: transition and transversion ones. Transition is a type of mutation that does not change the type of nucleotide (i.e. it occurs when a purine is substituted with another purine or when pyrimidine is substituted with another pyrimidine), while transversion is a change from a purine to a pyrimidine. Insertions are mutations taking place while nucleotides are inserted into the sequence. Contrarily, deletions are mutations that eliminate some part of the sequence from the DNA molecule.

Based on another classification, there are also spontaneous mutations and induced mutations. The spontaneous mutation rate in bacteria is around 10^{-10} to 10^{-9} (Fowler et al., 1974; Mackay et al., 1994; J. H. Miller, 1996). The genetic constitution of the organism influences its mutation rate. Many spontaneous errors are corrected by means of the cellular repair systems, and so do not emerge as constant in DNA. Spontaneous mutations are generated by replication errors, which can be caused by tautomeric shifts, wobble base pairing, strand slippage, unequal crossing over, and different kinds of chemical modifications such as depurination or deamination.

On the other hand, induced mutations are changes in the DNA sequence as a result of environmental factors like various toxic compounds or radiation. A number of environmental agents are capable of destructing DNA consisting of certain chemical substances (an agent that considerably increases the rate of mutation above the spontaneous rate) and radiation,

brought on mainly by means of base analogues, alkylating agents deamination, hydroxylamine, oxidative reactions, intercalating agents, radiation.

1.1.4. The average mutation frequency in bacteria and mechanism to overcome mutations in DNA

Even though the mutagenic capability of DNA replication mistakes as well as DNA damage is considerable, such as the rate for DNA polymerases misincorporation errors is 1 mistake per 1000 bases, the mutation rate is remarkably low. In E.coli, as an instance, the mutation rate is just around 10^{-9} in step with a site per replication and it's far even lower for humans, for every 10^{10} base pairs, 1 error occurs in the entire genome consistent with replication (J. H. Miller, 1996; Zimmerman, 2008). Even if some mutations in step with the genome can extensively lower the fitness, the mutation rate is extraordinarily low. Bacteria often have diverse mechanisms to limit both mutagenic and toxic effects from UV light or other radicals. In case of environmental damage or other factors that cause DNA damage, there are many mechanisms that work on it to fix the damage such as direct repair, excision repair or recombinational repair that are classified according to the overall mechanism of action. In terms of direct repair, any break of the phosphodiester bond in either single- or double-stranded DNA is restored by DNA ligation, which relegates the broken DNA backbone together. Excision repair works in a similar way with DNA repair, which firstly removes the section of DNA strand containing error then resynthesizes the removed section based on the other strand that is utilized as a template and ligates it into the place. The mutation frequency is low; as mentioned above, this is because of/ due to the mechanism recognizing and correcting the errors caused by DNA polymerase during replication or DNA damage. While two mechanisms above remove the damaged section and resynthesize, the recombinational repair is a special form of homologous recombination that exchanges the damaged section of DNA with an unaltered section present in the same chromosome to restore it. Even if there is no damage from outside happening with DNA, there is always a high possibility that some errors are caused by DNA polymerases during replication. There are two main mechanisms to detect and correct these errors known as proofreading and mismatch repair that share a common simple strategy: if any wrong base is inserted during DNA replication or few bases are inserted or deleted, the normal structure of double helix DNA will be distorted. While in proofreading, the machinery detects abnormalities, then the proofreading function of DNA polymerase will remove that error, and DNA replication can continue from that place. By using its own methylated mechanism, the mismatch repair system is able to differentiate the

old strand and the newly synthesized one, thereby correctly identifying the newly synthesized strand and fixing the mutation accordingly. The survival of bacteria is influenced by increasing environmental factors. Bacteria have universal response mechanisms that lead to drastic changes in gene expression and cellular metabolism to respond to these stresses and survive them. Such responses are regulated by master regulators, small molecule effectors, gene repressors, and inorganic molecules.

The stress response pathways vary greatly and are triggered by certain external stimuli of different dimensions. Such stresses compose of nutritional loss, DNA damage, temperature increase/decrease (temperature shock), antibiotic sensitivity, chemicals, inappropriate pH and hyperosmolarity. These global responses to stress have characteristics that may enhance genetic diversity. Up-regulating and triggering error-prone DNA polymerases, down-regulating error-correcting enzymes and shifting mobile genetic elements are typical features of different stress responses. As a result, bacteria are induced to evolutionary change, so-called mutations, under a number of stressful situations. For adaptive development, this transient mutator state might be essential.

1.2 Methods for monitoring the frequency of mutations in bacteria

There are two main methods used to monitor changes in DNA, which are sequencing, and specialized testing systems, usually based on the activation of tester genes. DNA sequencing is the technique of identifying the order of nucleotides in a DNA molecule. The target DNA is replicated several times in the Sanger sequencing, generating fragments of various lengths. Fluorescent nucleotides with "chain terminator" mark the fragments' ends and allow the determination of the sequence (Sanger et al., 1977). Modern, large-scale approaches to sequence technology in the next generation improve speed and reduce DNA sequencing costs. Next-generation sequencing (NGS) can be applied to any species (bacteria, plants, animals, humans, etc.) and DNA sources, including genomic DNA (such as genome sequencing), complementary DNA (RNA- Seq), methylated DNA (for gene sequencing) (Pareek et al., 2011) In sequencing, the number of colonies usually analyzed compared to the whole population is quite small, so this technique does not give the general picture of the mutation frequency of the whole population. It is also difficult to monitor the dynamics of mutation frequency caused by different stressors. Although there are a huge amount of mutations that are not taken into account during sequencing, it is useful to analyze the evolutionary process of the individual cell to get a deep understanding of changes that might be behind some new phenotypes. Tester genes, on the other hand, are specifically designed

systems that monitor the mutation frequency in concrete spots of the chromosome or in plasmids. The test system can be applied in many locations of the genome, but they only monitor the mutations appearing in the area of the test system. In spite of this disadvantage, these tester genes are well-usable in detecting mutational effects caused by the absence of different genes under investigation (L. P. Miller et al., 1994; Jatsenko *et al.*, 2010).

1.3 Test system for measuring the frequency of mutations in bacteria

1.3.1 Rifampicin (Rif^R) assay

In the laboratory, rifampicin assay is commonly used to estimate the frequency of spontaneous mutations in bacteria. Rifampicin binds to the β subunit within the DNA/RNA channel of RNA polymerase and inhibits the synthesis of RNA by physically blocking elongation, thereby preventing also the translation process. (Campbell et al., 2001) There are a number of mutations in *rpoB* gene that change the conformation of RNA polymerase in a way that inhibits the binding of rifampicin, thus enables transcription in a usual way, and bacterial colonies carrying these mutations can grow on rifampicin plates. This system has been previously used by T. Jatsenko to measure mutation frequency in *P. putida* PaW85 (Jatsenko *et al.,* 2010). She observed that there are many different types of mutations in *rpoB* genes that give *P. putida* the ability to grow on a rifampicin plate. The growth speed of colonies may vary due to different types of mutations. For example, besides the appearance of large colonies which can be seen after 24 hours of plating, small colonies grow on these plates in 48 hours (Jatsenko *et al*., 2010).

1.3.2 Assays based on phenol-degradation

Studies of mutational mechanisms have been made in mainly exponentially growing bacteria for a long time leading to the lack of information about this mechanism in the starving bacteria. So, in the last few years, researches have been focused on studying mutational processes in starved bacteria. Bacterial cell division during starvation is inhibited; however, under selective conditions, mutations overcoming starvation tend to appear, which has been termed as ‘‘adaptive’’, “starvation-associated” or “stationary-phase” mutation and has remained a lively controversial debate (Lenski and Mittler, 1993; Elsworth, 1993). Up to now, *E. coli* has been widely researched for mutation under starving condition, and the recovery of a frameshift mutation in an episomal lac allele has been mainly investigated as the controlled model system for the directed mutations. (J. Cairns and Foster, 1991; Foster and Cairns, 1992; Harris et al., 1994). It is evident that Lac^+ revertants did not appear on plates lacking a carbon source but rather on lactose-minimal plates in cultures of Lac^- *Escherichia coli* starving (John Cairns et al., 1988). In 1997, Kasak et al. reported mutations that create promoters, allowing *Pseudomonas* putida strains that have been starving to consume phenol as a sole source of carbon (Kasak et al., 1997). This phenol degradation test system is based on the phenol degrading genes *pheA* and *pheB*, which has been initially

cloned from plasmid pEST1001 and organized in a single operon. These phenol degrading gene *pheA* and *pheB* correspondingly encode for phenol monooxygenase and catechol 1,2-dioxygenase. (Kivisaar et al., 1991). The *pheA* and *pheB* (*pheBA*) insertion into *P. putida* strain PaW85, which is phenol non-degrading, facilitates the ability of this strain to consume phenol as the only carbon source. The phenol-consuming (Phe^+) mutants accumulated in the culture of P. putida, which is starving not in the lack of phenol but rather the presence of it. Apart from identifying the mutations occurring in starving bacteria, effects of the growth phase of cells on the rate of appearance of mutations were reported that bacteria derived from the stationary growth phase exhibited a higher frequency of Phe^+ mutations in comparison with bacteria derived from exponential growth phase. This implies that certain processes, primarily in stationary-phase cells, enable these mutations to be generated and fixed. Moreover, the lower accumulation rate of Phe^+ mutants with exponentially grown cells plated on selective plates does not imply the decreased cell viability on phenol-minimal plates. In Phe^+ mutants that emerged in the starving population selected for phenol use, one specific C to A transversion was prevalent. Different deletions were, however, the most common Phe^+ mutants in a growing culture of bacteria without any selection for growth on phenol (Kasak et al., 1997).

1.4 Test system based on lactose degradation

The most commonly used test systems for measuring mutation frequency are based on lactose degradation. These systems were developed for use with modified *E. coli*, which initially degrades lactose effectively, but the ability has been interrupted. The initial *lacZ* gene has been substituted with one carrying change, which results in the inactivation of β-galactosidase (Cupples and Miller, 1989). The modified sequences produce six or seven similar base pairs, generating a frameshift that corresponds to a Lac^- phenotype. Reversion to Lac^+ will only take place at certain positions in the sequence (Cupples and Miller, 1989). The table below describes the availability of different *lacZ* versions available for monitoring the frequency of reversion event restoring the Lac^+ phenotype (Cupples and Miller, 1989). However, these systems are not usable for strains incapable of using lactose as a carbon source. For instance, with well-known laboratory model organism *P. putida* strain KT2440, which is incapable of degrading lactose. These systems can be used by Pseudomonas species if they are previously modified to degrade galactose and transport lactose into the cells. *LacY*

transports lactose into the cells, and *gal ETKM* helps this kind of bacteria to catalyze the reversible conversion of galactose into glucose so that it can consume the energy source.

Table 1. The availability of different *lacZ* versions available for monitoring the frequency of reversion event restoring Lac^+ phenotype (Cupples and Miller, 1989)

Strain	Reversion event to restore Lac^+ the phenotype in strains CC101-CC111
CC101	A-T →C-G
CC102	G-C→A-T
CC103	G-C→C-G
CC104	G-C→T-A
CC105	A-T→T-A
CC106	A-T→G-C
CC107	+1G
CC108	-1G
CC109	-2(-C-G-)
CC110	+1A
CC111	-1A

1.5 Two-component signal transduction system GacA/GacS

GacS, which is a sensor kinase and originally named LemA, were initially recognized by this pathogenic strain on a bean as an integral factor for lesion manifestation plants in *Pseudomonas syringae* (Barta et al., 1992; Kitten et al., 1998). Mutagenesis inhibition of the *gacS* gene causes virulence deficiency and physiological fitness (Hirano et al., 1997; Hirano and Upper, 2000).

In *Pseudomonas fluorescens* biocontrol strain CHA0, the GacA response regulator has been identified as a global activator for growth under antibiotic and cyanide (Laville et al., 1992; Natsch et al., 1994). For the antibacterial performance and physical fitness of this strain, GacA's function is necessary (Laville et al., 1992; Natsch et al., 1994) .

Bacteria depend on many two-component systems to sense environmental or intracellular signals. The sensors kinase, GacS, and GacA, which is a transcriptional regulator, are a part of the two-component system for monitoring secondary metabolites and many other facets of bacterial physiology (Workentine et al., 2009). When the signal is obtained, the sensor is triggered and enabled by phosphorylating the reaction regulator.

Certain response regulators attach to RNA or proteins and also could conduct enzyme activities; however, DNA binding proteins often play an instrumental role as response regulators that can be used for transcriptional regulation (Stock et al., 2000, Gao et al., 2007). This two-factor regulatory system in bacteria is accountable for sensing and responding to external stimuli. Because of the function in virulence, subordinates sensing, production of biological regulatory factors, and the construction of biofilms, this two-component system has been previously researched in *Pseudomonas* sp. especially in *Pseudomonas fluorescens* (Haas and Amp; Defago, 2005) as well as *Pseudomonas aeruginosa* (Parkins et al., 2001; Goodman et al., 2004; Kay et al., 2006). Upon activation, GacA stimulates the synthesis of some small regulating RNA binding on the inhibitory protein RsmA/E, hence diminishing the target gene inhibition (Lapouge et al., 2008).

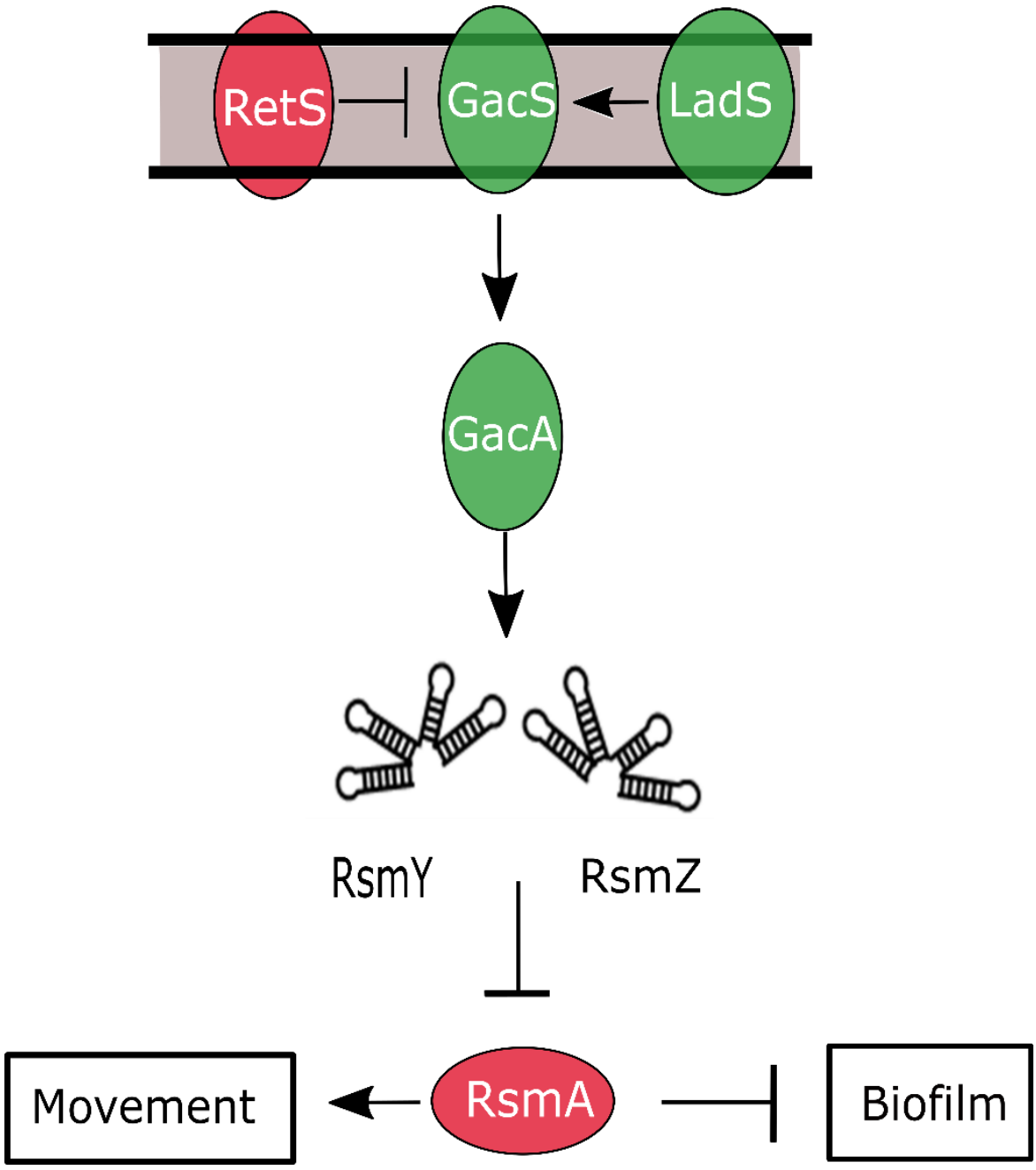

Figure 1. In *P. aeruginosa*, the pathway of GacA/S system and Rsm family proteins. Biofilm repressors (in red colour) and activators (in green) are indicated. GacS is known as a sensor kinase which through phosphorylating, recognizes an unknown signal and transmits it to GacA. LadS activates this transfer of signal, and RetS represses it. The transcription of RsmY and RsmZ, which are small noncoding RNA-s, is activated via phosphorylated GacA. These noncoding RNA-s bind to RsmA and hence inhibit posttranscriptional regulator RsmA. This prevents RsmA from activating genes needed for movement and facilitates the repression of biofilm genes

Although it is common that the lack of actual communication between signals may cause the absence of responsive signals, these signals cannot be activated if either one of the components of the system does not dissolve or build up. In *P. aeruginosa*, GacA/S system often communicates with RetS and LadS control systems (**Figure 1**). Such two-hybrid sensors are membrane-bound with reverse features. (Goodman et al., 2009; Ventre et al., 2006). RetS can develop GacS-based heterodimers, as well as prevent signalling pathways to GacA (Goodman et al., 2009) and LadS phosphorylate GacS to stimulate phosphorylation of GacA in effect. In biofilm development, both LadS, as well as GacA deficient strains, are significantly impacted while deletion of *retS* gene leads to the increased mass of biofilm.

2 THE AIMS OF THE THESIS

The aim of this thesis is to investigate the specific effect of the GacA/S system on the frequency of the different types of mutations. It is studied in a well-known laboratory reference strain *P. putida* KT2440Lac-test and its derivative strain lacking *gacA* gene; thus, the GacA/S signal transduction system is inactivated.

In order to test the effect of inactivation of the GacA/S system, Rif^R and Lac^+ test systems were used to monitor the frequency of mutations.

Two types of test systems based on lactose degradation were used in order to detect whether the frequency of frameshift mutations (CC107) or substitution mutations (CC104) has been affected by the absence of GacA/S system.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strains, plasmids and media

KT2440, which is an isogenic strain of P. putida strain PaW85 (Bayley et al., 1977). Complete media which have been used were Luria-Bertani (LB) medium (Cavalier-Smith 1980) and liquid minimal medium M9 (Blair, 1959). For obtaining a solid medium 1.5% Difco agar was added. At finally specified concentration of 0.2% and 0.4%, both glucose casamino acid (CAA) were applied correspondingly to a minimum medium. When need-ed, antibiotics were applied in final concentrations of rifampicin (Rif; 100 μg/ml), ampi-cillin (Amp; 100 μg /ml), penicillin (Pn; 200 mg/ml), streptomycin (Sm; 200 mg /ml), P. putida strain KT2440 was incubated at 30°C.

Table 2: Strains and plasmids in this work

Strain	Characterization	Source
KT2440Lac-test	Derivate of KT2440, which has been constructed in order to use test systems based on lactose utilization. Carries genes *lacY* and operon *galETKM* and is controlled by promoter Pgc.	MSc.Tanel Ilmjärv
KT2440Lac-test *gacA::Sm*	Derivate of KT2440Lac-test with inactivation of *gacA::Sm*	MSc. Tanel Ilmjärv
KT2440Lac-test + CC104	Derivate of KT2440Lac-test with introduced plasmid pKT_taclacZ*104CG	This work
KT2440Lac-test +CC107	Derivate of KT2440Lac-test with introduced plasmidpKT_taclacZ*107 -1	This work

KT2440Lac-test *gacA* ::Sm +CC104	Derivate of KT2440Lac-test with introduced plasmid pKT_taclacZ*104CG	This work
KT2440Lac-test *gacA*::Sm+CC107	Derivate of KT2440Lac-test with introduced plasmid pKT_taclacZ*107 -1	This work

KT2440 is an isogenic strain of P. putida strain PaW85. gacA::Sm indicates gacA inactivated by streptomycin resistance gene insertion. pKT_taclacZ*104CG is a plasmid that substitutes C-G base-pair in the 104th position in lacZ sequence by T-A base pair. pKT_taclacZ*107 -1 is a plasmid with a 1-bp deletion in the lacZ sequence in the 107th position.

Table 3. Plasmids used in this experiment

Plasmid	Characterization	Use
pKTlacZ*107-1	Derivate of pKTlacZ, which has Tac-promoter before mutated *lacZ* gene, designed based on Miller strain CC104 (Cupples and Miller, 1989); cells containing this plasmid will have a tag CC107	Converts Lac^{+} phenotype into Lac^{-} phenotype by creating deletion mutation in the 107th position
pKTlacZ*104CG	Derivate of pKTlacZ, which has Tac-promotor before mutated *lacZ* gene, designed based on Miller strain CC104 (Cupples and Miller, 1989); cells containing this plasmid will have a tag CC104.	Converts Lac^{+} phenotype into Lac^{-} phenotype by creating a substitution mutation in the 104th position

3.1.2 Measurement of spontaneous Rif^R mutants

3.1.2.1 Electroporation of bacterial cells

In order to insert the plasmid DNA into cells, electroporation was used. *P. putida* cells were grown overnight in LB liquid medium. For making competent cells, 250 µl of the overnight culture was taken and centrifuged (13400 rpm) followed by washing three times with 300 mM sucrose solution and suspended in 70 µl in sucrose solution.

0.2 µl of plasmid DNA dissolved in water was added to the competent cells. The cells were pipetted into electroporation cells and electroporated with a BioRad 2500 V electroporator. 900 µl of LB medium was added to the cells and washed out of the cuvette. Next the cells were grown for one hour on a shaker (*P. putida* cells grown at 30°C) and then plated on selective medium.

3.1.2.2 Polymerase chain reaction (PCR)

To identify the presence of desired plasmid or bacterial strain, colony PCR was made. The volume of the reaction was 20 µl. The oligonucleotides used as primers are lacZseq and lacZprtaq.

Component	**Amount**
Green Buffer PCR	2 µl
Forward primer (10 µM)	0,1 µl
Reverse primer (10 µM)	0,1 µl
dNTPs (0.2 mM)	0.4 µl
Taq DNA Polymerase	0,15 µl
Template DNA	picked colony

MQ water	Up to 20 µl

PCR was performed on a Biometra™ PCR machine to create the right conditions for the reaction:

	PCR settings		
Initial denaturation	96°C	5 min	
Denaturation	95°C	30 sec	25 cycles
Primers annealing	56°C	15 sec	
Extension	72°C	3 min	
Final extension	72°C	3min	

The amplied DNA segment length: 1 minute per 1000 bp.

Table 4. Primers used in this work

Primer	What was it used for	Sequence 5` → 3`	Position
lacZ-seq	Detection of plasmid pKTlacZ*	GGGGATGTGCTGCAAGGCG	From position 55 to 73 in the *lacZ* gene
yrghind	Detection of galactose degradation module	CCAAAGCTTTGTTTACGATCCAGGC	Complementary to the upper region of GC promotor

lacy-Rev-Kpn1	Detection of galactose degradation module	ATATGGTACCTGTTGGTCGGATAAGGCGCTC	Complementary to the region of +27 to +44 nucleotides from the end of *lacY*
prtac	Detection of plasmid pKTlacZ*	AATTAATCATCGGCTCGTATAA	Complementray to the Tac-Promotor region located before *lacZ* gene
gacAsaba	Detection of *gacA* interruption with *Sm* gene	GGACTCGAGGTTTTACAGGCTTGCGT	Complementary to the end of *gacA* gene from position 627 to +13 after the stop codon
smsplõpp	Detection of *gacA* interruption with Sm resistance gene	GCTGATCCGGTGGATGACCT	Complementary to the inverted terminal repeats of Sm resistance gene

3.1.2.3 Gel electrophoresis

Gel electrophoresis was utilized to detect PCR products. 5 µl of one PCR product was added to the sample, 2 µl of gel electrophoresis dye (0.04% bromophenol solution in 50% glycerol). Samples were transferred 1% agarose gel in TAE buffer (under pH = 8.2, 50 mM Tris-acetate, 1 mM EDTA). The gel contained ethidium bromide 0.33 µg/ml. The currency was used to determine the length of the DNA fragments. Electrophoresis was performed at 120 °C. The gel was imaged under UV light to visualize the DNA.

3.1.2.4 Measurement of spontaneous Rif^R mutants

The experiment was conducted at least in 40 independent cultures of *P. putida*. Cells were grown for six hours in M9 medium including glucose and CAA to late logarithmic growth phase then diluted this culture into newly fresh glucose and CAA-containing M9 medium with 10^5 dilution factor. After that, 2.3 ml aliquots were dispensed into test tubes to make independent cultures. For cells to reach saturation, they were grown overnight 20-24 h. After reaching saturation, in the vicinity of 0.5×10^9 cells (500 µl of culture) were plated on Rif-LB plates. The appearance of rifampicin resistant colonies has been counted after 24 hours of incubation. The frequency of the presence of Rif^R colonies was calculated independently applying the Lea-Coulson median test per 1 ml or 10^9 cells (Lea and Coulson, 1949; Rosche and Foster, 2000).

3.1.3 Measurement of the frequency of mutation based on lactose degradation system

Plasmids pKT/lacZ* 107 -1 and pKT/lacZ* 104 CG containing tester systems (CC104 and CC107) were introduced into KT2440 and KT2440 *gacA::Sm* strains, which were used in this experiment. Cells were grown on M9-based medium (Blair, 1959) supplemented with glc and CAA for 6 hours to late logarithmic growth phase then diluting this culture by 10^5 into newly fresh glucose and CAA-containing M9 medium and dispensing 2.3 ml aliquots into test tubes to make independent cultures. For cells to reach saturation, they were grown overnight. After reaching saturation, in the vicinity of 0.5×10^9 cells (500 µl of culture) were plated for strains on lactose-M9 plates. For strains containing CC107, a 10-fold dilution of the culture was made, and 10 µl of this dilution and 250 µl of KT2440 strain additionally to make the strains containing CC107, not over-grow was plated on lactose-M9 minimal plates.

The appearance of colonies was counted after 24 hours of incubation in 5 days continuously. In order to evaluate the viability of the plated cells, small plugs (around 1/100 of the plate) were cut out from the agar plates avoiding the formed colonies. Cells were suspended in 1 ml of M9 solution, and colony-forming units (CFU) were determined on LB plates to measure cell viability for 3 tubes in 3 different levels (10^{-7}, 10^{-8} and 10^{-9}) in the presence of penicillin to select for CC107 since KT2440 had also been added before in the plate. After incubating the plate at 30°C overnight (around 12 hours), colonies appearing in this plate were counted (usually 10^{-9} is taken into account when counting colonies in measuring the cell viability) and calculated per 1 ml.

3.2 RESULTS

3.2.1 The frequency of rifampicin-resistant Rif^R mutant is elevated in GacA-deficient strain

In order to identify the frequency of rifampicin-resistant mutants per 10^9 cells, five strains have been used, which are the plasmid-free wild type KT2440Lac-test (WT) strain and its derivate strain containing *gacA* inactivation with a streptomycin resistance gene. Into both of these strains, tester plasmid necessary for lactose degradation assay was inserted by electroporation. Strains introduced with plasmid pKT_taclacZ*104CG are marked as CC104, and cells containing pKT_taclacZ*107 -1 are marked with CC107 (**table 1**). The step by step experiments are described above; each strain has been grown on ten plates containing rifampicin. The experiments have been replicated three times.

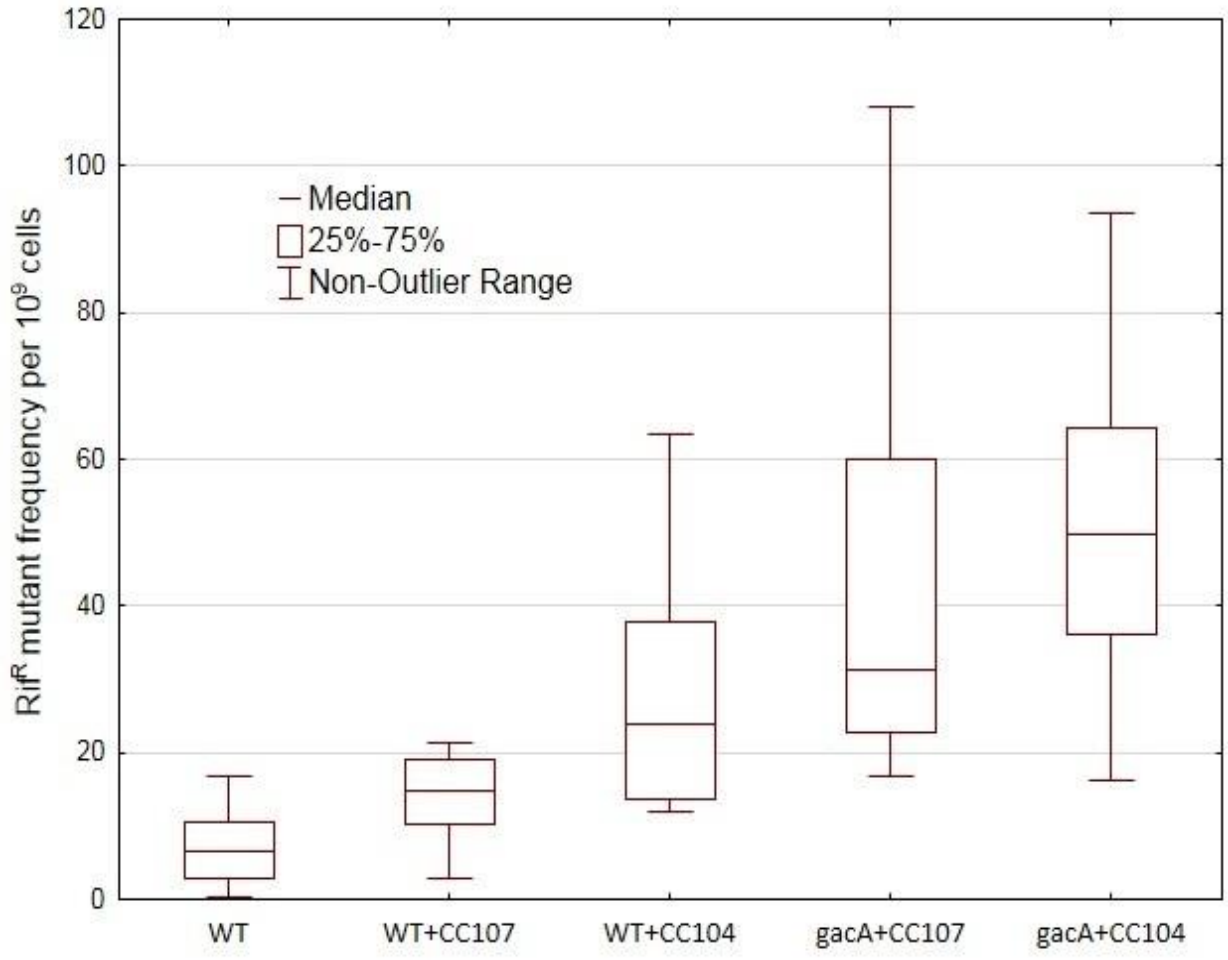

Figure 2. The frequency of rifampicin (Rif^R) mutant per 10^9 cells. The reference strain utilized in this experiment was *P. putida* KT2440Lac-test strain (Wild Type, WT). The median values for Rif^R mutants were calculated per 1 x 10^9 cells. The median values are shown by lines in the box. The first and third quartiles are represented by the upper and lower lines of boxes, respectively. All mutant frequencies were calculated for at least 3-4 independent experiments plated on LB agar with 100 μg/ml rifampicin with up to 40 plates for each strain.

In **Figure 2**, the results show that in the presence of the test system CC107, the frequency of Rif^R mutants is slightly increased compared to the wild type strain without test assay. Interestingly, CC104 had a stronger effect on the frequency of rifampicin mutation appearance. The results also confirm that the frequency of spontaneous mutations is elevated in the absence of the functional GacA/S system.

3.2.2 The frequency of lactose degrading Lac + revertants is elevated in GacA-deficient mutant

The WT+CC104 and gacA+CC104 strains were plated on lactose-containing minimal plates. Mutant colonies able to grow on minimal lactose plates (Lac^+ revertants) that appeared during the next five days were counted. On the second day, few colonies appeared, although most of them started to be clearly visible on the third day. Each strain was grown on ten different (technical replicates) lactose-containing plates. This experiment was repeated three times to gather more data. Obtained results were analyzed and converted into the graph (**Figure 3**). Based on these results, we can conclude that the absence of *gacA* leads to increased base substitution mutation frequency.

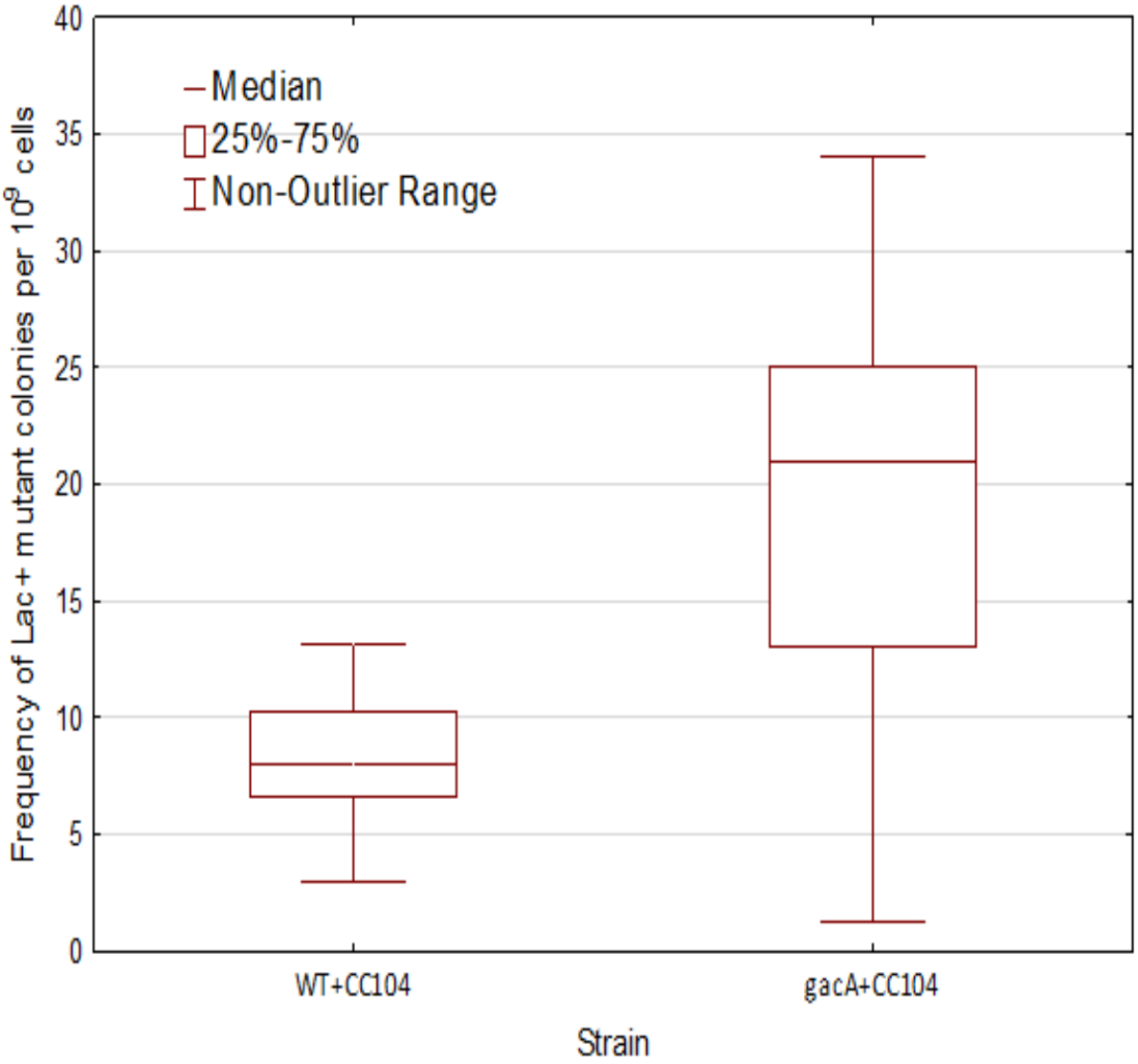

Figure 3. The frequency of Lac$^+$ mutant colonies per 10^9 cells with CC104 assay. The X-axis indicates the strains used in the experiment, which are the KT2440 Lac-test (WT+CC104) and KT2440Lac-test *gacA:: Sm* (gacA+CC104), both containing the plasmid pKT_taclacZ*104CG. Y-axis implies the frequency of Lac$^+$ mutant colonies per 10^9 cells. The Lac$^+$ mutant median values were calculated per billion cells. The line in the box shows the median value. The first and third quartile are represented by the upper and lower lines of boxes, respectively. All mutant frequencies were calculated for at least 3-4 independent experiments plated on LB agar of lactose containing plate up to 40 plates for each strain.

The frequency of frameshift mutations was analyzed with the test system CC107-carrying strain. The experiment was performed in the same way compared to the one with CC104 tester, which is described above (**Figure 3**), however with smaller amount of test system containing cells plated. Obtained results were analyzed and converted into the graph (**Figure 4**). The lack of *gacA* also results in the higher frequency of frameshift mutation. In other words, *gacA* inactivated system rises the mutation frequency in either substitution or deletion mutation frameshift test system based on lactose utilization.

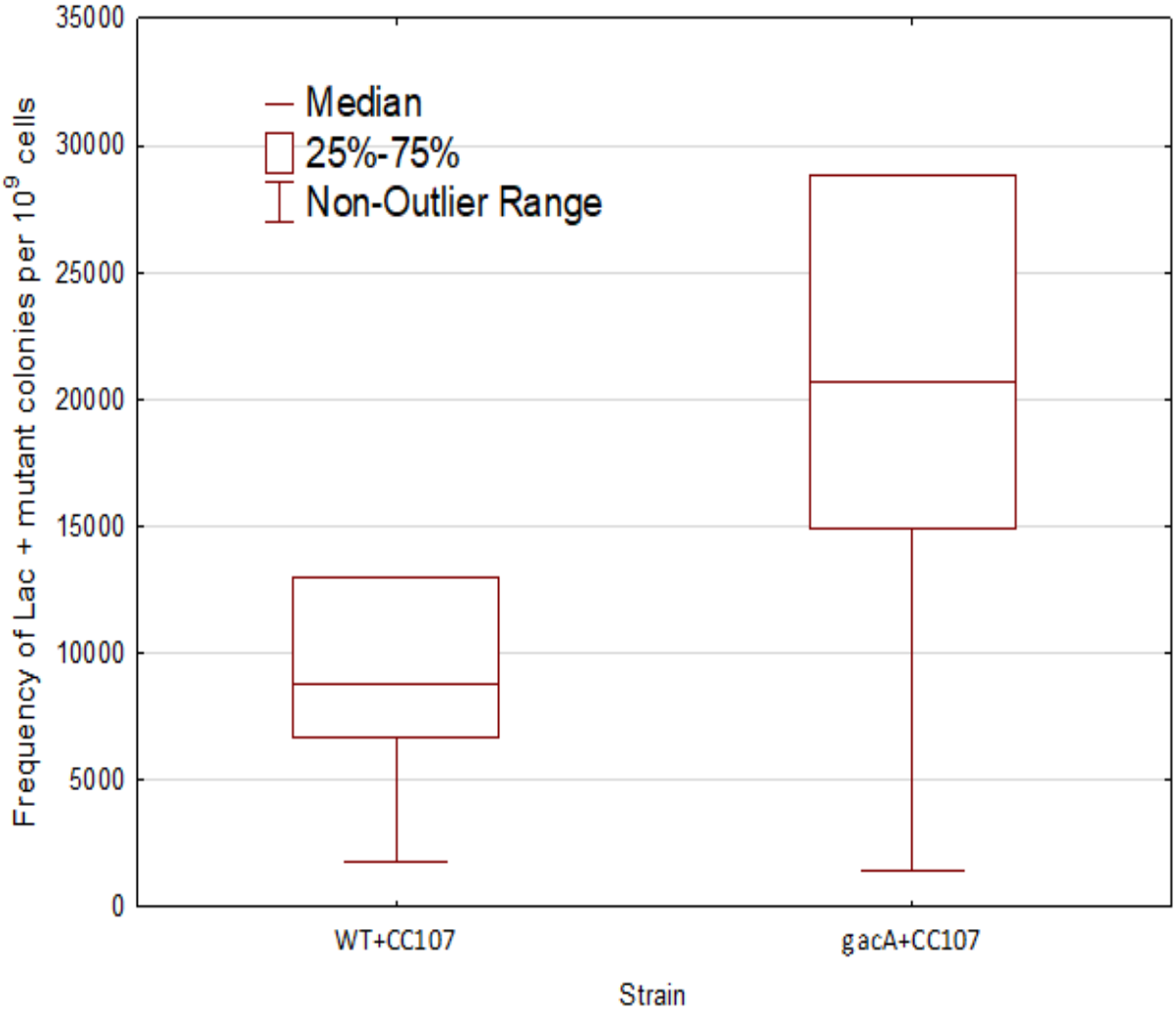

Figure 4. The frequency of Lac$^+$ mutant colonies per 10^9 cells in CC107 strain. The frequency of Lac$^+$ mutant colonies per 10^9 cells in CC107 strain. X-axis indicates the strains used in the experiment, which are the KT2440Lac (WT+CC104) and KT2440Lac-test *gacA::Sm* (gacA+CC104), both containing the plasmid pKT_taclacZ*104CG. Y-axis implies the frequency of Lac$^+$ mutant colonies per 10^9 cells. The Lac$^+$ mutant median values were calculated per 10^9 cells. The line in the box shows the median value. The first and third

quartile are represented by the upper and lower lines of boxes, respectively. All mutant frequencies were calculated for at least 3-4 independent experiments plated on LB agar of lactose containing plate up to 40 plates for each strain.

3.3 DISCUSSION

The absence of a two-component GacA and GacS system has been previously shown to increase the frequency of spontaneous Rif^R mutations in *P. putida* by Mari Tagel (Tagel et al., 2016). It is also proven by the same results in my work. Experiments that were done based on Rif^R assay allow us to identify a huge variation of mutations happening in the *rpoB* gene (Jatsenko et al. 2010). The occurrence of several (often more than eleven) Rif^R mutant colonies have been seen when streaking the bacterial colonies with a mutant phenotype (e.g., GacA inactivated strain) onto the plates containing Rif. On the other hand, few Rif^R mutants from the streaked colony of the wild type occur not many or even have not been observed at all. Hence, the existence of numerous Rif^R colonies could be considered as an indicator of a mutator phenotype.

The usage of Lac^+ assays also demonstrated that the frequency of mutations is elevated in the absence of GacA. In my experiments, the frequency of frameshift mutations (tester strain with a plasmid which contains CC107 mutation in *lacZ* gene) has also been compared with the frequency of base substitutions (tester strain with plasmid which includes CC104 mutation in *lacZ* gene). Compared to the frequency of base substitutions, the frequency of frameshift mutations was several orders of magnitude higher both in the wild type and GacA-deficient *P. putida* strains, demonstrating that the studied frameshift is a mutational hot spot. However, the increasing effect of the absence of GacA on mutation frequency was about two-fold in both for frameshift mutations and for base substitutions.

Interestingly, the wild type strain carrying the plasmid with CC104 mutation in the *lacZ* gene also exhibited slightly increased Rif^R mutant frequency, but for CC107, the effect was smaller (not statistically significant). The reason for this effect is unclear.

SUMMARY

The mutation is the driving force of evolution. In bacteria, mutational processes enable them to adapt and survive in a wide range of environmental conditions. Studying such mechanisms helps scientists get a better understanding of the long-term evolution of bacteria, i.e., how they change their life to adapt with a new environment. There are mainly two different kinds of point mutations, which are frameshift mutations (insertion, deletion) and point mutations (base substitution mutation). Although mutations usually happen during DNA replication, DNA has its own system to recognize and fix mutation before it gets fixed.

The aim of the current thesis was to investigate whether the two-component system GacA/GacS influences mutagenic processes in the bacterium *Pseudomonas putida*. There are two test systems used in this thesis to monitor the mutation frequency in bacteria, especially in *P. putida* strain KT2240. The first one was Rif^R assay, which was used to measure the frequency of spontaneous mutations occurring in *rpoB* gene in bacterial chromosomes on plates containing rifampicin. The second one was a test system based on the lactose degradation system (detection of Lac^+ revertants). This test was used to measure the frequency of more specific mutations on the plasmid. The mutations of interest in this second test are frameshift mutations (assay monitors reversion of deletion mutations) and base substitution mutation.

GacA/S two-component system was used to detect the effect of it when inactivated on the mutation frequency, based on the two test systems mentioned above. The obtained results obtained are the following:

1. In selective plates containing rifampicin, the strain with inactivated GacA exhibited the higher frequency of Rif^R mutations than the wild type strain,

2. In selective plates containing lactose as an only carbon source, when Lac^+ revertants were monitored, both frameshift mutations and base substitutions were elevated about two-fold in the absence of GacA.

Thus, the inactivation of the GacS/GacA system in *P. putida* elevates the frequency of mutations both in bacterial chromosomes and in the plasmid.

Kokkuvõte

Mutatsioonid on aluseks evolutsioonile. Uute mutatsioonide teke võimaldab bakteritel ellu jääda ja kohaneda ebasoodsates keskkonnatingimustes. Seega on mutatsiooniprotsesside uurimine vajalik mõistmaks evolutsioonimehhanisme, mis võimaldavad bakteritel kohaneda uute keskkonnatingimustega. Punktmutatsioonid jaotuvad kahte tüüpi – raaminihkemutatsioonid (insertsioonid ja deletsioonid) ning asendusmutatsioonid. Kuigi mutatsioonid tekivad DNA replikatsiooni käigus, on nende äratundmiseks ja fikseerimiseks rakus veel mitmeid kontrollsüsteeme.

Käesoleva bakalaureusetöö eesmärgiks oli selgitada välja, kas bakteris *Pseudomonas putida* asuv kahekomponendiline regulaatorsüsteem GacS/GacA mõjutab mutatsioonisagedust. Mutatsioonisageduse mõõtmiseks bakteritüves *P. putida* KT2440 on välja töötatud kaks testsüsteemi. Neist esimene põhineb bakteri kromosoomis *rpoB* geenis tekkivate Rif^R mutantide tuvastamisel rifampitsiini sisaldavatel selektiivplaatidel. Teine testsüsteem võimaldab tuvastada laktoosi lagundavate mutantide (Lac^+ revertandid) teket laktoosi sisaldavatel minimaalsöötmetel, jälgida saab nii raaminihke mutatsioonide kui ka asendusmutatsioonide teket plasmiidis. Neid kahte testsüsteemi kasutati mutatsioonisageduse mõõtmiseks *P. putida* metsiktüves ja *P. putida* mutandis, kus GacS/GacA kahekomponendilisest süsteemist oli inaktiveeritud transkriptsiooni regulaatorit GacA kodeeriv geen. Saadud tulemused olid järgmised:

Rifampitsiini sisaldavatel selektiivsöötmetel suurenes Rif^R mutantide tekkesagedus GacA inaktiveerimisel võrreldes Rif^R mutantide tekkesagedusega *P. putida* metsiktüves.

Laktoosi ainsa süsinikuallikana sisaldavatel selektiivsöötmetel suurenes Lac^+ revertantide teke GacA puudumisel ligikaudu kaks korda nii raaminihke mutatsioonide kui ka asendusmutatsioonide puhul.

Seega suureneb GacS/GacA süsteemi defektsuse puhul bakteris *P. putida* mutatsioonisagedus nii kromosoomis kui ka plasmiidis.

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