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LYSINE CATABOLISM AND *IN VIVO* SUBSTRATE SPECIFICITY OF D-AMINO ACID
DEHYDROGENASES IN *PSEUDOMONAS AERUGINOSA* PAO1

by

SAI MADHURI INDURTHI

Under the Direction of Chung-Dar Lu, PhD

ABSTRACT

Among multiple interconnected pathways for L-Lysine catabolism in pseudomonads, it has been reported that *Pseudomonas aeruginosa* PAO1 employs the decarboxylase and the transaminase pathways. However, knowledge of several genes involved in operation and regulation of these pathways was still missing. Transcriptome analyses coupled with promoter activity measurements and growth phenotype analyses led us to identify new members in L-Lys and D-Lys catabolism and regulation, including *gcdR-gcdHG* for glutarate utilization, *dpkA*, *amaR-amaAB* and PA2035 for D-Lys catabolism, *lysR-lysXE* for putative L-Lys efflux and *lysP* for putative L-Lys uptake. The *amaAB* operon is induced by L-Lys, D-Lys and pipecolate supporting the convergence of Lys catabolic pathways to pipecolate. Growth on pipecolate was retarded in the *gcdG* and *gcdH* mutants, suggesting the importance of glutarate in pipecolate and

2-aminoadipate utilization. Furthermore, this study indicated links in control of interconnected networks of lysine and arginine catabolism in *P. aeruginosa*.

Effect of D-amino acids and the genes involved in their metabolism are of great interest in both bacteria and mammals. D-Arg utilization in PAO1 requires the coupled dehydrogenases DauB and DauA. In this study, DauB was found to use only L-Arg as its substrate unlike its partner dehydrogenase DauA with wide substrate specificity. However, evidence from this study and previous studies suggest that the coupled enzymes DauB and DauA are unique for D-Arg catabolism.

The three D-amino acid dehydrogenases DguA, DadA and DauA were found to have somewhat limited *in vivo* substrate specificity compared to that found *in vitro* tested using purified enzymes. Many studies showed that D-amino acids are toxic to bacteria. The $\Delta dguA$, $\Delta dadA$ and $\Delta dauA$ triple mutant had two-fold lower minimum inhibition concentration of carbenicillin and tetracycline compared to wild-type PAO1. Both in the wild-type PAO1 and the triple mutant, synergy was observed between gentamicin or tetracycline (at concentrations below the MIC) and D-amino acids resulting in growth inhibition or reduction, respectively. However, no special synergistic or antagonistic effects were observed specifically in the $\Delta dguA$, $\Delta dadA$ and $\Delta dauA$ triple mutant as compared to the wild-type PAO1 when D-amino acids were given in combination with antibiotics.

INDEX WORDS: Pseudomonas, Lysine, Catabolism, Uptake, Regulation, Dehydrogenase, D-amino acids, Antibiotics, Synergy

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by

SAI MADHURI INDURTHI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

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2016

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December 2016

DEDICATION

To my dear husband Sreekanth Nutulapati, my beautiful son growing in my womb and my mother Vijayalakshmi Pasumarthi with love.

None of this would have been possible without the encouragement, love and patience of my husband and my family. I would like to express my heartfelt gratitude to my husband for all his encouragement and support; without him this dissertation would not have been possible. My gratitude is also extended to my mother who always believed in my abilities and has been my pillar of strength. I cannot forget my little niece Ishitha and nephew Pratheek, for they were my source to unwind and get rejuvenated. I would also like to thank my father Indurthi Venkata Lakshmana Rao, sister Anitha Indrakanti and brother-in-law Siddhartha Indrakanti for their encouragement.

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1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen found widely in the environment. It can cause mild infections in healthy individuals and serious life-threatening infections in hospitalized patients with low immunity. Its metabolic versatility helps the bacterium to thrive in different environments (58). Pseudomonads are known for their ability to utilize a number of organic compounds as carbon and nitrogen sources due to the presence of a large number of catabolic pathways. Understanding the regulation of these catabolic pathways helps gain insight into the strategies employed by this pathogen to survive in human hosts under conditions of varied nutrient availability and cause disease.

1.1 L-Lysine catabolism in *P. aeruginosa*

Unlike other pseudomonads, *P. aeruginosa* cannot utilize Lysine efficiently as a nutrient source. Multiple and interconnected catabolic pathways exist for the utilization of lysine in microbes. As shown in Figure 1.1, L-lysine is catabolized via the monooxygenase pathway in *Pseudomonas putida*, while in *P. aeruginosa* it is mainly mediated via the decarboxylase pathway (5, 49, 51). Expression of the lysine decarboxylase LdcA was found inducible by the arginine regulator ArgR and L-arginine but not L-lysine in *P. aeruginosa* PAO1 (5), making this initial step in the proposed pathway as a bottleneck for L-Lysine catabolism in this organism. Cadaverine, the product of LdcA, is further degraded to 5-aminovalerate through the γ -glutamylolation pathway for polyamine catabolism that is controlled by PauR (7, 65). Both monooxygenase and decarboxylase pathways converge at 5-aminovalerate, which is then converted to glutarate by a pair of transaminase and semialdehyde dehydrogenase enzymes encoded by the *gabDT* operon (7, 50). Glutarate is

proposed to produce acetyl-CoA that can enter the Krebs cycle (42), and glutaryl-CoA dehydrogenase enzyme (an enzyme in glutarate catabolism) activity has been reported in crude extracts of *P. aeruginosa* (15). However, none of the genes for glutarate catabolism and its regulation have been characterized in *Pseudomonas*.

1.2 Lysine racemase and D-lysine catabolism in *P. aeruginosa*

The presence of a lysine (Lys) racemase has been reported in *P. putida* to convert L-Lys to D-Lys (48, 49). The catabolic pathway for D-Lys catabolism in this organism (Figure 1.1) has been proposed to degrade D-Lys to 2-aminoadipate, which is then further degraded to α -ketoglutarate to enter the Krebs cycle. Several genes encoding enzymes of this proposed pathway in *P. putida* KT2440 have been identified, including PP3590 for D-Lys transaminase, PP3596 for D-Lys dehydrogenase, *dpkA* for Δ^1 -Piperidine-2-carboxylate reductase (38, 48, 49), *amaB* and *amaA* in conversion of pipercolate to 2-aminoadipate (48). In the case of *P. aeruginosa*, it does not possess the lysine racemase; however, L-Lys catabolism can potentially be connected to the D-Lys pathway via an arginine-pyruvate transaminase AruH that can take L-Lys as substrate (5, 64). One early report suggested the presence of another route through L-lysine 6-amino transferase to make L-pipercolate which again could possibly convert into glutarate (15). In *P. aeruginosa*, the alpha amino group of D-Lys can be deaminated by the FAD-dependent D-amino acid dehydrogenase DauA (33). It was not clear how D-Lys catabolism was operated in this organism due to very limiting information about potential players in the catabolic pathway.

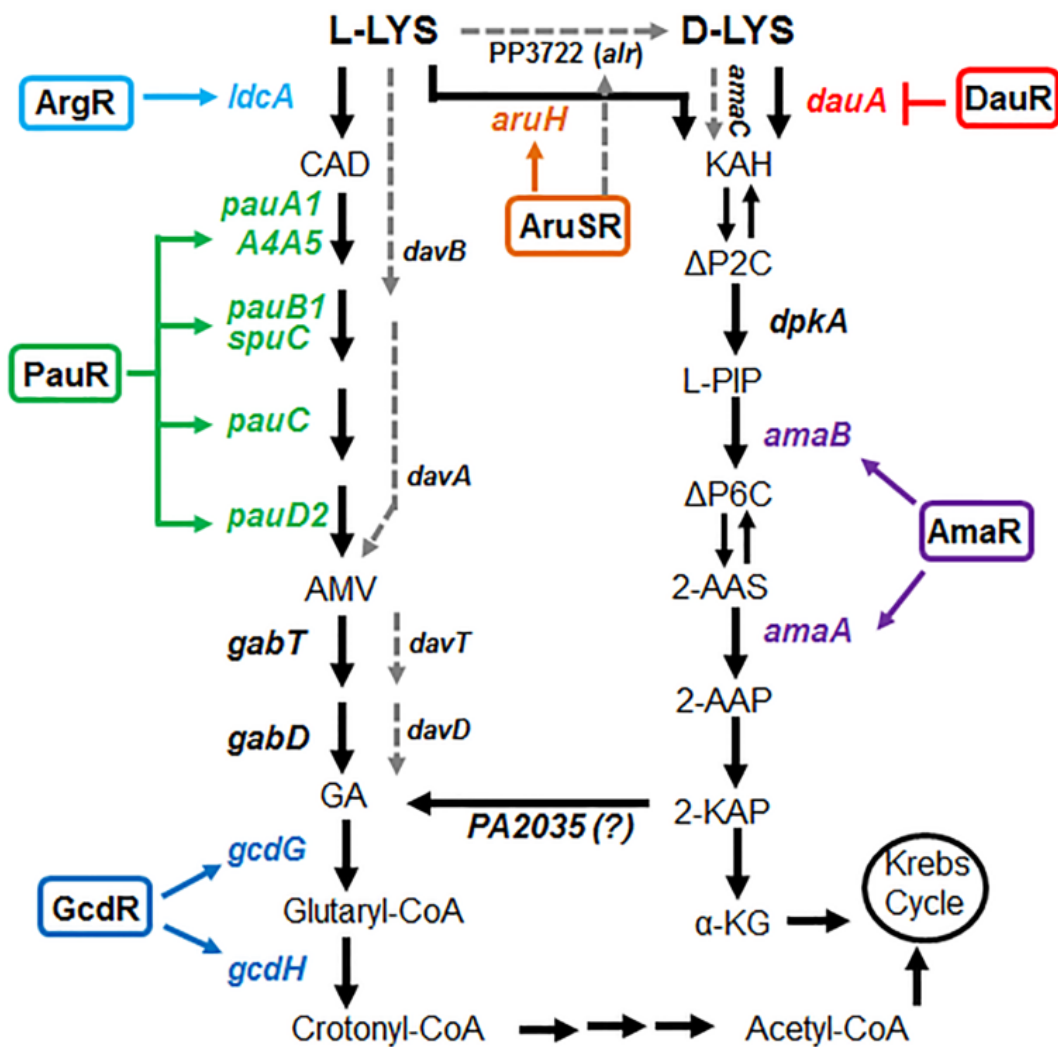


Figure 1.1 Lysine catabolism in *P. aeruginosa* PAO1 and *P. putida* KT2440.

Pathways in *P. aeruginosa* PAO1 and *P. putida* KT2440 were indicated in solid black arrows and dashed gray arrows, respectively. Genes that are subjected to control by transcriptional regulators ArgR, PauR, GcdR, DauR, and AmaR were labeled in different colors. LYS, lysine; CAD, cadaverine; AMV, δ -aminovalerate; GA, glutaric acid; KAH, α -keto- ϵ -aminohexanoate (keto-lysine); Δ P2C, Δ 1-piperidine-2-carboxylate; L-PIP, L-pipecolate; Δ P6C, Δ 1-piperidine-6-carboxylate; 2-AAS, 2-aminoadipate semialdehyde; 2-AAP, 2-aminoadipate; 2-KAP, 2-ketoadipate; α -KG, α -ketoglutarate.

1.3 D-Amino acids in human health and disease

A few decades ago D-amino acids were not believed to be present in mammals, until large amounts of free D-amino acids were detected in mammalian tissues (1). D-amino acids in humans can be derived either by racemization of the cognate L-forms, from the food ingested or from the intestinal normal flora (39). D-Serine (D-Ser) was shown to be associated with neurotransmission, cell migration, and neurotoxicity. Elevated levels of D-Ser have been detected in animal models of amyotrophic lateral sclerosis while a deficiency can trigger seizures (54, 1). Also, D-Ser has been implicated to have a role in Alzheimer's, a disease characterized by a decline in learning and memory. The neuronal networks of the hippocampus, center for memory, is affected by aging. Activation of N-Methyl-D-aspartate subtype of glutamate receptors (NMDAR) which regulates the strength of synaptic transmission is affected by D-serine related pathways (like lack of racemase to convert L-serine to D-form) (2). Elevated levels of D-Ser and D-Asp have implications in the possible development of Schizophrenia (1 & 13).

1.4 Significance of D-amino acids in bacteria

Amino acids exist as L and D-enantiomers. Proteins are composed of L-amino acids predominantly. However, the D-amino acids are also part of living organisms and are important for various cellular functions. Even though some D-amino acid containing peptides were discovered in bacteria, the wider importance of D-amino acids was not well studied or understood until a few decades ago (25). Recent studies showed that D-amino acids have important regulatory roles in the bacterial world in addition to serving as growth nutrient, being incorporated into the peptidoglycan layer (3) or inhibiting the bacterial spore germination (21). Many groups have

demonstrated that D-amino acids can be detrimental to the cell. Certain D-amino acids like D-Met were shown to be incorporated into the peptidoglycan and also inhibit peptidoglycan synthesis and cross-linking (3). D-amino acids in combination with β -lactam antibiotics were reported to alter cell morphology (4). *Bacillus subtilis* was shown to produce a mixture of D-Leu, D-Met, D-Tyr and D-Trp to prevent biofilm formation and also disassemble biofilms (26). Lam *et al*, (28) found unusual D-amino acids in *Vibrio cholerae* and *Bacillus subtilis* that seemed to modify the cell wall in stationary phase cultures. Excess D-amino acids were shown to be released into the environment and could serve as a means of communication to control and coordinate growth among the bacteria living in that environment (26). D-amino acids given in combination with antimicrobials has a synergistic effect on biofilm dispersal in some isolates of *P. aeruginosa* and *Staphylococcus aureus* (52).

1.5 Bacterial biosynthesis of D-amino acids

L-amino acids being predominant in nature, serve as the substrates for D-amino acid biosynthesis by racemization. **Amino acid racemases** are of two types: pyridoxal 5' phosphate (PLP) dependent and PLP independent enzymes. In the PLP dependent enzymes like the alanine and serine racemases, the α -carbon of the amino acid is first deprotonated. The anionic form thus formed is stabilized by the PLP moiety bound to the racemase after which the anionic form is re-protonated on the opposite side generating the other enantiomer of the same amino acid. In the case of PLP independent enzymes like the glutamate and proline racemases, even though they follow a similar mechanism as above they do not need a cofactor to stabilize the intermediate carbanion (67, 4). In addition to racemization from L-to-D form, D-amino acids can also be synthesized by **D-amino acid transaminases** using their respective keto acids and the amino

group donated by D-alanine. In many gram-positive bacteria like *Bacillus*, *Geobacillus* and *Staphylococcal* species D-glutamate (D-Glu) was found to be synthesized by D-amino acid transaminase enzymes. (47, 16, 30). In *P. aeruginosa*, the arginine: pyruvate transaminase AruH was shown to convert L-Lys to α -keto- ϵ -aminohexanoate (64, 5). This is one of the links connecting the L- and D-Lys pathway in *P. aeruginosa* PAO1.

1.6 Degradation of D-amino acids in bacteria

D-amino acids can serve as a nutrient source to support bacterial growth when provided in excess. Some organisms have genes to directly catabolize the D-amino acids while others require them to be converted into the L-forms before they can be catabolized by enzymes like transaminase and amidases (11, 12, 59). The first step in D-amino acid catabolism usually involves D-amino acid oxidases, dehydrogenases or aminotransferases to remove the amino group. The oxidase and dehydrogenase enzymes require cofactors like FAD^+ or NAD^+ as electron acceptors while the aminotransferases or transaminases can directly transfer the amino group from the α -amino acid to a α -keto acid (64, 31, 19, 35, 20).

1.7 D-amino acid catabolism in *P. aeruginosa* PAO1

P. aeruginosa has an enormous catabolic capacity to utilize a variety of D-amino acids as carbon and/or nitrogen sources. As described below, five different enzymes have been reported to facilitate the utilization of D-amino acids in this organism:

1.7.1 Arginine dehydrogenases, DauBA.

The utilization of D-arginine (D-Arg) in this organism is facilitated by the coupled arginine dehydrogenases, DauA and DauB (31). DauA catalyzes oxidative deamination of D-Arg into 2-

ketoarginine and ammonia. DauB then uses 2-ketoarginine and ammonia as substrates to convert them into L-arginine (L-Arg) in the presence of NADPH or NADH (Figure 1.2). This D-to-L racemization is an essential step for the utilization of D-Arg in this organism, taking advantage of four different catabolic pathways of L-Arg. However, it is not known if these two enzymes form a complex and if DauB also shares the wide substrate specificity of its partner enzyme DauA. Mutation of *dauA* abolishes growth on both D-Arg (carbon and nitrogen source) and D-Lys (as nitrogen source) while mutation of *dauB* abolishes growth on D-Arg (as carbon source). Enzymatic reactions using purified His-tagged protein showed that DauA can utilize a number of D-amino acids as the substrate generating their respective keto-acid and ammonia, while the *in vitro* substrate specificity of DauB is yet to be studied (31). In Chapter 2 of this study, we characterized the DauB enzyme and studied its substrate specificity using purified enzyme.

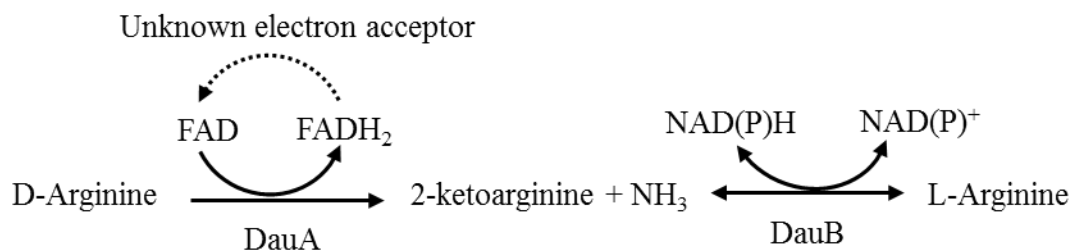


Figure 1.2 Arginine racemization by DauBA.

1.7.2 D-alanine dehydrogenase, DadA.

The *dadRAX* locus encodes the genes for alanine (Ala) catabolism and regulation in *P. aeruginosa* (19). DadX is an amino acid racemase that converts L-alanine to D-alanine while DadA is a dehydrogenase that catalyzes the breakdown of D-alanine into pyruvate and ammonia using FAD as a cofactor (see Figure 1.3). DadR is the transcriptional activator of *dadAX* operon

in the presence of L-alanine. DadA can utilize all D-amino acids as the substrate except for D-Glu and D-Gln as determined by the enzymatic reactions using purified His-tagged DadA. However, *in vivo* studies using the *dadA* mutant revealed that only the utilization of Ala, His, Phe, Ser, Thr and Val (all tested as a nitrogen source in glucose minimal medium) are dependent on DadA (19).

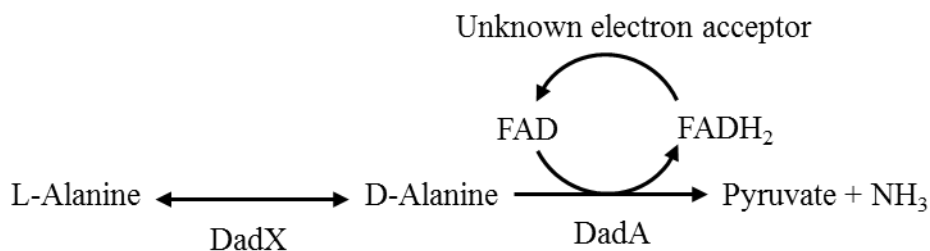


Figure 1.3 Alanine racemization and catabolism by DadXA.

1.7.3 D-glutamate dehydrogenase, DguA.

The divergent *dguR-dguABC* gene cluster was shown to participate in D-Glutamate (D-Glu) and D-Glutamine (D-Gln) catabolism and regulation (20). Both *dguA* and *dguR* mutants completely lost the ability to grow on D-Glu and grew very poorly on D-Gln as the sole source of carbon or nitrogen. DguR is the transcriptional activator of the *dguABC* operon in the presence of exogenous D-Glu and D-Gln. The *dguA* gene encodes a FAD-dependent D-amino acid dehydrogenase which deaminates D-Glu to generate α -ketoglutarate and ammonia in the presence of FAD as the cofactor (Figure 1.4). The *dguB* gene encodes a putative enamine/imine deaminase of the RidA family, but its role in D-Glu utilization is not known yet. The *dguC* gene encodes a periplasmic solute-binding protein, but was found not essential for D-Glu utilization. Instead, the acidic L-amino acid transported AatJQMP was found to be induced and essential for

D-Glu utilization. Enzymatic assays using crude cellular extracts *in vitro* showed that DguA has dehydrogenase activity against D-Glu, D-Pro, and D-Gln. (20).

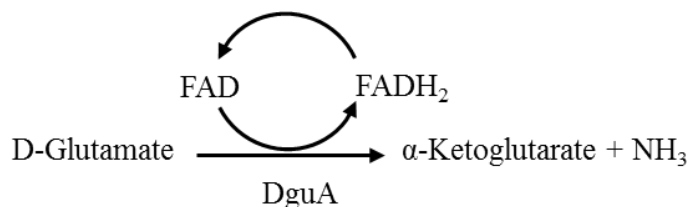


Figure 1.4 D-Glutamate catabolism by DguA.

1.7.4 D-serine dehydratase, DsdA.

D-Serine dehydratase DsdA is an orphan gene in the genome of *P. aeruginosa* PAO1. This gene has no regulatory elements resulting in no activation even in the presence of D-Ser. However, when over expressed it can support the growth of PAO1 on D-Ser as the sole source of carbon and nitrogen. Even though the D-alanine dehydrogenase DadA can utilize D-Ser as the substrate, it can only support growth on D-Ser as nitrogen source but not as a carbon source. These two enzymes have a very different enzymatic reaction. DsdA converts D-Ser into ammonia and pyruvate while DadA converts it into ammonia and β -hydroxy pyruvate. The DsdA is a PLP dependent enzyme (35). In *E. coli*, excess D-Ser can be toxic as it inhibits the biosynthesis of pantothenate and L-Ser. Pantothenate is essential for the biosynthesis of coenzyme A (10). DsdA rescues the cell from the effects of D-Ser by catabolizing it into pyruvate and ammonia. In *E. coli*, the *dsdCXA* locus encodes for the D-serine deaminase (*dsdA*), the D-serine inner membrane transporter (*dsdX*) and a divergently transcribed LysR-type transcriptional activator (*dsdC*). In addition, transcriptional activation of *dsdXA* gene is subject to catabolite repression and requires cyclic AMP and the cyclic AMP activator protein (37, 41).

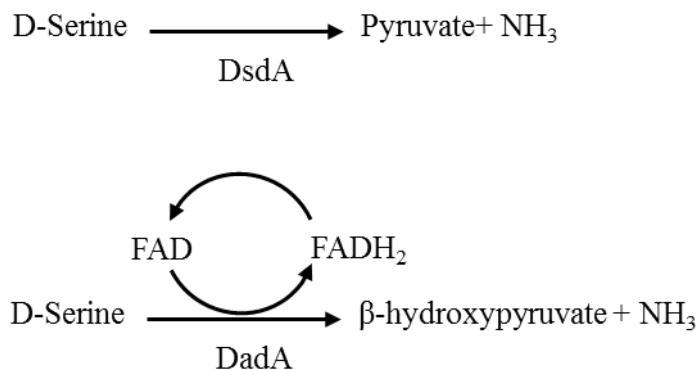


Figure 1.5 D-Serine catabolism by DsdA and DadA.

1.7.5 D-Hydroxyproline dehydrogenase, LhpBEF.

L-Hydroxyproline (L-Hyp), a non-proteogenic amino acid, is found in mammals and also in bacteria. In mammals, it is formed by post-translational modifications by the action of prolyl 4-hydroxylase and is an important constituent of collagen. In some bacteria, during the biosynthesis of secondary metabolites like actinomycin and etamycin, L-Hyp is synthesized from L-proline (29). L-Hyp can serve as a good carbon and nitrogen source in *P. aeruginosa*. This requires the L-Hyp to be converted into D-Hyp first by the epimerase LhpA. Then the D-Hyp is oxidized by the D-Hyp dehydrogenase LhpBEF into Δ^1 -pyrroline-2-carboxylate reductase and subsequently into α -ketoglutarate by the deaminase LhpC and aldehyde dehydrogenase LhpG (62, 34).

1.8 Research Strategy

My research project was focused mainly on identifying the missing players in Lysine utilization in *P. aeruginosa*. Previous research from our lab showed that L-Lysine is catabolized via the decarboxylase pathway to yield cadaverine which then is converted into 5-aminovalerate via the γ -glutamylolation pathway followed by its conversion into glutarate. Even though glutarate is

believed to be catabolized to yield intermediates of the Krebs cycle, the key players in this pathway are not known yet. In *P. aeruginosa*, the genes in D-lysine catabolic pathway and its connections to the L-lysine catabolism are far from being clear. This is the first report to identify the genes involved in glutarate catabolism, their regulation and also show that the D- and L-Lysine pathway are indeed connected in *P. aeruginosa* PAO1. All this work is discussed in Chapter 2.

Previous studies from our lab showed that racemization by the coupled DauA and DauB reactions is essential for D-arginine utilization in *P. aeruginosa* PAO1. They also showed that DauA, a catabolic D-arginine dehydrogenase, exhibits a wide substrate specificity in *in vitro* enzymatic studies. However, the DauB enzyme, an anabolic L-arginine dehydrogenase, has not been characterized. It is also not known if DauB reciprocates the substrate specificity exhibited by its partner dehydrogenase DauA. In this dissertation, we characterized the DauB enzyme and studied its substrate specificity as discussed in Chapter 3.

Even though the three known D-amino acid dehydrogenases (DauA, DadA, and DguA) were characterized previously, their substrate specificity *in vitro* and the effect of these respective mutants in the utilization of various D-amino acids differ greatly. This is possibly due to substrate redundancy as demonstrated by *in vitro* enzymatic studies. In order to identify the true or *in vivo* substrate specificity of each of these dehydrogenase enzymes, we designed a study. We constructed a triple mutant strain devoid of all these three known dehydrogenases discussed above and verified the utilization of various D-amino acids as the sole carbon and or nitrogen sources by complementing one dehydrogenase gene at a time. Further, as discussed above, the

availability of D-amino acids from nature is scarce and emerging research shows that D-amino acids have various regulatory roles. We also tested the hypothesis that the triple mutant devoid of three D-amino acid dehydrogenases will be more sensitive to antibiotics in the presence of D-amino acids. All this work is discussed in Chapter 4.

2 MOLECULAR CHARACTERIZATION OF *LYSR-LYSXE*, *GCDR-GCDHG*, AND *AMAR-AMAAB* OPERONS FOR LYSINE EXPORT AND CATABOLISM: A COMPREHENSIVE LYSINE CATABOLIC NETWORK IN *PSEUDOMONAS AERUGINOSA* PAO1

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2.1 Introduction

P. aeruginosa is a Gram-negative opportunistic human pathogen found widely in the environment. It can cause mild infections in healthy individuals and serious life-threatening infections in hospitalized patients with low immunity. Its metabolic versatility helps the bacterium to thrive in different environments (58). Pseudomonads are known for their ability to utilize a number of organic compounds as carbon and nitrogen sources due to the presence of a large number of catabolic pathways. Understanding the regulation of these catabolic pathways helps gain insight into the strategies employed by this pathogen to survive in human hosts under conditions of varied nutrient availability and cause disease.

2.1.1 L-lysine catabolism:

P. aeruginosa cannot utilize Lysine efficiently as a nutrient source (8). It was reported that this organism employs the decarboxylase pathway for L-lysine catabolism. The expression of the lysine decarboxylase LdcA is inducible by the arginine regulator ArgR and L-arginine but not by L-lysine in *P. aeruginosa* PAO1 (5), making this initial step in the proposed pathway a bottleneck for L-Lysine catabolism in this organism. Cadaverine, the product of LdcA, is further degraded to 5-aminovalerate through the γ -glutamylolation pathway for polyamine catabolism (7, 66). Then 5-aminovalerate is converted to glutarate by 4-aminobutyrate transferase and succinate-semialdehyde transferase enzymes (7, 50). Glutarate is proposed to be converted into acetyl-CoA that can enter the Krebs cycle (42), and glutaryl-CoA dehydrogenase enzyme (an enzyme in glutarate catabolism) activity has been reported in crude extracts of *P. aeruginosa* (15). However, none of the genes for glutarate catabolism and its regulation have been characterized in *Pseudomonas*. In addition to this, the genes for lysine uptake are also not known.

2.1.2 D-lysine catabolism:

P. aeruginosa can utilize D-Lys only as a nitrogen source but not as a carbon source. In the case of *P. putida* which can utilize D-Lys as a carbon and nitrogen source, D-Lys catabolism was reported to be mediated by the amino adipate pathway converting it into 2-amino adipate and further into α -ketoglutarate to enter the Krebs cycle. In addition, the presence of a lysine racemase has been reported in *P. putida* to convert L-Lys to D-Lys (49, 51) making the amino adipate pathway an alternate to L-Lys catabolism. In the case of *P. aeruginosa*, it does not possess the lysine racemase; however, L-Lys catabolism can potentially be connected to the D-Lys pathway via an arginine: pyruvate transaminase AruH (5, 64). L-Lys can also be taken up by L-lysine 6-amino transferase to make L-pipecolate (L-Pip) which again could convert into glutarate (15). In *P. aeruginosa*, the FAD-dependent D-amino acid dehydrogenase DauA can deaminate the alpha amino group of D-Lys (33). It was not clear how D-Lys catabolism was operated in this organism due to very limiting information about potential players in the catabolic pathway.

2.1.3 Research Strategy

Many of the intermediate compounds in the proposed lysine catabolic pathways in *P. aeruginosa* PAO1 can serve as better sources of carbon and nitrogen than lysine itself. It was known that lysine catabolism is composed of multiple modules of biochemical reactions, and the expression of each module is controlled by a specific intermediate compound and its corresponding regulators. To gain a better understanding of the interconnections of L-Lys and D-Lys catabolism in *P. aeruginosa*, we conducted transcriptome analysis to identify several possible missing links and new players, and these thus identified members were subjected to further characterization to tie in their physiological functions and regulatory mechanisms into an integrated metabolic network.

2.2 Materials and methods

2.2.1 Strains and growth conditions.

Bacterial strains used in this study were listed in Table 2.1. Luria–Bertani (LB) medium was used for bacterial growth with the following supplements as required: ampicillin at 100 µg/ml and carbenicillin at 100 µg/ml for both *E. coli* and *P. aeruginosa*. Minimal medium P (MMP) supplemented with specific carbon sources and nitrogen sources was used for the growth of *P. aeruginosa* (16). Unless specified, mutant strains with a single lesion by transposon insertion used in this study (Table 2.1) were obtained from the stock center at University of Washington, Seattle (24).

Table 2.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i>		
DH5α	F– Φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)	Bethesda
	U169 <i>recA1 endA1 hsdR17</i> (rK–, mK+) <i>phoA supE44</i> λ– <i>thi</i> –	Research Laboratories
TOP10	1 <i>gyrA96 relA1</i> F– <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1araD139</i> Δ(<i>ara leu</i>) 7697 <i>galU galK</i> <i>rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
<i>P. aeruginosa</i>		
PAO1	Wild type	(18)
PW1815	<i>gcdG</i> -F01::IS <i>lacZ</i> /hah	(24)
PW1817	<i>gcdH</i> -A02::IS <i>phoA</i> /hah	(24)
PW1819	<i>gcdR</i> -C02::IS <i>phoA</i> /hah	(24)
PW2858	PA1026-G12::IS <i>phoA</i> /hah	(24)
PW2859	<i>amaA</i> -E05::IS <i>phoA</i> /hah	(24)
PW2862	<i>amaB</i> -B10::IS <i>lacZ</i> /hah	(24)
PW3259	<i>dpkA</i> -D05::IS <i>phoA</i> /hah	(24)
PW4475	<i>dhcR</i> -G02::IS <i>lacZ</i> /hah	(24)

PW4478	<i>atoB</i> ::IS <i>lacZ</i> /hah	(24)
PW4480	PA2002-D03::IS <i>lacZ</i> /hah	(24)
PW4481	<i>bdhA</i> -H08::IS <i>phoA</i> /hah	(24)
PW4520	PA2035-D05::IS <i>phoA</i> /hah	(24)
PW8087	PA4181-F03::IS <i>lacZ</i> /hah	(24)
PW8089	PA4182-B01::IS <i>lacZ</i> /hah	(24)
PW8366	<i>lysR</i> -F10::IS <i>phoA</i> /hah	(24)
PW8368	<i>lysX</i> -G02::IS <i>lacZ</i> /hah	(24)
PW8370	<i>lysE</i> -A07::IS <i>phoA</i> /hah	(24)
PW9274	<i>amaR</i> -E05::IS <i>lacZ</i> /hah	(24)
PW9379	PA4979-G11::IS <i>phoA</i> /hah	(24)
PW9381	PA4980-B07::IS <i>phoA</i> /hah	(24)
PW9382	PA4981-C03::IS <i>phoA</i> /hah	(24)
PAO5720	<i>aruH</i> ::Tc ^r	(5)
PAO501	<i>argR</i> ::Gm ^r	(44)
PAO5508	<i>aruS</i> ::Tc ^r	(65)
PAO5509	<i>aruR</i> ::Tc ^r	(65)
MJ 27	Parental strain of MJ99	(61)
MJ 99	$\Delta dhcAB$	(61)

Plasmids

pQF50	<i>bla lacZ</i> transcriptional fusion vector	(14)
pLYS 1-5	<i>lysX</i> :: <i>lacZ</i> fusion of pQF50	This study
pGCD 1-5	<i>gcdH</i> :: <i>lacZ</i> fusion of pQF50	This study
pAMA1-8	<i>amaA</i> :: <i>lacZ</i> fusion of pQF50	This study
pPA4181	PA4181:: <i>lacZ</i> fusion of pQF50	This study
pLYSP	<i>lysP</i> :: <i>lacZ</i> fusion of pQF50	This study
pBAD-HisE	Vector modified from pBAD-HisC	(31)
pGCDR-HisC	6His-tagged GcdR protein expression vector	This study
pLYSR-HisC	6His-tagged LysR protein expression vector	This study
pAMAR-HisC	6His-tagged AmaR protein expression vector	This study

Tc^r, tetracycline resistance; Gm^r, gentamicin resistance

2.2.2 Transcriptome analysis.

Two independent sets of *P. aeruginosa* PAO1 cultures were grown aerobically in MMP media with 10 mM L-Glu with 10 mM L-Lys, cadaverine (Cad), 5-amino valerate (AMV), glutaric acid (GA), D-Lys or 5 mM L-pipecolate (L-Pip). Cells in the exponential growth phase were harvested, and RNA samples were extracted from cells with RNeasy *Plus* Mini Kit (Qiagen). Following the protocol of Affymetrix, cDNA was synthesized, fragmented, and labeled. Labeled cDNA was used

for GeneChip Microarrays with *P. aeruginosa* Genome Array. Data were collected and analyzed by comparing gene expression under each test condition to that by L-Glu following the same parameters as described previously (6). Data were processed by Microarray Suite 5.0 software, normalizing the absolute expression signal values of all chips to a target intensity of 500. Only genes showing consistent expression profiles in duplicates were selected for further analysis. All GeneChip data used by this study have been deposited in NCBI database (GEO accession number GSE75502).

2.2.3 Construction of gene promoter::*lacZ* fusions.

For the construction of promoter *lacZ* fusions, the regulatory regions of the genes were amplified from the genomic DNA of *P. aeruginosa* PAO1 by PCR with specific primer pairs as following: GCD1-F and GCD1-R for pGCD1; LYS1-F and LYS1-R for pLYS1; AMA1-F and AMA1-R for pAMA1; PA4181-F and PA4181-R for pPA4181; and LYSP-F and LYSP-R for pLYSP. Purified PCR products were digested with specific restriction enzymes and ligated to pQF50 (13) digested with the same enzymes before they were transformed into *E. coli* DH5 α . The positive clones were selected on LB plates containing ampicillin, and the nucleotide sequence of the inserts was confirmed by DNA sequencing. Plasmids with serial deletions from the full-length inserts were constructed by the same approach with specific primers.

2.2.4 Cloning, expression, and purification of hexahistidine-tagged LysR and GcdR.

The structural genes of *lysR*, *gcdR* and *amaR* were amplified by PCR from the genomic DNA of *P. aeruginosa* PAO1 using the following primer pairs: LysR-F and LysR-R for *lysR*; GcdR-F and

GcdR-R for *gcdR*; and AmaR-F and AmaR-R for *amaR*. The resulting PCR products were digested with specific restriction enzymes and cloned into the expression vector pBAD-HisE (31) so that the C-terminus of LysR, GdhR and AmaR was fused in-frame with the His6-tag while the N-terminus is preceded by a ribosome binding site and an arabinose-inducible promoter in the plasmid. The resulting plasmids, pLYSR, pGCDR, and pAMAR were introduced into *E. coli* Top10 (Invitrogen). For overexpression of LysR, GcdR and AmaR proteins, the recombinant strains of *E. coli* were grown in LB medium containing ampicillin at 37 °C. For over expression, 0.2 % L-arabinose (w/v) was added to the culture when the OD₆₀₀ was approximately 0.5-0.6. After 4h of vigorous shaking at 18 °C, cells were harvested by centrifugation and stored at -80 °C until use.

The cell pellets were suspended in phosphate buffer A (20 mM Na₂HPO₄, 0.5 M NaCl, 20 mM imidazole, pH 7.5) with PMSF (1 mM) as a protease inhibitor and the cells were ruptured by an Aminco French press. Cell debris was removed by centrifugation at 30,000 g for 30 min. The supernatant was applied to a HisTrap HP column (GE Health Care), and the His-tagged LysR was eluted at 20-60 % buffer B and His-tagged GcdR was eluted at 40-70 % buffer B (20 mM Na₂HPO₄, 0.5 M NaCl, 1 M imidazole, pH 7.5). The target fractions were analyzed by SDS-PAGE, pooled and concentrated using an Amicon Ultra-15 centrifugal filter unit (molecular mass cut-off, 30 kDa; Millipore) to change the buffer to 20 mM Tris-HCl (pH 7.5). Protein concentration was determined by the method of Bradford (27). In the case of AmaR, all the protein was in the pellet fraction (insoluble), and nothing was eluted in the fractions collected from the nickel column after purification.

Table 2.2 Primers used in this study.

Primer name	Primer sequence	Reference
GCD1-F	5'-ATGCGGATCCGGGTCAGGGACAACCTCCTCC-3'	This study
GCD1-R	5'-ATGCAAGCTTCCGGTCTCGCCCATCTCGCG-3'	This study
LYS1-F	5'-ATGCGGATCCGCAGGCGTTCGGGGCACC-3'	This study
LYS1-R	5'-ATGCAAGCTTCCGTGGCGAAGGCGGCGGTCAG-3'	This study
AMA1-F	5'-ATGCGGATCCGTCGGCGTTC GCAGGGTG-3'	This study
AMA1-R	5'-ATGCGGATCCGCCCTCCAGTCGCAC GCA-3'	This study
PA4181-F	5'-ATGCGGATCCGCGCAGGGACTCCAGCAGGT-3'	This study
PA4181-R	5'-ATGCAAGCTTGGCGCTCTACGGCGCGGCCG-3'	This study
LysR-F	5'-ATCTCATGATTTTGTTCGACTACAAGTTGCTCG CCGC-3'	This study
LysR-R	5'-GGGGCCTGACACCCGCACCAAGCC-3'	This study
GcdR-F	5'-ATCTCATGAGACGCAAGATCCCTTCCACCG-3'	This study
GcdR-R	5'- GGGGCCAGCCCGTTGGCCTCGC-3'	This study
AmaR-F	5'-ATCGGATCCATGCTGAACAAACGCCGCCTGC-3'	This study
AmaR-R	5'-GGGAACCGGGCCGTGGCGCGTC-3'	This study
LYSP-F	5'-ATGCGGATCCCGCCCATCGCCTGTCCGGAA-3'	This study
LYSP-R	5'-ATGCAAGCTTCCGCGGAGCCCATGAACAGC-3'	This study

2.2.5 Electrophoretic mobility shift assays (EMSA).

The DNA probes (2 ng) were allowed to interact with different concentrations of GcdR or LysR in the reaction buffer containing 50 mM Tris-HCl (pH 7.5 or pH 8.5, respectively), 50 mM KCl, 1 mM EDTA, 5 % (v/v) glycerol, 4 mM dithiothreitol (DTT) and 200 µg/ml acetylated bovine serum albumin. After incubation for 10 min at room temperature, 20 µl of each reaction mixture was loaded on a polyacrylamide gel (6 %) in Tris/borate-EDTA buffer (pH 8.7). The gels were stained with SYBR Gold solution (Invitrogen) for 20 min, washed twice with deionized H₂O, and scanned.

2.3 Results

2.3.1 Transcriptome profiling.

Many of the intermediate compounds in the proposed lysine catabolic pathways in *P. aeruginosa* PAO1 can serve as better sources of carbon and nitrogen than lysine itself. We hypothesized that lysine catabolism is composed of multiple modules of biochemical reactions, and each module is controlled by a specific intermediate compound and its corresponding regulatory mechanism at the genetic level. To get a more detailed understanding of lysine catabolism and regulation, DNA microarray experiments were conducted to get a snapshot of gene expression during exponential growth phase in the wild type strain PAO1 grown in glutamate (L-Glu) minimal medium supplemented with L-Lys, CAD, AMV, GA, D-Lys or L-Pip. The expression profiling under each test condition was compared to that of L-Glu and the genes that displayed over three-fold induction with significant signal levels in duplicates were selected for further analyses.

Listed in Table 2.3 is a selected group of genes that was subjected to more detailed characterization in this study. Some genes that passed the initial screening parameters have been characterized in our previous reports as members of the γ -glutamylolation pathway for CAD and polyamine catabolism (7, 66), and hence were intentionally omitted from the list. Therefore in this table, we mainly focused on genes that are either new or less characterized members in the Lys metabolic network. In particular, this study characterized three operons (*lysXE-R*, *gcdHG-R* and *amaAB-R*) that play significant roles in Lys utilization in PAO1, apart from some other genes that are activated by L-Lysine.

Table 2.3 Genes selected from DNA microarray analysis for L-Lys catabolism.

PA ID*	Gene Name	Signal Intensity§							Description
		Glu	L-Lys	CAD	AMV	GA	D-Lys	L-Pip	
PA0446	<i>gcdG</i>	266	1263	1698	2294	11738	615	2256	Acyl-coA transferase
PA0447	<i>gcdH</i>	242	9746	7201	9183	19218	1572	4215	Glutaryl-coA dehydrogenase
PA0448	<i>gcdR</i>	115	83	69	91	156	56	58	Transcriptional regulator
PA1027	<i>amaA</i>	139	1230	197	238	81	4858	6105	NAD-dependent aldehyde dehydrogenase
PA1028	<i>amaB</i>	75	483	231	144	136	2460	3521	FAD-dependent oxidoreductase
PA1252	<i>dpkA</i>	113	205	465	279	105	285	252	L-malate dehydrogenase
PA1998	<i>dchR</i>	126	284	351	322	165	221	238	Transcriptional regulator
PA1999	<i>dchA</i>	54	72	215	371	7081	161	153	Dehydrocarnitine CoA transferase
PA2000	<i>dchB</i>	63	156	380	624	5767	157	165	Dehydrocarnitine CoA transferase
PA2001	<i>atoB</i>	101	165	175	259	3763	95	73	Acetyl-CoA acetyltransferase
PA2002		38	165	185	136	542	95	103	Conserved hypothetical protein
PA2003	<i>bdhA</i>	137	402	411	375	1387	315	257	3-hydroxybutyrate dehydrogenase
PA2035		51	198	167	35	82	1659	2974	Probable decarboxylase
PA4181		169	5721	432	385	191	344	416	Hypothetical protein
PA4182		234	8589	423	457	282	276	250	Transcriptional factor
PA4363	<i>lysR</i>	54	59	35	104	45	65	28	Inhibitor of chromosome initiation IciA
PA4364	<i>lysX</i>	38	2448	125	30	60	182	162	Hypothetical protein
PA4365	<i>lysE</i>	33	2304	181	111	37	89	115	Lysine efflux permease
PA4914	<i>amaR</i>	187	321	299	337	137	239	142	Transcriptional regulator
PA4976	<i>aruH</i>	130	630	130	53	111	180	149	Arginine: pyruvate transaminase
PA4981	<i>lysP</i>	38	5855	189	39	61	100	93	Amino acid permease

* The PA ID numbers were from the PAO1 genome annotation project (WWW.pseudomonas.com).

§ GeneChip raw data were mean values of two independent sets of cultures.

2.3.2 Growth phenotypes.

Mutants of various genes under study were obtained from the transposon mutant library stock center (University of Washington). These mutants were checked for growth on L-Lys, CAD, AMV, GA, L-Pip or L-Arg as the sole source of carbon and nitrogen on minimal medium plates

and the growth was recorded as in Table 2.4. In the case of glutarate which can only serve as a carbon source, ammonium chloride (0.5 %) was included in the growth medium as the nitrogen source.

Table 2.4 Growth phenotype of various *P. aeruginosa* knockout mutants.

PA ID	Strain	Genotype	Transposon direction§	Growth with indicated supplement					
				L-Arg	L-Lys	CAD	AMV	GA	L-Pip
	PAO1	Wild-type	NA	++	++	++	++	++	++
PA0446	PW1815	<i>gcdG</i>	R	++	-	-	-	-	-
PA0447	PW1817	<i>gcdH</i>	R	++	-	-	-	-	-
PA0448	PW1819	<i>gcdR</i>	F	++	-	-	-	-	NT
PA1026	PW2858		R	++	++	++	++	++	++
PA1027	PW2859	<i>amaA</i>	R	++	+	++	++	++	-
PA1028	PW2862	<i>amaB</i>	F	++	+	++	++	++	-
PA1252	PW3259	<i>dpkA</i>	R	++	++	++	++	++	NT
PA1998	PW4475	<i>dhcR</i>	R	++	++	++	++	++	++
PA2001	PW4478	<i>atoB</i>	F	++	++	++	++	++	++
PA2002	PW4480		R	++	++	++	++	++	++
PA2003	PW4481	<i>bdhA</i>	R	++	++	++	++	++	++
PA2035	PW4520		R	++	+	++	++	++	-
PA4181	PW8087		F	++	++	++	++	++	NT
PA4182	PW8089		R	++	++	++	++	++	NT
PA4363	PW8366	<i>lysR</i>	F	++	+++	++	++	++	NT
PA4364	PW8368	<i>lysX</i>	F	++	+++	++	++	++	NT
PA4365	PW8370	<i>lysE</i>	F	++	+++	++	++	++	NT
PA4914	PW9274	<i>amaR</i>	F	++	+	++	++	++	-
PA4976	PAO5503	<i>aruH</i>		++	++	++	++	++	NT
PA4979	PW9379		F	++	++	++	++	++	NT
PA4980	PW9381		R	++	++	++	++	++	NT
PA4981	PW9382	<i>lysP</i>	R	++	+/-	++	++	++	NT
	*MJ27	Wild-type		++	++	++	++	++	++
PA1999-2000	*MJ99	<i>dhcAB</i>		++	++	++	++	++	++

Cells from two independent colonies were grown in MMP with the compounds indicated as the sole source of carbon and nitrogen (20 mM) except for GA (10 mM) where 0.5 % NH₄Cl was added as the nitrogen source. Aerobic growth at 37 °C was followed for 4 days and recorded as follows under each growth condition: +++, better growth than wild-type PAO1; ++, same growth as wild-type PAO1; +, weaker growth than wild-type PAO1; +/-, very poor growth compared with wild-type PAO1; 2, no growth even after 4 days. NT, Not tested; NA, not applicable.

Significant changes in the growth phenotype were marked in gray. The PW strains of transposon insertion mutants were obtained from the University of Washington Pseudomonas stock center. MJ27 and MJ99 were gifted by Dr. M. J. Wargo (University of Vermont).

*PA ID numbers were from the PAO1 genome annotation project (<http://www.pseudomonas.com>). †Transposon direction refers to the absolute direction of the transposon and transcriptional direction of a strong internal promoter carried by the transposon in the genome. F, forward; R, reverse. Forward is oriented parallel to increasing nucleotide base number and PA gene annotation number.
‡MJ27 is the parental strain of MJ99 and the comparison is made between these two and not the wild-type PAO1 above.

It was found that mutants of *gcdG*, *gcdH* or *gcdR* were completely defective for growth on L-Lys, CAD, AMV, GA and L-Pip but grew normally on L-Arg. Based on the genome annotation, the divergent *gcdR-gcdHG* operon (Figure 2. 4a) encodes a transcriptional regulator, the glutaryl-CoA dehydrogenase, and a putative acyl-CoA transferase, respectively. The results of growth phenotype analyses support the proposed functions of these genes in glutarate utilization. Surprisingly, it was found that these mutants cannot grow on L-Pip that is the intermediate compound in D-lysine catabolism and was proposed to be converted into 2-aminoadipate (Figure 1.1). The results would, therefore, support the route for converting 2-aminoadipate into glutarate, but not α -ketoglutarate as has been suggested by early reports (46, 49).

In the proposed pathway, conversion of L-Pip into 2-aminoadipate requires two sequential reactions catalyzed by AmaB and AmaA. Indeed the *amaA* and *amaB* mutants were completely defective for growth on L-Pip, supporting their role in L-Pip and hence D-Lys catabolism. Furthermore, these two mutations also affected growth on L-Lys, suggesting the presence of a metabolic flow that is branched out from L-Lys catabolism (Figure 1.1). A similar pattern of growth phenotypes was also observed for the mutant of *amaR* encoding a putative transcriptional regulator of the *amaAB* operon.

Among genes that were induced specifically by L-lysine, PA4981 (*lysP*) encodes a putative amino acid permease, and the lesion in this gene exhibited strong growth defect on L-Lys. We also compared the growth phenotype of the *lysP* mutant and the wild-type strain PAO1 on glucose minimal medium with different nitrogen sources, including ammonium, L-Arg, D-Arg, L-Lys, and D-Lys. The apparent growth defect of the *lysP* mutant was observed with D-Lys and L-Lys but not D-Arg and L-Arg. It suggested the significant role of LysP as a potential Lys transporter.

On the contrary, among all mutants tested, it was found that the *lysR*, *lysX*, and *lysE* mutants grew better than the parental strain on L-Lys. The *lysX* gene codes for a protein of unknown function while the *lysE* gene codes for a putative amino acid exporter of the LysE/ArgO family, showing 45% sequence identity to the arginine exporter ArgO and the lysine exporter LysO of *E. coli* (40, 45). Upstream of the *lysXE* operon, the divergent *lysR* gene was annotated as *iciA* (60) by the genome project, and the encoding transcriptional regulator exhibited 41% sequence identity to ArgP of *E. coli* in control of ArgO expression. As supported by other lines evidence described below, the enhanced growth on L-Lys of the *lysR* mutant was very likely due to its activation effect on *lysXE* expression.

2.3.3 Induction of the *gcdHG* promoter.

To study regulation of the *gcdHG* operon, a *gcdH::lacZ* transcriptional fusion plasmid (pGCD1; Figure. 2.3a) was constructed and introduced into wild-type PAO1 as described in Materials and Methods. The ability of various compounds to activate the *gcdH* promoter was tested. Consistent with the results of GeneChip analysis (Table 2.3), a similar pattern of promoter induction by L-

Lys, CAD, AMV, GA, D-Lys and L-Pip was observed in PAO1 harboring pGCD1 by measurements of β -galactosidase activities (Figure 2.1).

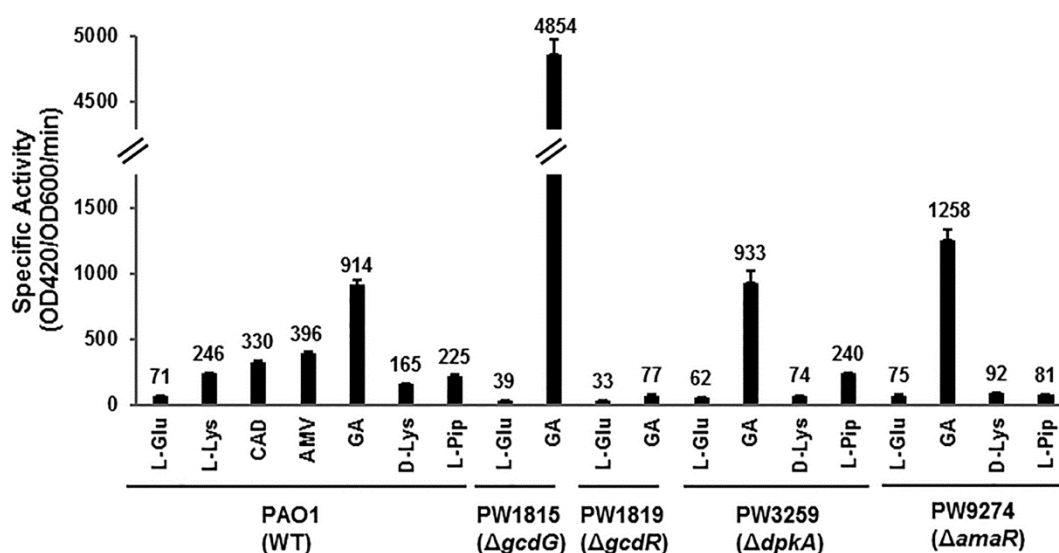


Figure 2.1 Expression profile of the *gcdH* promoter *in vivo*.

The *gcdH* promoter activity was monitored by measuring β -galactosidase activity in the wild-type (WT) PAO1 and its mutants harboring the pGCD1 plasmid. The cells were grown in L-Glu (10 mM) minimal medium in the presence of various compounds as indicated above (10 mM). Specific activity values represent the mean \pm SD of three measurements for each growth condition. L-Glu, L-glutamate; L-Lys, L-lysine; CAD, cadaverine; AMV, 5-aminovalerate; GA, glutarate; D-Lys, D-lysine; L-Pip, L-pipecolate.

Among compounds tested, exogenous glutarate exerts the strongest effect on promoter induction, suggesting glutarate as the potential effector compound for promoter activation. To test this hypothesis, the levels of *gcdH* promoter activation by glutarate were also measured in the *gcdG* and *gcdR* mutants harboring pGCD1. In comparison to the level in the wild-type PAO1, this promoter was activated more than five-fold higher in the *gcdG* mutant, which was expected to accumulate glutarate inside the cell without the acyl-CoA transferase activity. On the contrary, this promoter showed no sign of induction in the *gcdR* mutant (Figure 2.1). These results support the

hypothesis of GcdR as the transcriptional activator of the *gcdHG* operon in response to glutarate as the signal molecule.

2.3.4 DNA binding activity of GcdR.

The results of growth phenotype analysis and promoter measurements indicated the potential function of GcdR as the transcriptional regulator of the *gcdHG* operon in glutarate catabolism. Recombinant GcdR with a hexahistidine tag at its carboxyl terminus was expressed and purified from a strain of *E. coli* as described in Materials and Methods. This recombinant GcdR was used to demonstrate its interactions with the *gcdH* promoter region by electrophoretic mobility shift assays (EMSA). As shown in Figure 2.2, GcdR forms nucleoprotein complexes with DNA fragments covering the *gcdHG* promoter region carried by pGCD1. Although the presence of glutarate in the binding reaction did not cause any change in affinity of GcdR to the probe DNA, it seemed to favor nucleoprotein complexes that migrate slower on the gel in comparison to those without glutarate.

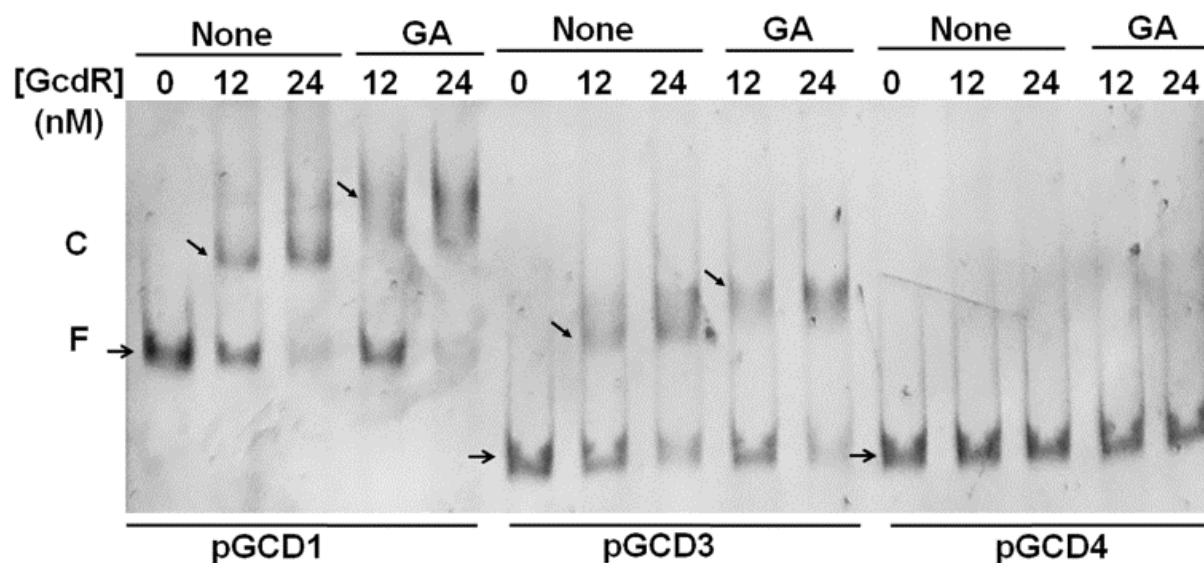


Figure 2.2 Demonstration of GcdR-DNA interactions.

The *gcdH* promoter regions as in pGCD1, pGCD3 or pGCD4 were used as the probe. The purified GcdR-His(6) of the concentrations indicated was used with or without 5 mM glutaric acid (GA) as indicated in each reaction. F, free probe; C, nucleoprotein complexes. Locations of free probes and major nucleoprotein complexes are also marked with arrows.

In order to further narrow down the GcdR binding site, shorter probes carried by pGCD2, pGCD3, pGCD4 and pGCD5 (Figure 2.3a) were generated and tested for interactions with GcdR (Figure 2.2). The result of serial deletions led us to identify a region of 30 bp that may carry the GcdR operator site as described below. It was also supported by the promoter activity measurements (Figure 2.3b) carrying the same series of promoter fragments as in the EMSA. Site-directed mutagenesis of the proposed GcdR operator in pGCD4 or absence of the proposed GcdR operator in pGCD5 resulted in complete abolishment of glutarate-dependent induction of the *gcdH* promoter activity.

2.3.5 Multiple sequence alignment of *gcdHG* promoters in *Pseudomonas*.

Information from comparative genomics indicated that *gcdR-gcdHG* genes are highly conserved among various species of *Pseudomonas* (www.pseudomonas.com). Multiple sequence alignment was conducted for the *gcdHG* regulatory regions from representative species which revealed a highly conserved sequence motif. In accordance with the results of promoter analysis and gel shift assays shown in Figure 2.1 and 2.2, it appeared that the sequence motif (5'-GTGAGAAAATAGCAC-3') 109 bp upstream of the *gcdH* initiation codon might be a potential candidate of GcdR operator. A second conserved sequence motif was found downstream of the proposed GcdR operator site (Figure 2.3d). However, this site was excluded as the GcdR operator site as binding of GcdR to the pGCD1 probe was retained after digestion with the restriction enzyme *EcoRI*, which is located in the middle of the second conserved sequence motif (data not shown). Site-directed mutagenesis of the first three bases in the proposed GcdR operator from GTG in pGCD3 to ACA in pGCD4 resulted in complete loss of GcdR binding *in vitro* (Figure 2.2) and loss of promoter activation *in vivo* (Figure 2.1).

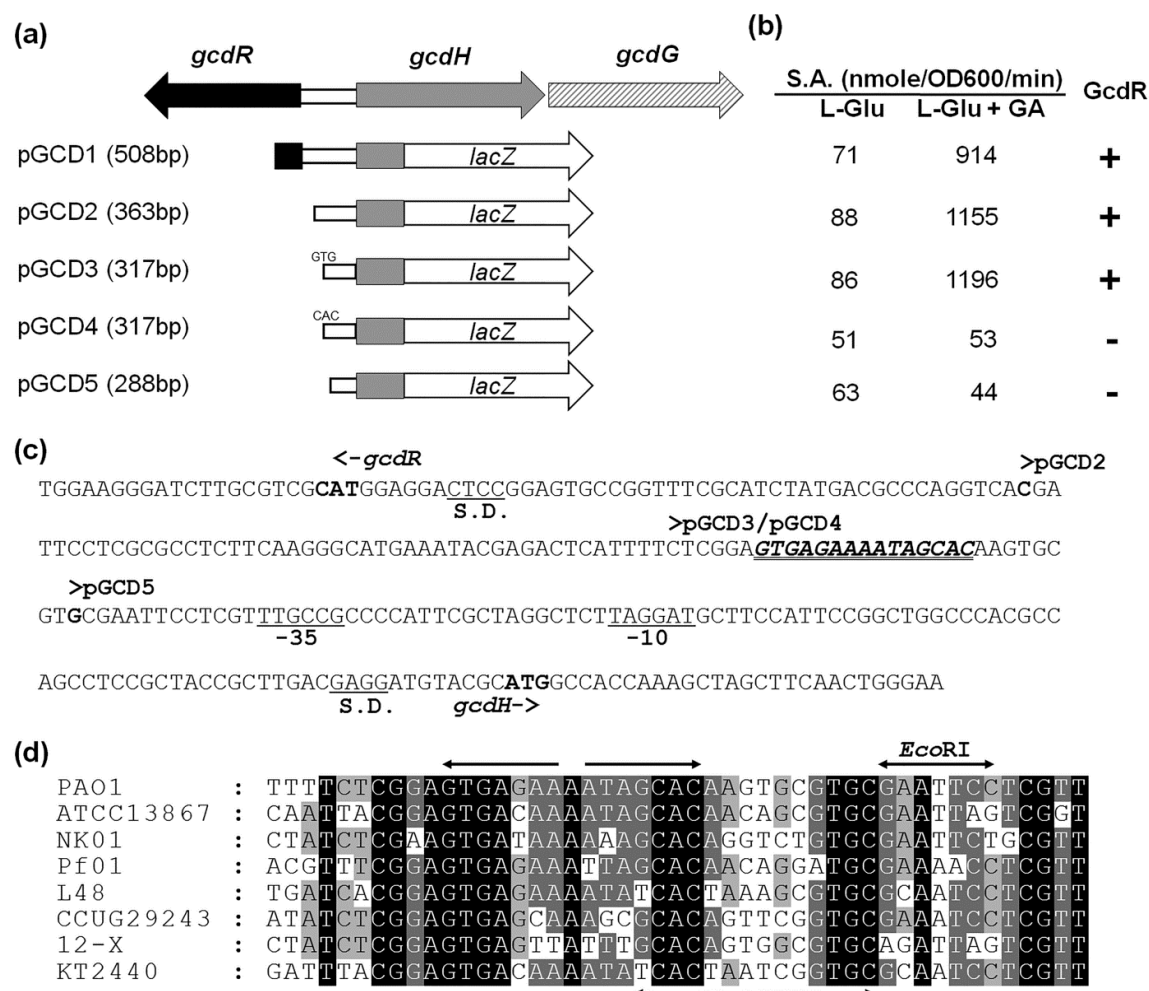


Figure 2.3 Identification of GcdR binding site on the *gcdH* promoter region.

(a) Schematic presentations of the divergent *gcdR-gcdGH* operons and constructs of the *gcdH::lacZ* fusions. Five plasmids of serial deletions in the regulatory regions were labeled with plasmid names.

(b) Measurements of β -galactosidase activity from fusion plasmids and summary of GcdR binding activity with the *gcdH* regulatory region carried by these plasmids. L-Glu, L-glutamate; GA, glutaric acid. Binding of GcdR to the region was indicated by “+” signs and no binding was indicated by “-”.

(c) The nucleotide sequence of the *gcdH* regulatory region. Locations of 5'-end for pGCD2, pGCD3, pGCD4 and pGCD5 and ATG initiation codons for *gcdH* and *gcdR* were indicated with arrow signs and labels. The -10 and -35 sequences for the putative promoter were also labeled. The proposed GcdR operator site was in bold italicized letters with double underline. S.D., Shine-Dalgarno sequence for ribosomal binding.

(d) Multiple sequence alignments for the putative GcdR operators in the *gcdH* regulatory regions of representative species of *Pseudomonas* in the following order: *P. aeruginosa* (PAO1), *P. denitrificans* (ATCC13867), *P. mendocina* (NK01), *P. fluorescens* (Pf01), *P. entomophila* (L48), *P. stutzeri* (CCUG29243), *P. fulva* (12-X), and *P. putida* (KT2440).

2.3.6 Induction of *lysXE* by L-Lys.

As described above, *lysE* was proposed to encode the putative L-Lys exporter, and it forms a putative operon with the upstream *lysX* of unknown function. To test the expression profiling of *lysXE*, a *lysX::lacZ* transcriptional fusion plasmid (pLYS1) was constructed and introduced into wild-type PAO1. The ability of various compounds to activate this promoter was tested. As shown in Figure 2.4, it was found that this promoter was induced by L-Lys only, consistent with the results of the DNA microarray experiments (Table 2.3).

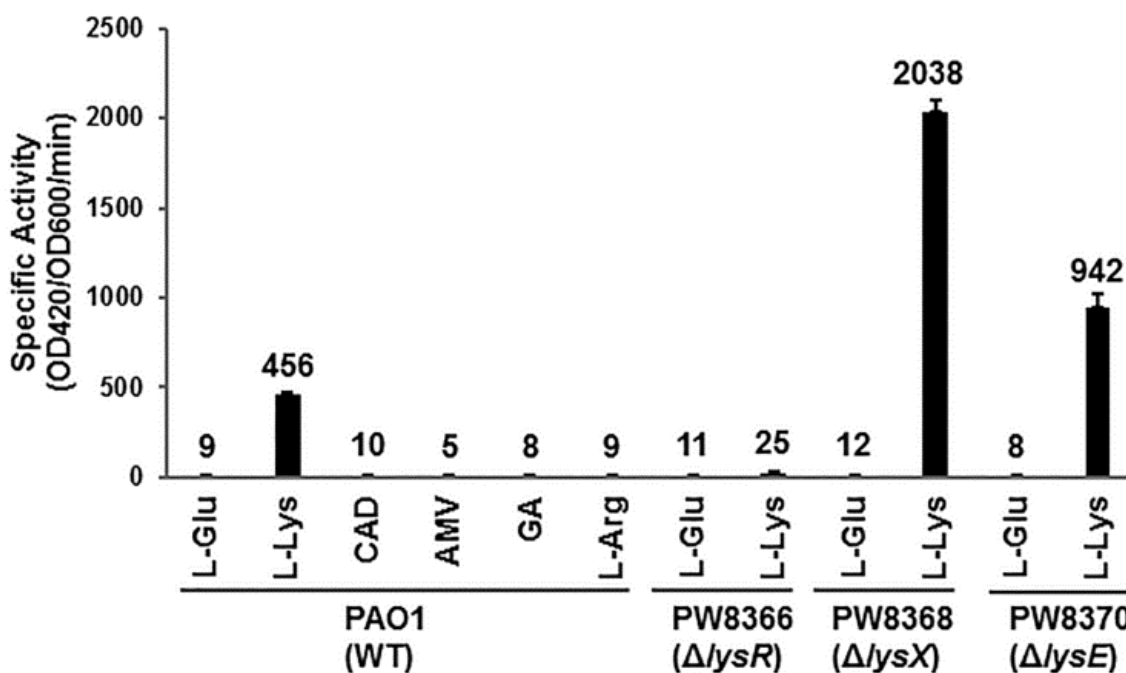


Figure 2.4 Expression profile of the *lysX* promoter *in vivo*.

The *lysXE* promoter activity was monitored by measuring β -galactosidase activity in the wild-type & mutant PAO1 harboring the pLYS1 plasmid. The cells were grown in L-Glu alone (20 mM) or L-Glu + L-Lys (10 mM each) minimal medium. Specific activity values represent the mean \pm SD of three measurements for each growth condition. L-Glu, L-glutamate; L-Lys, L-lysine; CAD, cadaverine; AMV, 5-aminovalerate; GA, glutarate; L-Arg, L-arginine.

The activity of *lysX* promoter was also measured in various mutants in order to understand the regulatory mechanism of the *lysXE* operon. It was found that lysine-dependent induction of the *lysX* promoter was completely abolished in strain PW8366 carrying a lesion on *lysR* (Figure 2.4). On the contrary, the level of induction by L-Lys in the *lysX* or *lysE* mutant was even higher than that in the wild-type strain PAO1. These results supported the proposed functions of LysE and LysR as L-Lys exporter and lysine-responsive transcriptional activator, and suggested a link of LysX to the lysine export system.

2.3.7 DNA binding activity of LysR.

LysR was overexpressed and purified with a hexahistidine tag at its carboxyl terminus as described in Materials and Methods. This recombinant LysR was used to demonstrate its interaction with the *lysX* promoter region by EMSA. LysR alone was able to bind to a DNA fragment carrying the *lysX* promoter region in pLYS1 to form nucleoprotein complexes (C1 and C2; Figure 2.5). We also tested the potential effects of two amino acids, L-Lys and L-Arg, on LysR-DNA interactions. However, only minimal effects can be detected by the presence of these two amino acids *in vitro*, which cannot be explained for the observed effects *in vivo*.

In an attempt to identify the LysR operator sites, multiple sequence alignments were conducted with the *lysX* promoter sequences from PAO1 and representative strains from different gene clusters. As shown in Figure 2.6c, a conserved sequence motif was found in this regulatory region that could potentially be the Lys operator site. This finding is further supported by the loss of LysR binding in EMSA with *lysX* promoter DNA fragments lacking this motif in pLYS4 and pLYS5

(Figure 2.6b) and also by the lack of transcriptional activation in these two plasmids in the presence of L-Lys in the wild-type PAO1 in Figure 2.6b.

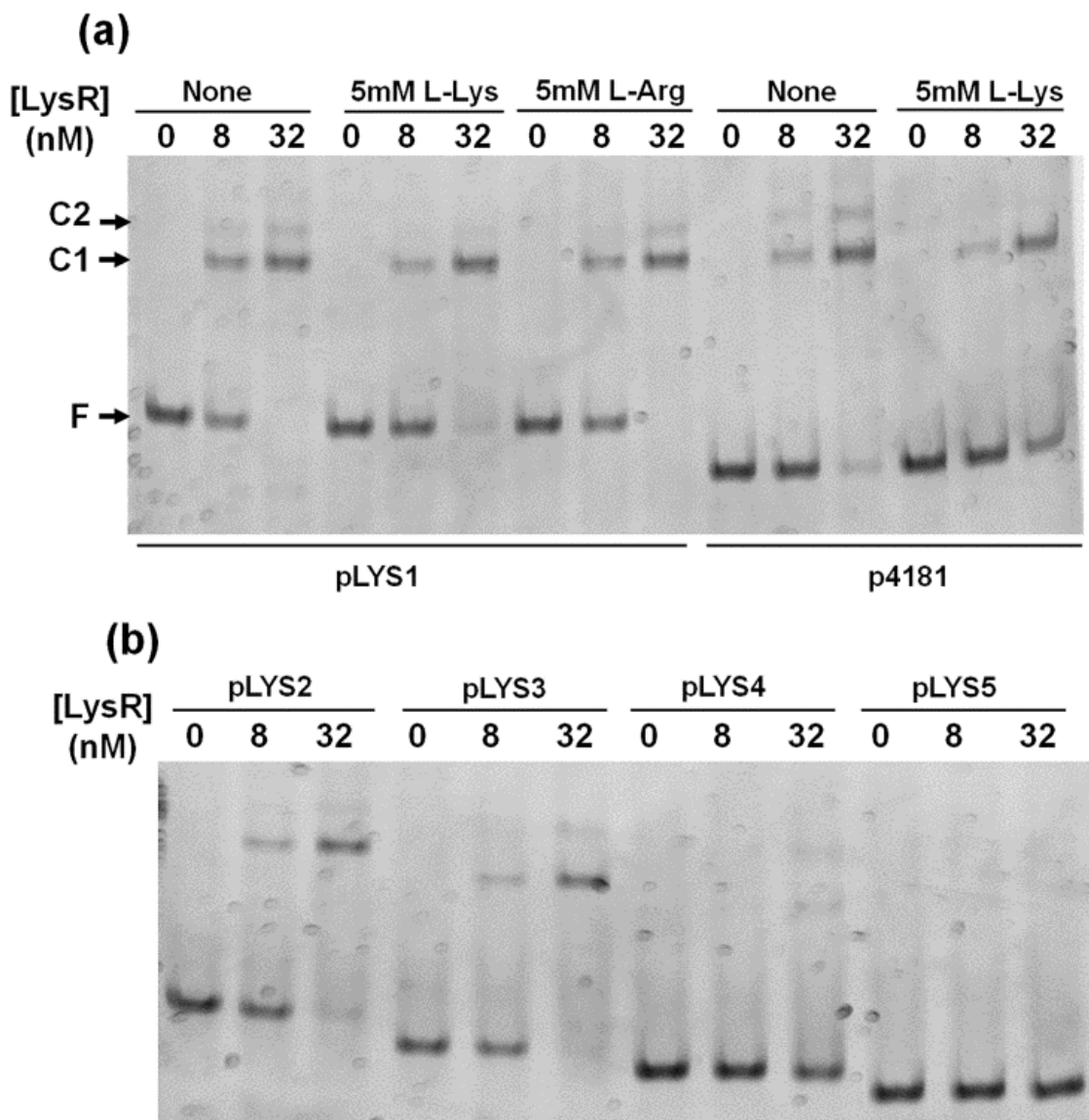


Figure 2.5 Demonstration of LysR-DNA interactions.

(a) Promoter DNA as in pLYS1 or pPA4181 was used as the probe. The purified LysR-His(6) of indicated concentrations was used with or without 5 mM L-lysine (L-Lys) or L-arginine (L-Arg) in the reaction.

(b) Promoter DNA as in pLYS2, pLYS3, pLYS4 or pLYS5 was used as the probe. All reactions were carried out in the presence of 5 mM L-Lys. F, free probe; C1 and C2, nucleoprotein complexes.

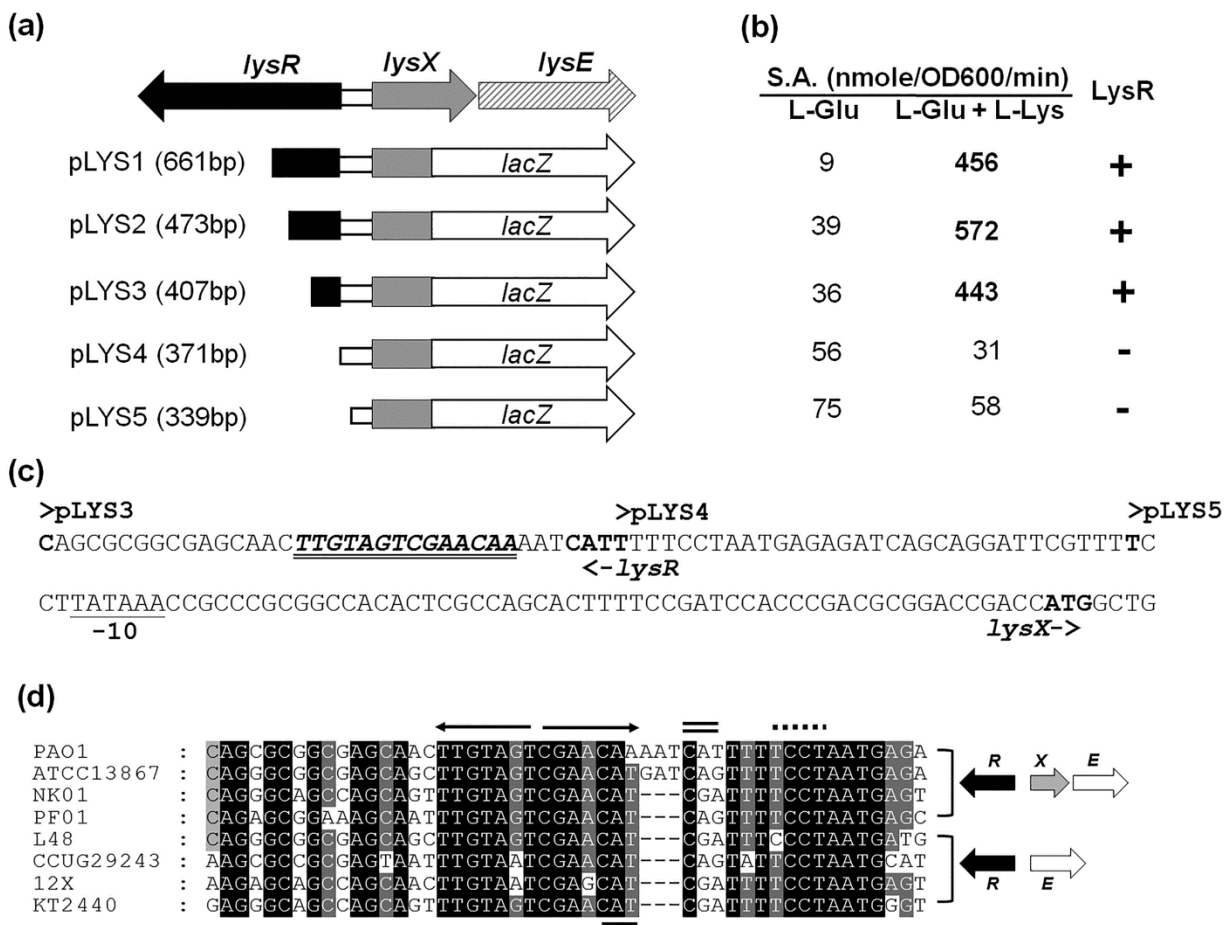


Figure 2.6 Identification of LysR operator on the *lysXE* promoter region.

(a) Schematic presentations of the divergent *lysR-lysXE* operons and constructs of the *lysX::lacZ* fusions. Five plasmids of serial deletions in the regulatory regions were labeled with plasmid names.

(b) Measurements of β -galactosidase activity from fusion plasmids and summary of LysR binding activity with the *lysXE* regulatory region carried by these plasmids. L-Glu, L-glutamate; L-Lys, L-lysine. Binding of LysR to the region was indicated by “+” signs and no binding was indicated by “-”.

(c) The nucleotide sequence of the *lysXE* regulatory region. Locations of 5'-end for pLYS3, pLYS4, and pLYS5 and ATG initiation codons for *gcdH* and *gcdR* were indicated with arrow signs and labels. The -10 sequence for the putative promoter were also labeled. The proposed LysR operator site was in bold italicized letters with double underline.

(d) Multiple sequence alignments for the putative LysR operators in the *lysXE* regulatory regions of representative species of *Pseudomonas* in the following order: *P. aeruginosa* (PAO1), *P. denitrificans* (ATCC13867), *P. mendocina* (NK01), *P. fluorescens* (Pf01), *P. entomophila* (L48), *P. stutzeri* (CCUG29243), *P. fulva* (12-X), and *P. putida* (KT2440). The proposed LysR site was marked with divergent arrows. The ATG initiation codon for PAO1 was marked with double lines, and single line with other species. The dashed line denotes the ribosomal binding site. Also shown on the right side of this panel were the two types of gene organizations for lysine exporter and transcriptional regulator in these species of *Pseudomonas*.

2.3.8 Induction of the *amaAB* promoter.

From transcriptome analysis, *amaAB* of *P. aeruginosa* PAO1 was found inducible to a comparable level by D-Lys and L-Pip. This operon was also induced by L-Lys but to a relatively low level. To substantiate this finding, the *amaA::lacZ* transcriptional fusion plasmid (pAMA1) was constructed and introduced into the wild type strain PAO1 to analyze the expression profiling of this promoter. As shown in Figure 2.7, the *amaA* promoter was induced by L-Lys, D-Lys, and L-Pip, consistent with the DNA microarray experiments (see Table 2.3).

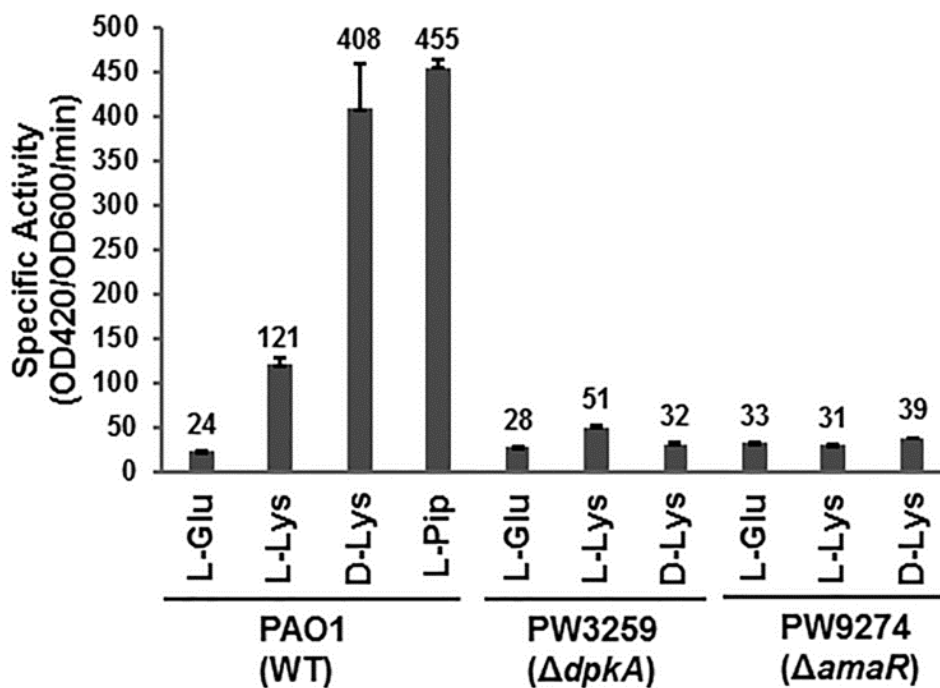


Figure 2.7 Expression of the *amaA* promoter *in vivo*.

The *amaA* promoter activity was monitored by measuring β -galactosidase activity in the wild-type and mutant PAO1 harboring the pAMA1 plasmid. The cells were grown in glutamate minimal medium in the presence of various compounds as indicated above (10 mM). Specific activity values represent the mean \pm standard deviation of three measurements for each growth condition. L-Glu, L-glutamate; L-Lys, L-lysine; D-Lys, D-lysine; L-Pip, L-pipecolate.

Divergent from the *amaAB* operon, the PA1026 gene codes for a putative transcriptional elongation factor of the GreA/GreB family (www.pseudomonas.com). However, the knockout mutant of this gene grew normally on all compounds tested in this study (Table 2.4), and it did not affect the expression profiling of the *amaA* promoter (data not shown). These results led us to exclude PA1026 as the regulatory gene of *amaAB*. In the case of *P. putida* KT2440, the PP5259 gene encoding a transcriptional regulator of the LysR family was also divergently transcribed from the *amaAB* operon in this bacterium (49). Based on sequence similarity we found PA4914 of PAO1 as the possible orthologue of PP5259. As shown in Figure 2.7, activation of the *amaAB* promoter by L-Lys or D-Lys was lost completely in the PA4914 knockout mutant (PW9274) suggesting that PA4914 is the transcriptional activator of the *amaAB* operon. We, therefore, designated PA4914 as *amaR*. Consistent with its role in activation of *amaAB*, the *amaR* mutant exhibited the same pattern of growth defect on L-Lys and L-Pip as the *amaA* and *amaB* mutants (Table 2.4).

2.3.9 Induction of the *amaAB* operon by L-Lys was abolished in the PA1252 mutant.

Although L-Lys catabolism in *P. aeruginosa* PAO1 is mainly through the decarboxylase pathway, it can also be channeled into the D-Lys pathway via the transaminase pathway (3). As shown in Figure 1.1, L-Lys and D-Lys catabolism may converge at α -keto- ϵ -aminohexanoate (aka keto-lysine), which then spontaneously cyclizes to form Δ' -piperidine-2-carboxylate. This convergence was further supported by our findings that L-Lys is able to induce the *amaAB* operon (Table 2.3 and Figure 2.7), which encodes enzymes for the subsequent degradation of Δ' -piperidine-2-carboxylate to 2-aminoadipate. One missing link in the converged pathway is the enzyme converting Δ' -piperidine-2-carboxylate to L-Pip. One potential candidate of this enzyme was

encoded by the PA1252, based on its sequence homology to DpkA (PP3591) of *P. putida* KT2440 that has been previously shown to catalyze this reaction in D-Lys and D-Pro catabolism (25, 33). From the results of transcriptome analysis (Table 2.3), the PA1252 gene was found not inducible by L-Lys, D-Lys, or L-Pip. However, activation of the *amaA* promoter by L-Lys, and D-Lys was completely lost in the PA1252 mutant (Figure 2.7), supporting its role in the conversion of Δ' -piperidine-2-carboxylate to L-Pip and thereby in Lys catabolism in PAO1.

2.3.10 DNA binding activity of AmaR and the potential AmaR operator site.

Even though the cloning and expression of AmaR-HisC were successful, the purification of this protein was not successful. SDS-PAGE analysis showed that all the target protein is in the pellet fraction (inclusion bodies) and none was seen in the fractions collected during purification. The protein purification was repeated again under the same conditions. The collected fractions that are free of other major proteins were pooled and checked on SDS-PAGE to see if there was any small amount of AmaR-HisC. No protein was detected on the gel after staining. Since the protein purification was not successful, the plan to test the DNA binding of AmaR was dismissed. However, based on multiple sequence alignment of *amaAB* regulatory regions from representative pseudomonas species, a potential AmaR binding site was identified (as shown in Figure 2.8c). A total of eight plasmids of serial deletions were tested for the promoter activation using β -galactosidase assay. However, we could not confirm the potential AmaR binding site which was possibly disrupted in pAMA6 and hence had no promoter activation (see Figure 2.8b)

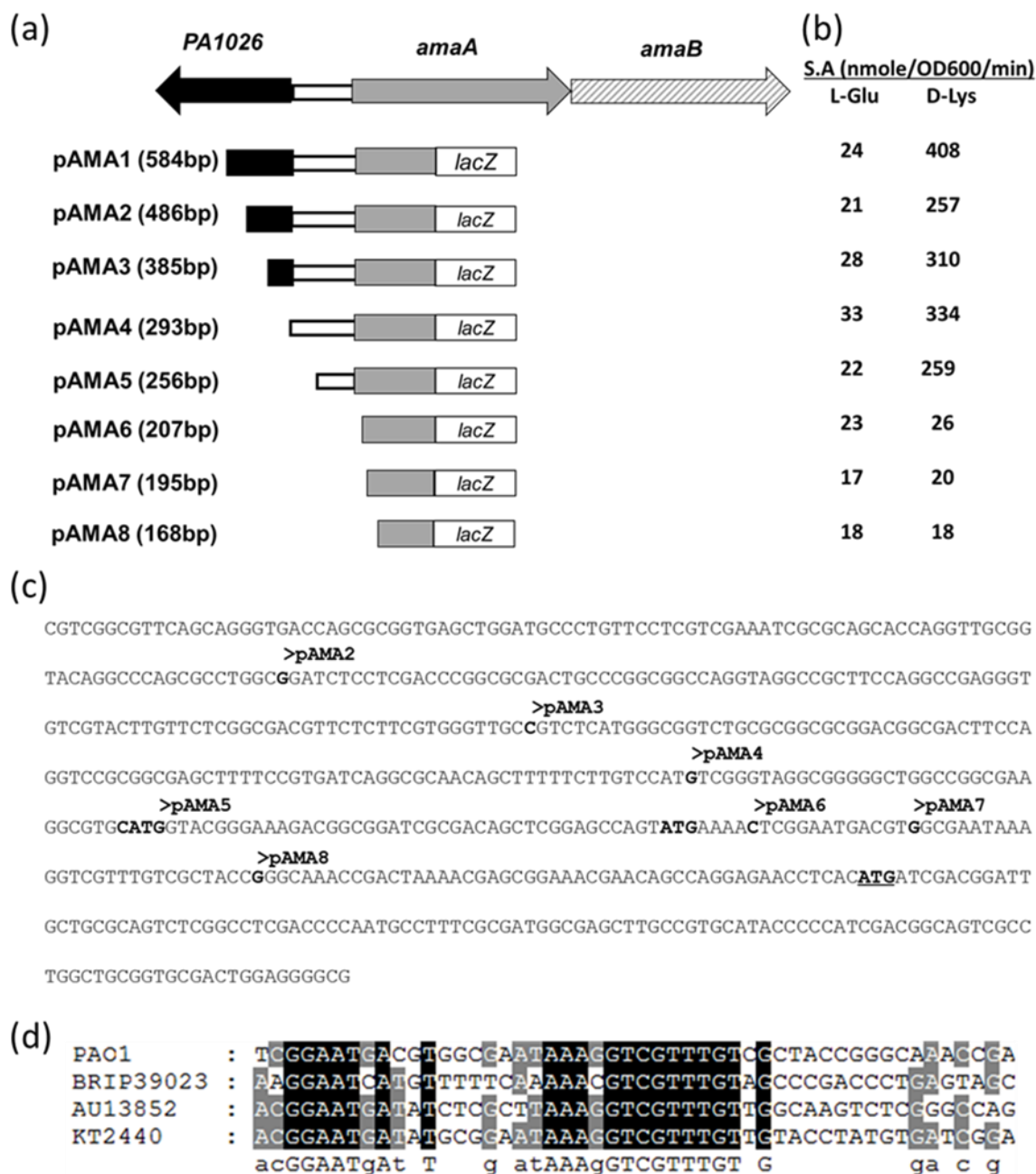


Figure 2.8 Identification of AmaR operator on the *amaAB* promoter region.

(a) Schematic presentations of the *amaAB* operon and constructs of the *amaA::lacZ* fusions. A total of eight plasmids of serial deletions in the regulatory regions were labeled with plasmid names. (b) Measurements of β -galactosidase activity from fusion plasmids. (c) The nucleotide sequence of the *amaA* regulatory region. Locations of the 5' ends for pAMA2, pAMA3, pAMA4, pAMA5, pAMA6, pAMA7, and pAMA8 are indicated by arrowheads and the ATG initiation codon for *amaA* is underlined and bolded. (d) Multiple sequence alignment of *amaA* regulatory regions of representative species *P. aeruginosa* (PAO1), *P. syringae* (BRIP39023), *P. fluorescens* (AU1382) and *P. putida* (KT2440).

2.3.11 The PA4181-PA4182 operon of unknown functions was inducible by L-Lys.

The putative PA4181-PA4182 operon was identified from transcriptome analysis as genes specifically induced by L-Lys (Table 2.3). To substantiate this finding, the PA4181::*lacZ* fusion plasmid (pPA4181) was constructed and introduced into wild-type PAO1. The ability of various compounds to activate this promoter was tested. It was found that this operon was induced by L-Lys only (Figure 2.9) consistent with the DNA microarray experiments. Genome annotations suggested the presence of a B3/B4 tRNA-binding domain in the PA4181 peptide, and the PA4182 peptide was predicted as transcriptional factor with an FMN-binding domain. To test if the induction effect of L-Lys on the PA4181 promoter depends on PA4182 or *lysR*, the pPA4181 plasmid was introduced into the PA4181 and *lysR* mutants to measure the promoter activities. The results showed that promoter activation of pPA4181 by L-Lys was completely lost in the *lysR* mutant indicating that *lysR* is the transcriptional activator of this operon. The promoter region of PA4181 carried by the pPA4181 plasmid was used as the probe to test the binding of *lysR* as described in Materials & Methods. Indeed purified His-tagged LysR did bind to this DNA probe of PA4181 as shown in Figure 2.5a, confirming the results of promoter activation experiments (Figure 2.9). Similar to results with the *lysE* probe, at least two nucleoprotein complexes (C1 and C2) can be observed by EMSA, and the addition of L-Lys to the binding reaction also led to the preferential formation of C1 complex (Figure 2.5a).

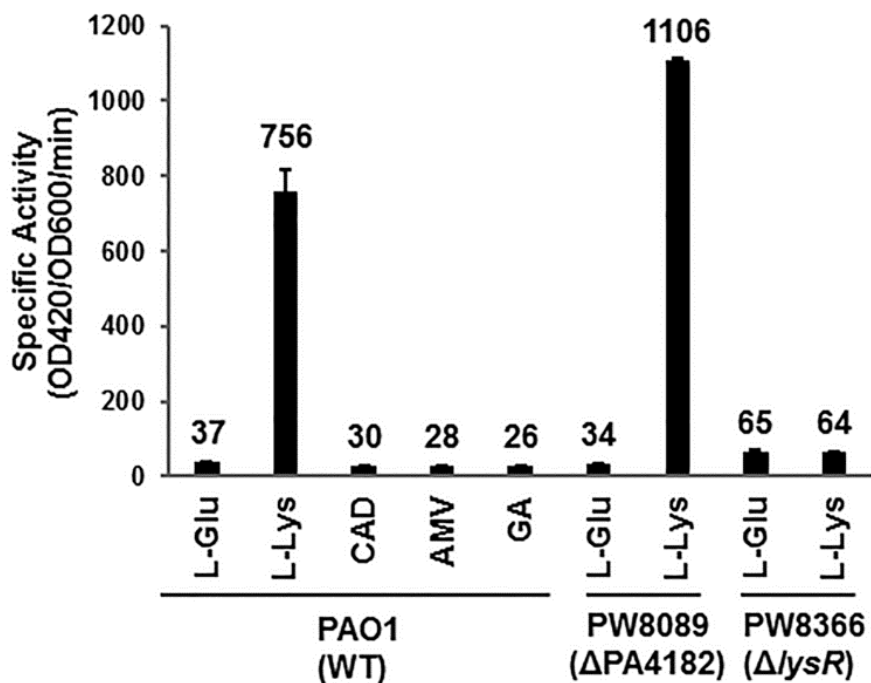


Figure 2.9 Expression of the PA4181 promoter *in vivo*.

The promoter activity of PA4181 was monitored by measuring β -galactosidase activity in the wild-type & mutant PAO1 harboring the pPA4181 plasmid. The cells were grown in glutamate minimal medium in presence of various compounds as indicated above (10 mM). Specific activity values represent the mean \pm standard deviation of three measurements for each growth condition. L-Glu, L-glutamate; L-Lys, L-lysine; CAD, cadaverine; AMV, 5-aminovalerate; GA, glutaric acid.

2.3.12 Induction of *lysP* promoter.

The *lysP* is an orphan gene next to the *aruSR* two component system on the *P. aeruginosa* genome. This gene was found to be induced by L-Lys during transcriptome analysis (Table 2.3). Further, growth phenotype results (Table 2.4) showed that the *lysP* mutant grew very poorly on L-Lys suggesting the importance of *lysP* for L-Lys uptake in PAO1. Previous studies showed that *lysP* is induced by L-Arg and ArgR is a repressor for *lysP*. Promoter activity assay was conducted *in vitro* using pLYSP in wild-type PAO1 and various mutants. It was found that *lysP* was induced by L-Arg and L-Lys and the promoter activity was increased by several folds in PAO501 (Figure 2.10) confirming that ArgR is indeed a transcriptional repressor of *lysP*. The

expression of *lysP* was not affected in PW8366 indicating that LysR is not a transcriptional regulator of this gene. Further, it was observed that *lysP* activation was completely lost in *aruS* and *aruR* mutants. The *aruSR* two component system was shown to be induced by L-Arg. The uptake system *lysP* being under the control of two arginine regulatory systems explains for the poor growth of PAO1 on L-Lys suggesting that the uptake is one bottleneck in L-Lys utilization.

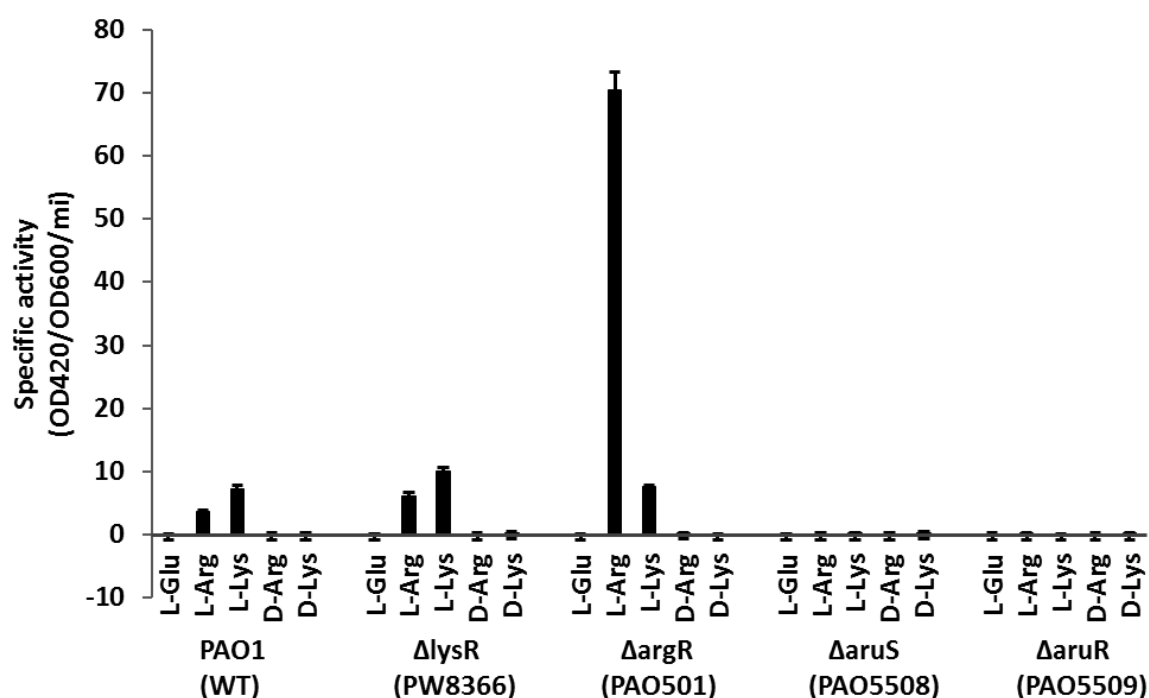


Figure 2.10 Expression of *lysP* promoter *in vivo*.

The promoter activity of *lysP* was monitored by measuring β -galactosidase activity in the wild-type & mutant PAO1 harboring the pLysP plasmid. The cells were grown in glutamate minimal medium in presence of various compounds as indicated above (10 mM). Specific activity values represent the mean \pm standard deviation of three measurements for each growth condition. L-Glu, L-glutamate; L-Lys, L-lysine; L-Arg, L-arginine; D-Lys, D-lysine.

2.4 Discussion

2.4.1 Glutarate catabolism plays a pivotal role in lysine catabolism.

L-Lys catabolism in *P. aeruginosa* is mainly mediated by the lysine decarboxylase pathway, which converts L-Lys into glutarate. Previous reports from our group have characterized all genes encoding enzymes in this process. In the current study, the *gcdHG* operon was identified to play essential roles in glutarate catabolism from the results of transcriptome analysis (Table 2.3) and growth phenotype analysis (Table 2.4). Genome annotations predicted GcdH as glutaryl-CoA dehydrogenase and GcdG as putative acyl-CoA transferase (www.pseudomonas.com). In conjunction with our results presented here, this information supported GcdG and GcdH as glutaryl-CoA transferase and glutaryl-CoA dehydrogenase that catalyze two contiguous reactions to convert glutarate into crotonyl-CoA (42, 46).

Not only essential for L-Lys catabolism, it was found in our study that the *gcdG* and *gcdH* mutants cannot grow on L-Pip, suggesting that L-Pip is also degraded via glutarate. L-Pip is an intermediate compound of D-Lys catabolism (Figure 1.1), followed by the generation of 2-aminoadipate. Enzymes and their coding genes that are involved in the conversion of L-Pip to 2-aminoadipate have been identified in *P. putida* (34) and *P. aeruginosa* PAO1 in this study. However, information regarding degradation of 2-aminoadipate to glutarate was still missing. One possible player in this process might be PA2035 encoding a TPP-dependent decarboxylase. The PA2035 gene found inducible by D-Lys and L-Pip in transcriptome analyses (Table 2.3), and the knockout mutant of PA2035 completely lost the ability to grow on L-Pip (Table 2.4) suggesting that PA2035 is essential for L-Pip utilization in PAO1.

2.4.2 The GcdR transcriptional activator responds to its signaling compound glutarate.

Several lines of evidence support the conclusion that GcdR is the transcriptional activator for the *gcdH* promoter. First, the *gcdR* mutant cannot grow on L-Lys or its catabolic intermediates Cad, AMV or GA. Second, induction of the *gcdH* promoter by exogenous GA was completely abolished in the *gcdR* mutant. Third, the purified recombinant GcdR protein binds to the regulatory region of the *gcdH* promoter. A combination of serial deletion analysis and multiple sequence alignments led us to identify a highly conserved sequence motif among species of *Pseudomonas* as the GcdR operator site in the *gcdH* promoter region (Figure 2.3).

2.4.3 Lysine uptake and export are two bottlenecks for growth on L-Lys.

P. aeruginosa PAO1 can utilize L-Lys as the sole source of carbon and nitrogen. However, it has been observed that under this growth condition with L-Lys, the wild-type strain PAO1 spent a very long lag phase before resuming logarithmic growth. We have reported that the lysine decarboxylase LdcA was subjected to induction by L-arginine and the ArgR transcriptional regulator (5), and hence considered the lack of lysine-dependent induction of *ldcA* as the bottleneck for L-Lys catabolism.

In the current study, we found that the presence of a lysine-inducible exporter posed another major limiting factor for growth on L-Lys. From the transcriptome analysis, the *lysXE* genes were found to be induced specifically by exogenous L-Lys and this was confirmed by promoter activity measurements. The *lysXE* genes encode an uncharacterized protein with one Pfam ATC3 domain and a putative lysine efflux protein, respectively, while the divergent *lysR* gene encodes a

transcriptional regulator. The *lysE* gene shares 45 % sequence similarity with the arginine exporter ArgO of *E. coli*. Knockout mutants of *lysR*, *lysX*, and *lysE* were found to grow much better than the wild-type PAO1 on MMP agar with L-Lys as the sole source of carbon and nitrogen. In liquid media, the *lysR*, *lysX* and *lysE* mutants had a much shorter lag-phase compared with the wild-type PAO1. Also, the estimated generation time of these mutants was shorter (9-13 h) than that of the WT (19 h). We believe that the shorter lag phase and generation time observed in *lysR*, *lysX*, and *lysE* mutants was due to the lack of a functional lysine exporter, resulting in higher intracellular concentrations of L-Lys compared with the wild-type PAO1.

Previous studies from our lab showed that the AotJQMP transporter for L-Arg uptake is also induced by L-Lys. The first enzyme in L-Lys catabolism is under the control of ArgR, an arginine responsive regulator, and L-Arg. These two things lead to the belief that L-Lys uptake is also mediated by the AotJQMP transporter. However, growth phenotype results in this study showed that the *lysP* mutant almost lost the ability to grow on L-Lys as the sole carbon and nitrogen source indicating its importance in L-Lys uptake. Further, we found that *lysP* promoter was induced by both L-Arg and L-Lys. This induction effect by L-Arg was enhanced several folds in the *argR* mutant confirming its repression effect on *lysP* transcription. In addition, we found that the induction of *lysP* was completely abolished in the *aruS* and *aruR* mutants which are again known to respond to exogenous L-Arg. This suggests that the uptake via an L-Arg responsive and ArgR regulated transporter also is another reason for the poor growth of PAO1 on Lysine.

2.4.4 The LysR transcriptional activator responds to its signaling compound L-Lys.

Several lines of evidence support the conclusion that LysR is the transcriptional activator for the *lysX* promoter. First, the *lysR* mutants grow much better than the wild-type PAO1 (possibly due to the absence of LysX and LysE). Second, the induction of the *lysX* promoter by exogenous L-Lys was completely abolished in the *lysR* mutant. Third, the purified recombinant LysR protein binds to the regulatory region of the *lysX* promoter. The LysR operator site in the *lysX* promoter region was also deduced from the results of serial deletions and multiple sequence alignments (Figures 2.4 & 2.6)

2.4.5 Interconnected regulatory systems for ArgR and Lysine metabolism.

Findings from the current study and previous reports indicated the presence of a sophisticated regulatory network of Arg and Lys uptake and catabolism in *Pseudomonas*. As shown in Figure 2.11, there are at least four transcriptional regulators that respond to Arg and Lys – LysR (L-Lys), AruSR (L-Lys and L-Arg), ArgR (L-Arg) and DauR (D-Lys and D-Arg). *P. aeruginosa* is well equipped to grow more efficiently on Arg than on Lys, with the ArgR regulator in response to L-Arg as the major player to control the entire arginine network (33, 36), including the AotJQMP transporter for L-Arg and L-Lys uptake. Furthermore, the ArgR extended its influence to Lys catabolism through its control on *ldcA* for L-Lys decarboxylase (5), *dauBAR-dauT* for D-Lys/D-Arg dehydrogenase and uptake, and potentially on *lysP* and *aruH* for L-Lys uptake and L-Lys/L-Arg aminotransferase (65). The overall efficiency of L-Lys catabolism was further dampened by the presence of L-Lys exporter LysXE that is subjected to positive regulation by the LysR regulator in response to L-Lys as depicted in this study. The physiological significance of this observed

nutritional preference for Arg over Lys in *Pseudomonas* was a very intriguing question for future investigation.

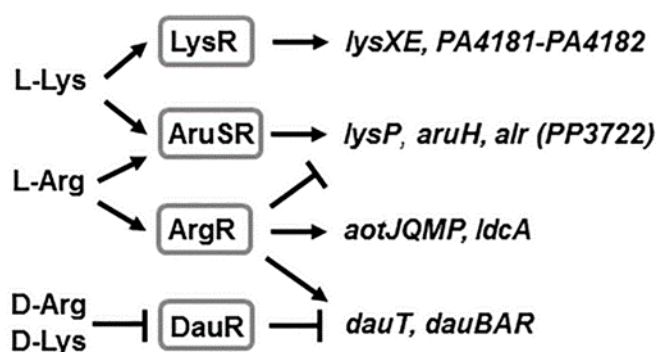


Figure 2.11 Schematic summary of arginine and lysine interconnected regulatory networks.

The transcriptional regulators along with their signal compounds and the genes under their control are connected with either an arrow for positive effect ‘ \rightarrow ’ or for negative effect.

In conclusion, we have characterized several new members of the L-Lys and D-Lys catabolic pathways and their regulatory elements, including *gcdR-gcdHG* for glutarate utilization, *dpkA*, *amaR-amaAB* and PA2035 for D-Lys catabolism, *lysR-lysXE* for putative L-Lys efflux, *lysP* (PA4981) for putative L-Lys uptake. Integration of these new data with information from previous reports led us to discover an inducible amino acid exporter as a bottleneck for L-Lys catabolism and to decipher a tightly interconnected metabolic network for D-Lys and L-Lys in *Pseudomonas*.

3 CHARACTERIZATION OF ANABOLIC L-ARGININE DEHYDROGENASE DAUB OF *PSEUDOMONAS AERUGINOSA* PAO1

3.1 Introduction

Proteins are composed of L-amino acids predominantly. However, the D-amino acids are also part of living organisms and are important for various cellular functions. D-amino acids are essential components of bacterial cell walls (53). D-serine and D-aspartate were shown to be involved in cellular aging and neural signaling, respectively (41, 42). Amino acid racemases catalyze the conversion of L-forms of the amino acids into the D-forms for these purposes. Some D-amino acids can be used for growth by bacteria when in excess with the help of these racemases. *P. aeruginosa* PAO1 has been shown to possess coupled dehydrogenases DauA and DauB that facilitate the racemization of D-arginine (D-Arg) to L-arginine (L-Arg) (31). The *dauA* gene encodes for the enzyme catabolic D-arginine dehydrogenase while *dauB* gene encodes for the enzyme anabolic L-arginine dehydrogenase. The proposed biochemical reaction of these coupled enzymes is as follows:

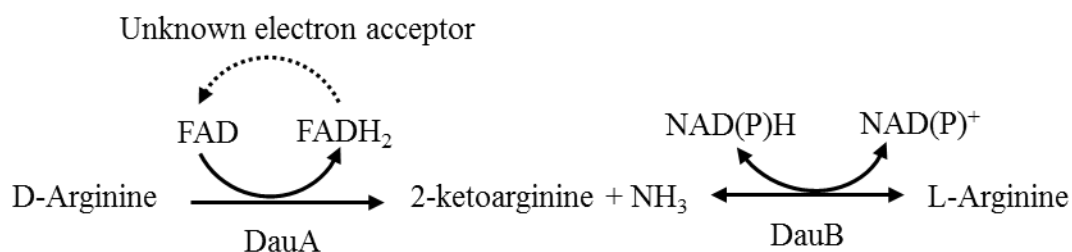


Figure 3.1 Schematic representation of arginine racemization.

DauA can utilize a number of D-amino acids as the substrate in addition to D-Arg to generate their respective keto-acid and ammonia (33) as shown in Figure 3.2

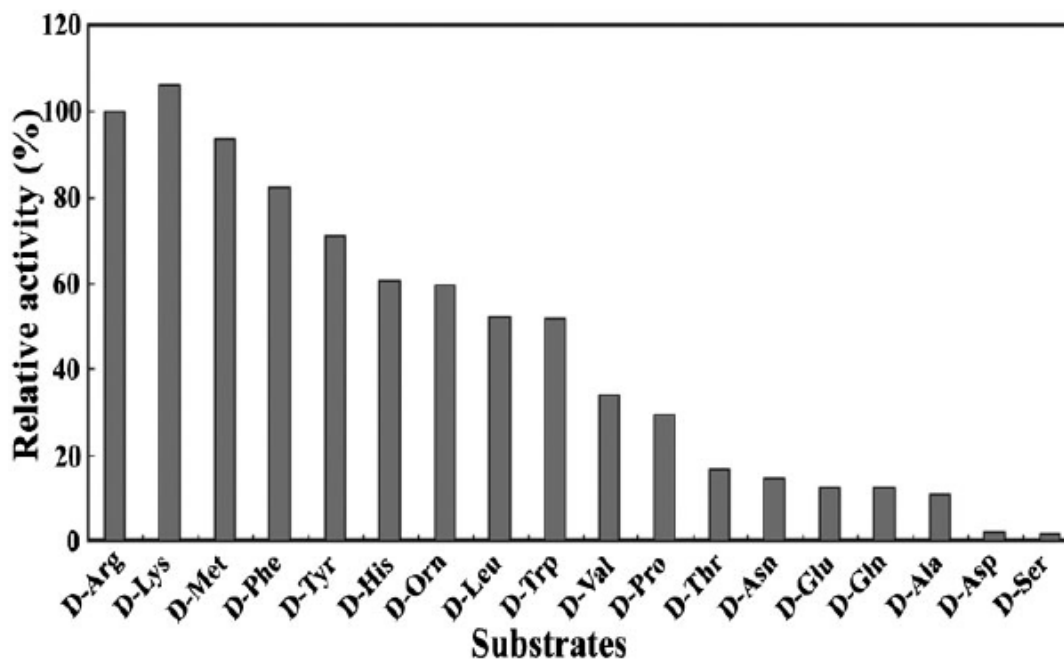


Figure 3.2 Substrate specificity of DauA.

The substrate specificity of DauA as determined from the D-amino acid dehydrogenase activity of the purified enzyme. The enzyme activity towards D-Arg was taken as 100 %, and with respect to it, the relative activity towards each D-amino acid was calculated. This figure is adapted from Li *et al*, 2010 (33).

DauB is a bidirectional enzyme that can use either 2-ketoarginine + NH_4Cl in a forward reaction or L-Arg in a reverse reaction with NADH/NADPH or $\text{NAD}^+/\text{NADP}^+$ as the cofactors respectively. In this research, we attempted to characterize the enzyme DauB. I hypothesized that, if DauA and DauB are coupled enzymes, then DauB should also be able to utilize other D-amino acids as a substrate like its partner enzyme DauA. We also attempted to characterize the DauB enzyme kinetics.

3.2 Materials and methods

3.2.1 Bacterial strains, plasmids and growth conditions.

P. aeruginosa PAO1 wild-type (8), *E. coli* DH5 α (Bethesda Research Laboratories) and Top10 (Invitrogen) were used in this study. Both *E. coli* and *P. aeruginosa* were routinely grown and maintained in Luria–Bertani (LB) medium. Ampicillin at 100 μ g/ml was supplemented in the growth medium when required for *E. coli*. The protein expression vector pBAD-HisE (32) was used in this study to clone and express recombinant DauB protein.

3.2.2 Cloning, expression and purification of hexahistidine tagged DauB.

The *dauB* gene excluding the translational stop codon was amplified from the genomic DNA of *P. aeruginosa* PAO1 wild type using the primers DauB-HisCF: 5'-CGCTCATGAGCGCCGCGCCACTCCC-3' and DauB-HisCR: 5'-TCCCCGGGGCCTGCCTGGCGCAGGCG-3'. The resulting PCR fragments were digested with *Bsp*HI/*Sma*I restriction enzymes and cloned into pBAD HisE plasmid vector (31) so that the C-terminus of DauB was fused in-frame with the His6-tag while the N-terminus is preceded by a ribosome binding site and an arabinose-inducible promoter in the plasmid. pDAUB was introduced into *E. coli* Top10 (Invitrogen). For overexpression of DauB-HisC, the recombinant strains of *E. coli* were grown in LB medium containing ampicillin at 37°C. For over expression, 0.2 % L-arabinose (w/v) was added to the culture when the OD₆₀₀ was approximately 0.5-0.6. After 4 h of vigorous shaking at 37 °C, cells were harvested by centrifugation and stored at -80 °C until use. The cell pellets were suspended in phosphate buffer A (20 mM Na₂HPO₄, 0.5 M NaCl, 20 mM imidazole, pH 7.5) with PMSF (1 mM) as a protease inhibitor and the cells were ruptured by an Aminco French press. Cell debris was removed by centrifugation at 30,000 g for 30 min. The

supernatant was applied to a HisTrap HP column (GE Health Care) and the His-tagged DauB was eluted at 20-60 % buffer B (20 mM Na₂HPO₄, 0.5 M NaCl, 1 M imidazole, pH 7.5). The target fractions were analyzed by SDS-PAGE, pooled and concentrated using an Amicon Ultra-15 centrifugal filter unit (molecular mass cut-off, 30 kDa; Millipore) to change the buffer to 20 mM Tris-HCl (pH 7.5). Protein concentration was determined by the method of Bradford (27).

3.2.3 DauB enzyme assay.

DauB enzyme activity was measured using the reverse reaction as described earlier (31, 33). L-Arg was used as the substrate and the formation of NADH was measured at 340 nm using Cary 3E (Varian). The reaction mixture contained L-Arg, NAD⁺, and purified DauB in 50 mM Tris-HCl in a total volume of 2 ml. The reaction mix lacking L-arginine, NAD⁺ or DauB-HisC was pre-incubated at 37 °C. The reaction was initiated by adding the missing component and followed for up to 15 min.

3.3 Results

3.3.1 DauB pH profile.

Little to no enzyme activity, as determined by the formation of NADH, was detected at near neutral pH. As the pH increased, the enzyme activity increased and peaked at pH 9.0 and then started to reduce (Figure 3.3). Hence, pH 9.0 was taken as the optimal pH for the DauB enzymatic reaction and was used for all subsequent assays.

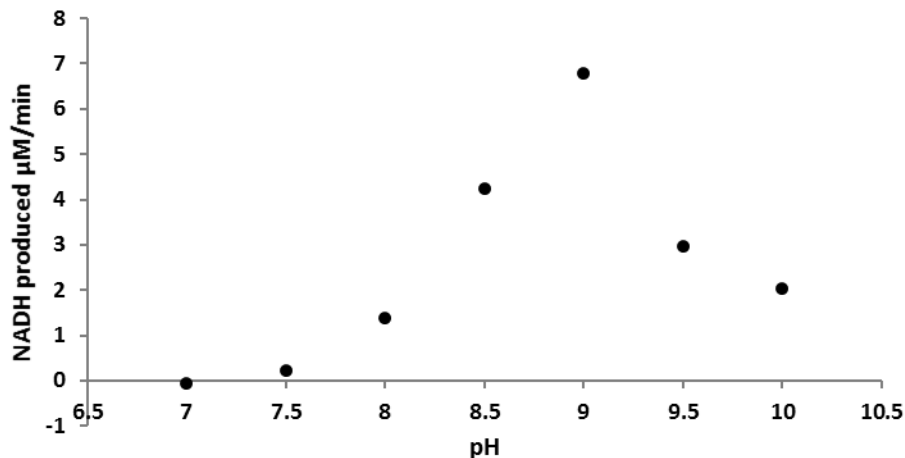


Figure 3.3 DauB pH profile.

Each reaction included 141 µg of purified DauB-HisC, 10 mM L-Arg, 0.5 mM NAD⁺, 50mM Tris-HCl (pH 7, 7.5, 8, 8.5, 9, 9.5 and 10) in 2 ml. The reaction was followed for 5 min after adding the amino acid at 37°C to the pre-incubated reaction mixture.

3.3.2 DauB enzyme activity with varied enzyme concentrations.

In order to find the optimal enzyme concentration under the given experimental conditions, DauB enzyme activity was measured with fixed concentrations of L-Arg (10 mM), NAD⁺ (0.5 mM) and varied enzyme concentration. The reaction was initiated by the addition of the enzyme to the pre-warmed reaction mix and the reaction was followed for 5 min by measuring the formation of NADH. It was found that 70.5 µg of DauB-HisC gives a reaction velocity in the linear range (Figure 3.4). Hence this concentration was used for further analyses.

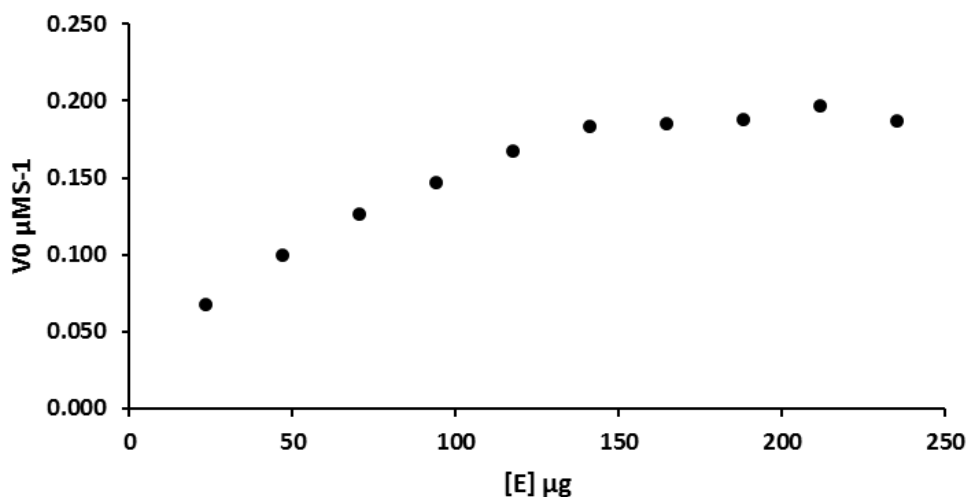


Figure 3.4 DauB enzyme activity with varied enzyme concentrations.

Each reaction included DauB-HisC (23.7, 47, 70.5, 94, 117.5, 141, 164.5, 188, 211.5 and 235 μg), 10 mM L-Arg, 0.5 mM NAD⁺ and 50 mM Tris-HCl pH 9 in 2 ml. The reaction was followed for 5 min at 37 °C after adding enzyme to the pre-incubated reaction mix.

3.3.3 DauB enzyme activity with varied L-arginine concentrations.

This experiment was conducted to find the kinetic parameters for L-Arg used in the reaction. The reaction was initiated by the addition of the enzyme to pre-incubated reaction mix and was followed for 5 min. It was found that the K_m was 15.45 mM of L-Arg and the V_{max} for DauB under these reaction conditions was 1.03 μM/S (Figure 3.5).

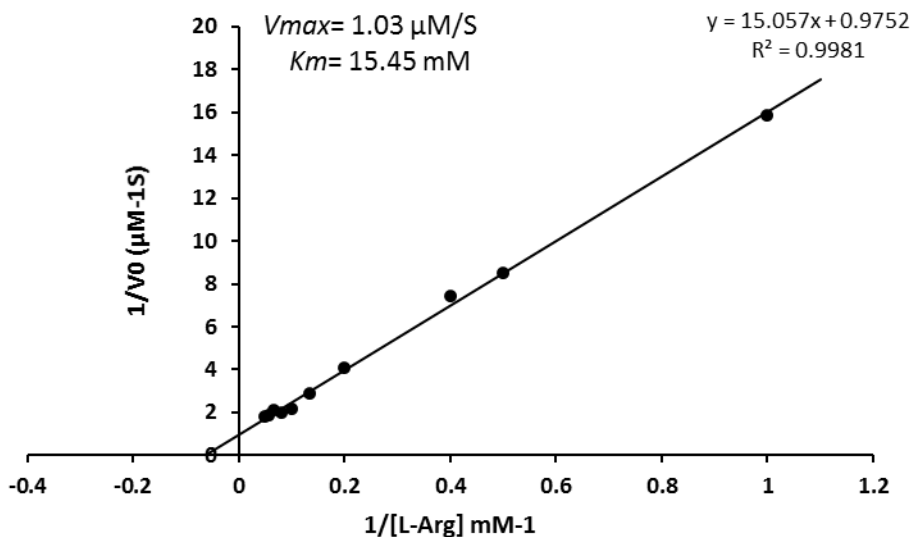


Figure 3.5 DauB enzyme activity with varied L-arginine concentrations.

Each reaction included L-Arg (1, 2, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 mM), 70.5 μg of DauB-HisC, 0.5 mM NAD^+ , 50 mM Tris-HCl pH 9 in 2 ml. Each reaction was followed for 5 min after the addition of enzyme to the pre-incubated reaction mix.

3.3.4 DauB enzyme activity with varied NAD^+ concentrations.

This experiment was conducted to find the kinetic parameters for NAD^+ used in the reaction. The reaction was initiated by the addition of the enzyme to pre-incubated reaction mix and was followed for 5 min. The concentration of L-Arg used in this reaction was increased to 40 mM from 10 mM based on the calculated K_m for L-Arg. It was found that the K_m was 1.88 mM of NAD^+ and the V_{max} for DauB under these reaction conditions was 0.80 $\mu\text{M/S}$ (Figure 3.6).

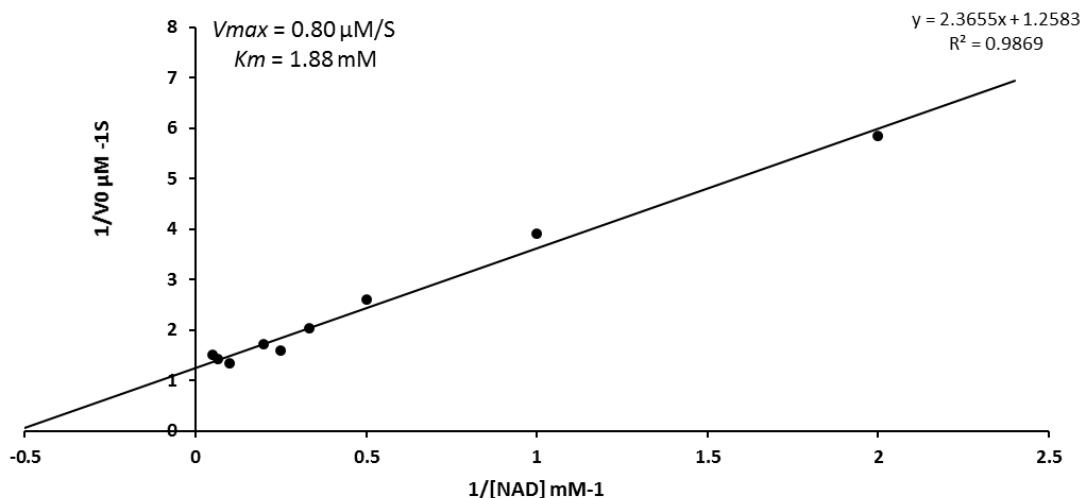


Figure 3.6 DauB enzyme activity with varied NAD⁺ concentrations.

Each reaction included NAD⁺ (0.5, 1, 2, 3, 4, 5, 10, 15 and 20 mM), 70.5 μg of DauB-HisC, 40 mM L-Arg, 50 mM Tris-HCl pH 9 in 2 ml. Each reaction was followed for 5 min after the addition of enzyme to the pre-incubated reaction mix.

3.3.5 Substrate specificity of DauB.

DauA was shown to utilize a number of D-amino acids very well as its substrate. Since DauBA are coupled dehydrogenases, we set to determine if DauB will complement the substrate specificity shown by DauA. Each reaction was initiated by the addition of the addition of DauB to pre-incubated reaction mix and was followed for 15 min. It was found that unlike its partner enzyme DauA, DauB can utilize only L-Arg as a good substrate (Figure 3.7). Hence, DauB is an arginine-specific dehydrogenase enzyme.

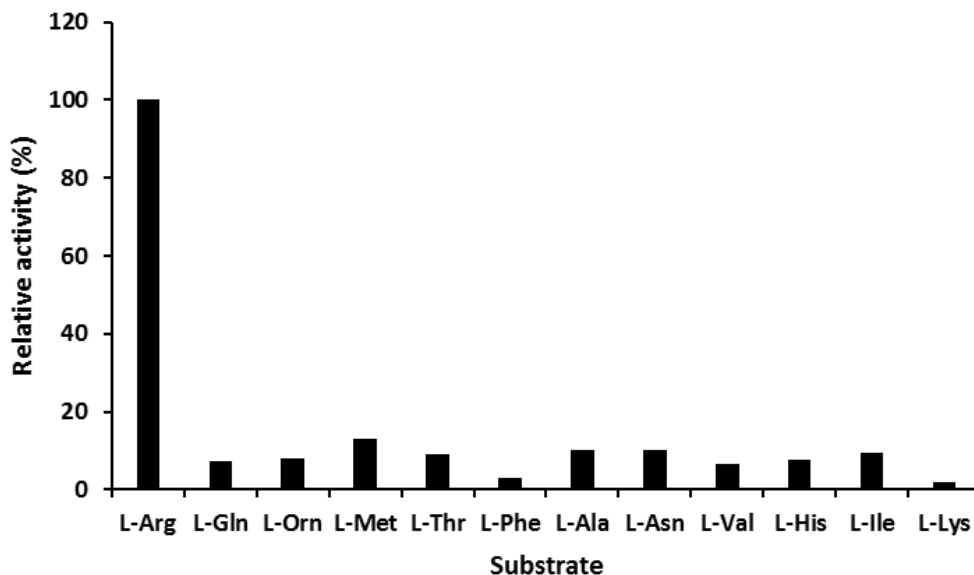


Figure 3.7 *In vitro* substrate specificity of DauB enzyme.

Each reaction included 70.5 μg of DauB-HisC, 0.5 mM NAD^+ , 10 mM L-amino acid, 50 mM Tris-HCl pH 9.0 in 2 ml. The reaction was followed for 15 min after adding DauB at 37 $^{\circ}\text{C}$ to the pre-incubated reaction mixture.

3.4 Discussion

3.4.1 DauB is an arginine-specific dehydrogenase.

In order to identify the optimal reaction conditions and kinetic parameters, we used purified C-terminal hexahistidine tagged DauB enzyme. As shown in Figure 2.3, at pH 9.0, DauB exhibited maximal enzyme activity under our experimental conditions. The K_m value for L-Arg was 15.45 mM using 70.5 μg of DauB and 0.5 mM of NAD^+ and the V_{max} for DauB under these reaction conditions was 1.03 $\mu\text{M}/\text{S}$ (Figure 2.5). The K_m of NAD^+ was 1.88 mM 70.5 μg of DauB and 40mM L-Arg and the V_{max} for DauB under these reaction conditions was 0.8 $\mu\text{M}/\text{S}$ (Figure 3.6). DauB showed relatively little to no activity using other L-amino acids as substrates (shown in Figure 3.7) as compared to that against L-Arg (taken as 100%). This indicates that DauB is an

arginine-specific dehydrogenase unlike its partner, DauA dehydrogenase, which can utilize a number of D-amino acids as the substrate (Figure 3.2). In addition, it is very likely that the potential DauAB complex is either formed in presence of D-Arg only or is limited to the racemization of D-Arg to L-Arg considering that these two enzymes vary widely in their substrate specificity.

Transcription of the *dauBAR* operon is induced by D-Arg and D-Lys and this is further enhanced by L-Arg in the presence of ArgR (33). Even though DauA was shown to utilize a number of D-amino acids as its substrates *in vitro*, only utilization of D-Arg as carbon and or nitrogen source was abolished in the *dauA* mutant (33). In the case of DauB, in agreement with the *in vitro* enzymatic assays in the current study and the previous studies (31), the *dauB* mutant can utilize D-Arg as a nitrogen source but not as carbon. All these support the unique role of DauBA in D-Arg catabolism.

4 *IN VIVO* SUBSTRATE SPECIFICITY OF D-AMINO ACID DEHYDROGENASES IN *PSEUDOMONAS AERUGINOSA* PAO1

4.1 Introduction

D-amino acids are formed by racemization from cognate L-enantiomers. Until recent times the physiological importance of D-amino acids was not well known other than the presence of D-Ala and D-Glu in bacterial cell wall. However, recent studies revealed that the D-amino acids play many other important functions. Gramicidin is an antimicrobial peptide produced by *Bacillus brevis* that contains D-amino acids (4). DadA, D-alanine dehydrogenase, is involved in multiple virulence factor production in *P. aeruginosa* (43). In addition, many groups have demonstrated that D-amino acids can be detrimental to the cell. D-amino acids in combination with β -lactam antibiotics were shown to alter cell morphology (1). *Bacillus subtilis* was shown to produce a mixture of D-Leu, D-Met, D-Tyr and D-Trp that prevented biofilm formation and also disassemble existing biofilms (26). Lam *et al.*, (28) found unusual D-amino acids in *Vibrio cholerae* and *Bacillus subtilis* that seemed to modify the cell wall in stationary phase cultures. D-amino acids given in combination with antimicrobials has a synergistic effect on biofilm dispersal in some isolates of *P. aeruginosa* and *Staphylococcus aureus* (52)

P. aeruginosa PAO1 has three known D-amino acid dehydrogenases. D-Arginine dehydrogenase encoded by the gene *dauA* is part of a two-component racemase that converts D-arginine to L-arginine (31, 33). This gene is indispensable for the utilization of D-Arg in PAO1. It was shown to utilize a number of D-amino acids as the substrate *in vitro*. D-Alanine dehydrogenase encoded by *dadA* gene is involved in the conversion of D-Ala to pyruvate and ammonia and was also shown

to utilize many D-amino acids as its substrate. DadA is essential for the utilization of D-Ala, D-Val, D-Ser, D-Thr, D-Phe and D-His in PAO1. Although the *dadA* promoter is induced by several L-amino acids and by D-Ala, L-Ala was proposed as the signaling molecule for its transcriptional regulator DadR (19). D-Glutamate dehydrogenase encoded by the gene *dguA* is involved in the deamination of D-Glu and D-Gln (20). These three genes are essential for the utilization of the above mentioned D-amino acids as carbon and nitrogen sources. Since, the natural occurrence of D-amino acids is much less compared to the L-forms, we hypothesized that there could be a much important role for these dehydrogenases other than aiding the cell utilize these D-amino acids as nutrient sources. We hypothesized that the lack of the *dauA*, *dadA*, and *dguA* genes might weaken the cell and make it more susceptible to antibiotics when given in combination with D-amino acids.

4.2 Materials and methods

4.2.1 Strains and growth conditions.

Bacterial strains used in this study were listed in Table 3.1. Luria–Bertani (LB) medium was used for bacterial growth with the following supplements as required: ampicillin at 100 µg/ml, carbenicillin at 100 µg/ml for *E. coli*, and streptomycin at 500 µg/ml and gentamicin at 30 µg/ml for *P. aeruginosa*. Minimal medium P (MMP) supplemented with specific carbon sources and nitrogen sources was used for the growth of *P. aeruginosa* (17).

Table 4.1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source
<i>E. coli</i>		
DH5 α	F- Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK-, mK+) <i>phoA</i> <i>supE44</i> λ - <i>thi-</i> 1 <i>gyrA96 relA1</i>	Bethesda Research Laboratories
TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139 Δ(<i>ara leu</i>) 7697 <i>galU</i> <i>galK rpsL</i> (StrR) <i>endA1 nupG</i></i>	Invitrogen
SM10	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i> (Kmr)	(56)
<i>P. aeruginosa</i>		
PAO1	Wild type	(18)
PAO1-Sm ^r	Spontaneous Sm ^r mutant strain of PAO1	(23)
PAO5731	Δ <i>dguA</i>	(20)
PAO5434	Δ <i>dguA</i> ; Δ <i>dadA</i> ; Δ <i>dauA</i>	This study
Plasmids		
pRTP2	Amp ^r Sm ^s conjugation vector derived from pRTP1	(31)
pPS856	Amp ^r , Gm ^r cloning vector	(22)
pFLP2	Amp ^r , <i>sacB</i> ⁺ containing broad host range cloning vector	(22)
pUCP18	<i>Escherichia-Pseudomonas</i> shuttle vector	(55)
pRTP2 Δ <i>dauA::Gm</i>	pRTP2 with upstream and downstream of <i>dauA</i> linked by Gm cassette from pPS856	This study
pRTP2 Δ <i>dadA::Gm</i>	pRTP2 with upstream and downstream of <i>dadA</i> linked by Gm cassette from pPS856	This study
pUCP18D <i>gUA</i>	pCUP18 derivative carrying the <i>dguA</i> gene	This study
pUCP18D <i>auA</i>	pCUP18 derivative carrying the <i>dauA</i> gene	Congran Li (CR5.1)
pUCP18D <i>auBA</i>	pCUP18 derivative carrying the <i>dauBA</i> genes	This study
pUCP18D <i>adA</i>	pCUP18 derivative carrying the <i>dadA</i> gene	This study

Sm^r, streptomycin resistance; Amp^r, ampicillin resistance; Gm, gentamicin resistance; *sacB*⁺, sucrose-sensitive

4.2.2 Construction of D-amino acid dehydrogenase triple mutant.

A Δ *dguA*, Δ *dadA*, and Δ *dauA* triple mutant of PAO1 devoid of the three D-amino acid dehydrogenases, was constructed using a markerless gene deletion technique described previously (22). PAO5731 (Δ *dguA*), a derivative of PAO1 was used as the host to construct this triple dehydrogenase mutant. Briefly, the flanking regions of the *dauA* or *dadA* were amplified

by PCR, and restriction enzymes sites were introduced into the primers so that the PCR products possess configurations of 5'-*Bam*HI-[left arm]-*Sma*I-3' and 5'-*Sma*I-[right arm]-*Hind*III-3'. A 1.6-kb *Eco*RI fragment containing the gentamicin resistance (Gm^r) cassette was excised from the plasmid pPS856 by digesting with *Sma*I. After restriction enzyme digestion, these three DNA fragments were ligated and cloned into the *Bam*HI and *Hind*III sites of pRTP2. The final plasmid construct was introduced into *E. coli* SM10 to serve as the donor in the biparental conjugation, with PAO1- Sm^r as the recipient. After incubation at 37 °C for 6 h, the transconjugants were spread and selected on LB plates supplemented with streptomycin and gentamicin. The protocol for gene replacement and *in vivo* excision by the Flp-FRT recombination system was used to remove the gentamicin resistance marker facilitating the generation of unmarked mutants of PAO1 with deletions on multiple genes. Expected deletions in these mutants were confirmed by PCR and by growth in specific D-amino acids that require these genes for catabolism.

4.2.3 Construction and verification of D-amino acid dehydrogenase complementation clones.

The *dguA*, *dada*, *dauA* or *dauBA* genes were amplified from the genomic DNA of PAO1 along with their ribosome binding site (RBS) and were cloned into pUCP18 plasmid vector. The recombinant plasmids were verified by DNA sequencing and introduced into the $\Delta dguA$, $\Delta dada$, and $\Delta dauA$ triple mutant independently. Two single colonies of the triple mutant with or without the gene complementation clone were resuspended in phosphate buffered saline and inoculated into MMP broth supplemented 20 mM L-Glu/10 mM D-Glucose + 10 mM D-Glu, D-Arg or D-Ala/20 mM D-Glu, D-Arg or D-Ala for carbon and or nitrogen sources. Growth was followed by visual inspection for turbidity at 37 °C, 300 rpm for up to 3 days.

4.2.4 Determination of MIC for wild-type PAO1 and the $\Delta dguA$, $\Delta dadA$, and $\Delta dauA$ triple mutant.

Minimal inhibition concentrations (MICs) of antibiotics was determined by a liquid microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (8). Serial two-fold dilutions of the antibiotics in LB broth were prepared in a 96-well microtiter plate. LB broth without any antibiotic was used for both positive (inoculated) and negative (uninoculated) controls. Fresh overnight cultures of wild-type PAO1 and the $\Delta dguA$, $\Delta dadA$, and $\Delta dauA$ triple mutant (two independent cultures each) were diluted and inoculated with approximate 5×10^4 CFU/well. Cells were incubated without shaking at 37 °C for 24 h. The lowest concentration of antimicrobial agent at which cells were not able to grow was defined as its MIC.

4.2.5 *In vivo* substrate specificity of D-amino acid dehydrogenases.

MMP broth supplemented with various D-amino acids as C and N source (20 mM) or as N source (5 mM) were prepared. When testing for nitrogen utilization, 10 mM D-Glucose was supplemented as a carbon source. The pH of the final medium was neutralized using HCl or NaOH. About 5×10^4 CFU of wild-type PAO1 and the $\Delta dguA$, $\Delta dadA$, and $\Delta dauA$ triple mutant with or without the gene complementation clones (two independent cultures for each genotype tested) were inoculated. MMP medium with 10 mM D-Glucose and 0.5 % NH_4Cl was used as positive (inoculated) and negative (un-inoculated) controls. The plates were incubated at 37 °C and growth was noted by visual inspection 2 and 4 days post-inoculation.

4.2.6 Effect of D-amino acids given in conjunction with antibiotics.

LB medium was supplemented with 5 mM of various D-amino acids in combination with 1X, 0.5X or 0.25X MIC of carbenicillin, gentamicin or tetracycline. About 5×10^4 CFU of wild-type PAO1 or the $\Delta dguA$, $\Delta dadA$, and $\Delta dauA$ triple mutant were inoculated into each well. The plates were incubated at 37 °C and growth was recorded by measuring OD₆₀₀ 24 h post-inoculation. LB medium without any antibiotic or D-amino acid was used as positive control while the same without any bacterial cells served as negative control.

4.3 Results

4.3.1 Verification of $\Delta dguA$, $\Delta dadA$, and $\Delta dauA$ triple mutant.

The triple mutant was tested for the loss of *dauA*, *dadA*, and *dguA* genes by PCR. The loss of these three genes was confirmed by testing the ability of the wild-type PAO1 and the triple mutant to utilize D-Glu, D-Arg and D-Ala as sole carbon and or nitrogen sources in minimal medium P. The wild-type PAO1 was able to utilize all the three D-amino acids tested as both carbon and nitrogen sources. The triple mutant could not use any of these compounds as either carbon or nitrogen sources indicating the loss of *dauA*, *dadA*, and *dguA* genes as expected. The gene organization of the three dehydrogenase genes in wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant along with the gene complementation clones is shown in Figure 4.1 below.

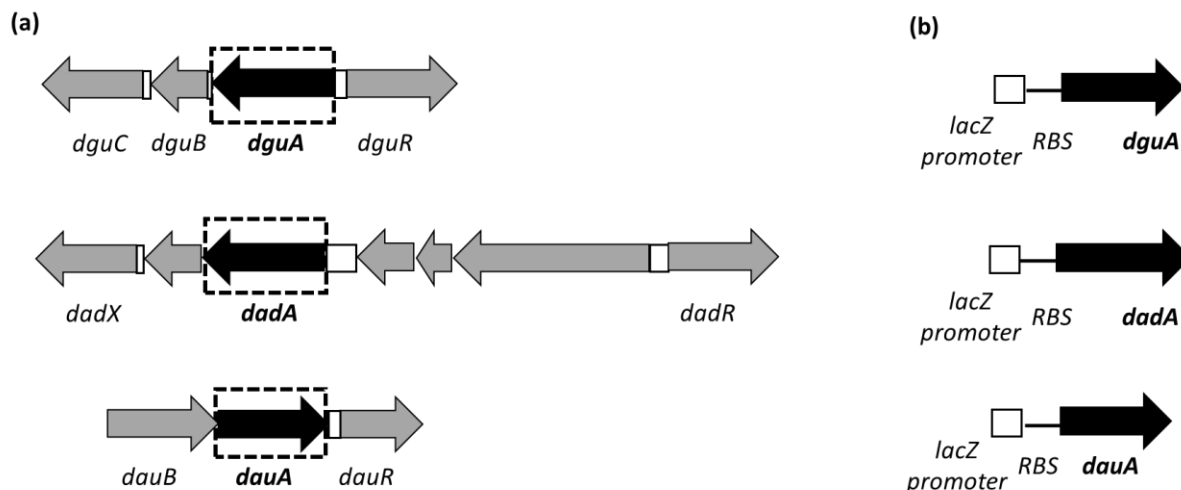


Figure 4.1 Dehydrogenase gene organization and gene complementation.

(a) Organization of the three dehydrogenase genes in wild-type PAO1. The dashed outline indicates the genomic region deleted in each locus to construct the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant. (b) Gene complementation clones constructed in the pUCP18 plasmid to complement each dehydrogenase gene in the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant.

4.3.2 D-amino acid dehydrogenase gene complementation.

In order to test for the *in vivo* substrate specificity of the D-amino acid dehydrogenases, gene complementation clones were constructed using the pUCP18 plasmid, an *E. coli-Pseudomonas* shuttle vector. Each D-amino acid dehydrogenase was cloned along with its ribosome binding site such that the gene expression is driven by the *lacZ* promoter on the pCUP18 plasmid (see Figure 4.1). These plasmids can reach 10 to 25 copies per cell with constitutive expression in *P. aeruginosa* (14). During the testing of gene complementation, we observed that the complementation of *dauA* gene could only restore utilization of D-Arg as a nitrogen source. This could be due to a non-functional *dauB* gene or DauB protein which may have got disrupted in part during the deletion of *dauA*. Hence, the *dauBA* complementation clone was also included in the analysis. It was found that the *dguA*, *dadA*, *dauA*, and *dauBA* gene clones all were able to

support growth on D-Glu, D-Ala, and D-Arg, as anticipated indicating that the gene complementation worked.

4.3.3 Antibiotic susceptibility of wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant.

The susceptibility of wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant were tested for the antibiotic commonly used in the laboratory. It was found that the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant had two-fold lower minimum inhibition concentration (MIC) for carbenicillin and tetracycline while gentamicin remained same as compared to the wild-type PAO1 (see Table 4.2). This supports our hypothesis that the triple mutant is weakened though by unknown mechanism(s).

Table 4.2 MIC for wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant.

	Minimum inhibitory concentration (MIC) ($\mu\text{g/mL}$)		
	Carbenicillin	Gentamicin	Tetracycline
WT PAO1	64	8	16
PAO5434	32	8	8

4.3.4 *In vivo* substrate specificity of D-amino acid dehydrogenases.

Previous studies used purified His-tagged proteins and tested the substrate specificity of D-amino acid dehydrogenases *in vitro*. Each D-amino acid dehydrogenase was shown to utilize more than one D-amino acid as the substrate. Hence, the mutant growth phenotypes may or may not be an accurate reflection of the *in vivo* characteristics of these enzymes. Here, we tried to see the *in vivo* substrate specificity of the D-amino acid dehydrogenases. It was found that DadA can utilize D-Ala, D-Val, D-His, D-Lys, D-Arg, D-Ser, D-Pro, D-Met, and D-Phe as the substrates. DguA can utilize D-Glu, D-Gln, and D-Ser as substrates. DauA can utilize D-His, D-Orn, D-Lys, D-Arg, D-Pro, D-Met, and D-Phe as substrates (see Table 4.3).

Table 4.3 *In vivo* substrate specificity of D-amino acid dehydrogenases.

	Wild-type		PAO5434 ($\Delta dguA$, $\Delta dauA$, and $\Delta dadaA$) + gene complement									
	PAO1		None		<i>dguA</i>		<i>dadaA</i>		<i>dauA</i>		<i>dauBA</i>	
	N	CN	N	CN	N	CN	N	CN	N	CN	N	CN
D-Ala	+	+	-	-	-	-	+	+	-	-	-	-
D-Val	+	+	-	-	-	-	+	+	-	-	-	-
D-Glu	+	+	-	-	+	+	-	-	-	-	-	-
D-Gln	+	+	+	+	++	++	+	+	+	+	+	+
D-His	+	-	+	-	+	-	++	-	++	-	++	-
D-Asn	+	-	+	-	+	-	+	-	+	-	+	-
D-Asp	-	-	-	-	-	-	-	-	-	-	-	-
D-Orn	+	-	-	-	-	-	-	-	+	-	+	-
D-Lys	+	-	-	-	-	-	+	-	++	-	++	-
D-Arg	+	+	-	-	-	-	+	-	++	-	++	++
D-Ser	+	-	+	-	++	-	++	+	+	-	+	-
D-Thr	+	-	+	-	+	-	+	-	+	-	+	-
D-Pro	+	-	-	-	-	-	+	-	+	-	+	-
D-Met	+	-	+	-	+	-	++	-	++	-	++	-
D-Phe	+	-	+	-	+	-	+	+	+	+	+	+

The data represents at least two independent sets of culture under each condition tested. Inoculated and un-inoculated LB medium was used as the positive and negative controls (not shown in table). Results were observed 2 days and 4 days post inoculation. Growth is by + and – is no growth even after 4 days. The ++ sign indicates that these cells grew better than or on par with the wild-type by 2 days itself indicating that the complemented gene enhances growth on that substrate. Grey colored cells indicate that the enzyme encoded by complemented gene can utilize that D-amino acid as a substrate.

4.3.5 Effect of D-amino acids given in conjunction with antibiotics.

Based on previous reports from various groups it was expected that D-amino acids might weaken the cell wall making it more permeable to antibiotics. Alternatively, the lack of the *dguA*, *dadA*, and *dauA* genes may also make the cell more resistant as a coping mechanism. When the cells were exposed to 1X MIC of carbenicillin, presence of D-Phe and D-Pro resulted in cell growth both in the wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$ and $\Delta dadA$ triple mutant. At lower concentrations of carbenicillin (0.5X and 0.25X MIC), both the wild-type and the triple mutant cells grew normally like the respective control groups. When the cells were exposed to gentamicin, growth was completely abolished at 1X MIC. Further, even at 0.5X MIC the presence of any D-amino acid tested resulted in complete growth inhibition both in the wild-type and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant cells indicating synergy between gentamicin and D-amino acids. When the cells were exposed to tetracycline, growth was completely abolished at 1X MIC. Further both at 0.5X and 0.25X MIC, a significant growth reduction was noted both in the wild-type and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant cells as compared to the respective control groups indicating synergy between tetracycline and the D-amino acids tested. However, no special synergistic or antagonistic effects were observed specifically in the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant as compared to the wild-type PAO1 when D-amino acids were given in combination with antibiotics (see Tables 4.4 and 4.5).

Table 4.4 Effect of D-amino acids given in conjunction with antibiotics in wild-type PAO1.

MIC	Antibiotic	Wild-type PAO1							
	Carbenicillin								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.62
0.5X	32	0.34	0.29	0.38	0.41	0.88	0.46	0.37	0.55
0.25X	16	0.24	0.23	0.28	0.40	0.28	0.27	0.21	0.53
	Carbenicillin								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	64	0.00	0.00	0.00	0.07	0.02	0.91	0.32	0.46
0.5X	32	0.44	0.73	0.82	0.18	0.25	0.33	0.27	0.57
0.25X	16	0.24	0.23	0.32	0.27	0.18	0.25	0.25	0.50
	Gentamicin								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.68
0.5X	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.66
0.25X	2	0.62	0.41	0.47	0.53	0.61	0.62	0.70	0.80
	Gentamicin								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.73
0.5X	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.65
0.25X	2	0.57	0.68	0.43	0.40	0.51	0.66	0.45	0.89
	Tetracycline								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83
0.5X	8	0.15	0.34	0.43	0.38	0.21	0.01	0.00	0.75
0.25X	4	0.42	0.38	0.57	0.47	0.45	0.33	0.22	0.85
	Tetracycline								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	16	0.00	-0.01	0.00	0.00	0.00	0.00	-0.01	0.89
0.5X	8	0.40	0.13	0.10	0.19	0.00	0.25	0.12	0.75
0.25X	4	0.61	0.34	0.33	0.32	0.47	0.50	0.38	1.10

About 5×10^4 cells of WT PAO1 were inoculated into LB broth supplemented with 5 mM of various D-Amino acids in combination with 1X, 0.5X or 0.25X MIC of carbenicillin, gentamicin or tetracycline. The 96-well plates were incubated at 37 °C and growth was recorded by measuring OD₆₀₀ 24 h post inoculation. These results are an average of two independent sets of culture under each condition. LB medium without any antibiotic or D-amino acid was used as positive control. Negative control (not shown in table) was LB medium without any antibiotic, D-amino acid or bacterial cells.

Table 4.5 Effect of D-amino acids given in conjunction with antibiotics in $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant.

MIC	Antibiotic	PAO5434 ($\Delta dguA$, $\Delta dauA$, and $\Delta dadA$)							
	Carbenicillin								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	32	0.01	0.13	0.20	0.12	0.00	0.00	0.01	0.71
0.5X	16	0.19	0.22	0.28	0.16	0.21	0.25	0.41	0.87
0.25X	8	0.44	0.51	0.43	0.37	0.36	0.52	0.59	0.89
	Carbenicillin								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	32	0.06	0.00	0.04	0.07	0.01	0.10	0.42	0.43
0.5X	16	0.47	0.22	0.16	0.30	0.28	0.44	0.27	0.42
0.25X	8	0.77	0.55	0.56	0.63	0.64	0.69	0.58	0.44
	Gentamicin								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.82
0.5X	4	0.10	0.09	0.11	0.15	0.01	0.04	0.01	0.86
0.25X	2	0.30	0.33	0.40	0.41	0.43	0.33	0.39	0.84
	Gentamicin								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	8	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.83
0.5X	4	0.04	0.06	0.02	0.07	0.14	0.03	0.05	0.86
0.25X	2	0.43	0.33	0.32	0.35	0.36	0.40	0.30	0.84
	Tetracycline								Positive control
	($\mu\text{g/mL}$)	Ala	Val	Glu	Gln	His	Asn	Asp	
1X	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.93
0.5X	4	0.11	0.17	0.29	0.33	0.35	0.14	0.43	0.97
0.25X	2	0.49	0.49	0.49	0.50	0.58	0.53	0.43	0.92
	Tetracycline								Positive control
	($\mu\text{g/mL}$)	Orn	Lys	Arg	Ser	Thr	Pro	Phe	
1X	8	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.95
0.5X	4	0.28	0.26	0.22	0.20	0.16	0.33	0.36	0.96
0.25X	2	0.48	0.51	0.44	0.47	0.54	0.50	0.38	0.95

About 5×10^4 CFU of $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant cells were inoculated into LB broth supplemented with 5 mM of various D-Amino acids in combination with 1X, 0.5X or 0.25X MIC of carbenicillin, gentamicin or tetracycline. The 96-well plates were incubated at 37 °C and growth was recorded by measuring OD₆₀₀ 24 h post inoculation. These results are an average of two independent sets of culture under each condition. LB medium without any antibiotic or D-amino acid was used as positive control. Negative control (not shown in table) was LB medium without any antibiotic, D-amino acid or bacterial cells.

4.4 Discussion

4.4.1 *In vivo* substrate specificity of the D-amino acid dehydrogenases.

The *in vivo* substrate specificity of the D-amino acid dehydrogenase is different from that reported using *in vitro* studies.

Table 4.6 *In vitro* and *in vivo* substrate specificities of D-amino acid dehydrogenases.

Enzyme	Amino acids that induce transcription	<i>In vitro</i> D-amino acid specificity of enzyme	<i>In vivo</i> D-amino acid utilization observed (this study)
DguA	D-Glu, D-Gln	Glu, Gln, Pro	Glu, Gln, Ser
DadA	L-Ala, L-Val, L-Trp, L-Ser, L-Thr, L-Lys, L-Gly, L-Cys, D-Ala, D-Thr	Ala, Arg, Asn, Asp, His, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Trp, Tyr, Val	Ala, Val, His, Lys, Arg, Ser, Pro, Met, Phe
DauA	D-Arg, D-Lys	Arg, Lys, Met, Phe, Tyr, His, Orn, Leu, Trp, Val, Pro, Thr, Asn, Glu, Gln, Ala	His, Orn, Lys, Arg, Pro, Met, Phe

The *in vitro* substrate specificities were pooled from previously published reports (19, 20, 33) and the *in vivo* substrate specificities are from this study. The amino acids that are utilized by each enzyme either only *in vitro* or *in vivo* are highlighted with a gray background. D-Trp and D-Tyr were not tested *in vivo* and hence not highlighted.

Purified His-tagged DguA was shown to utilize D-Glu, D-Gln and D-Pro *in vitro* (20). However, in our study using *in vivo* gene complementation, it was found that DguA can utilize D-Glu, D-Gln, and D-Ser as substrates. DadA was shown to utilize 16 of the 18 D-amino acids tested *in*

vitro (19). But, we found that *in vivo* substrate utilization by DadA is limited to D-Ala, D-Val, D-His, D-Lys, D-Arg, D-Ser, D-Pro, D-Met, and D-Phe. Similarly, DauA was also shown to utilize 9 D-amino acids very well, 9 other somewhat poorly (31). However, we found that DauA can utilize D-His, D-Orn, D-Lys, D-Arg, D-Pro, D-Met, and D-Phe as substrates (see Table 4.7). Overall, the enhanced growth on D-amino acids by the presence of recombinant clones was due to increased levels of enzymes with broad substrate specificity. In the wild type strain, expression of these enzymes is subjected to induction by specific D-amino acids or related compounds. However, expression of these enzymes is constitutively active from the recombinant clones.

4.4.2 Utilization of other D-amino acids.

If the wild type strain needed to convert D to L via the traditional racemase, then utilization of D amino acids will not be affected in the triple mutant. Both the wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant were able to utilize D-Gln as the sole source of carbon and nitrogen while D-His, Asn, Ser, Thr, Met and Phe served only as a nitrogen source. D-Glutamine degradation can proceed in two possible ways: it can be converted into the L-form by a racemase or it can be directly deaminated to form glutamate and ammonia. In the case of the triple mutant, since D-glutamate degradation is abolished (requires DguA), I presumed that D-Gln is being converted into L-Gln by an unknown racemase followed by deamination to form L-Glu (a good carbon and nitrogen source for PAO1) and ammonia. However, enhanced growth by *dguA* gene complementation suggests that the alternate route is also possible. The utilization of D-Asn could be following similar suite. Both L-Gln and L-Asn can be deaminated by the glutaminase and asparaginase enzymes to remove the amine group and produce L-Glu and L-Asp respectively.

Both these enzymes have been previously detected and purified from Pseudomonads and PAO1 has two genes (*ansA* and *ansB*) that have high sequence similarity to the glutaminase and asparaginase enzymes (59, 11, 12). Utilization of D-Ser and D-Thr can be attributed to D-Ser dehydratase even though *dadA* gene complementation enhanced growth on D-Ser as nitrogen. In *E. coli*, D-Met was suggested to be converted into keto-methionine and then into L-Met (9). The arginine: pyruvate transaminase AruH of *P. aeruginosa* PAO1 was shown to utilize L-Met as a substrate (64). Even though not studied experimentally, it is possible that AruH is responsible for the utilization of D-Met as a nitrogen source in the triple mutant. However, as demonstrated by others previously we also observed an enhanced growth on D-Met when complemented by *dadA* and *dauA* genes. In case of D-His, even though *dadA* and *dauA* gene complementation seemed to contribute to its utilization as a nitrogen source, there are other unknown enzymes that we are not aware of allowing the triple mutant to harvest nitrogen from D-His. The wild-type and $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant strains were able to utilize D-Phe as only a nitrogen source indicating the involvement of another unknown enzyme. *dadA* and *dauA* or *dauAB* gene complementation allowed the triple mutant to utilize D-Phe as both carbon and nitrogen sources confirming previous reports (19, 31). The wild-type PAO1 was also able to utilize D-Pro as a nitrogen source which was completely abolished in the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant. Complementation with any of the three dehydrogenase genes did not restore the utilization of D-Pro. D-Pro utilization was shown to be dependent on the *dpkA* gene in *P. putida*. Since the *dpkA* gene was not mutated, it is not clear as to why the utilization D-Pro was abolished in the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant (38).

4.4.3 The triple mutant is weakened by an unknown mechanism.

The triple dehydrogenase mutant is weakened by an unknown mechanism. As a result, the triple mutant exhibited increased susceptibility to some of the common laboratory antibiotics. Both for Carbenicillin and tetracycline, the triple mutant had two-fold lower MIC than the wild-type PAO1. The mechanism by which the loss of *dauA*, *dadA*, and *dguA* genes affects the cell is yet to be studied. This might pave way for better strategies to combat *P. aeruginosa* infections.

4.4.4 No special synergy was observed between D-amino acids and antibiotics against the triple mutant.

Previous studies by other groups showed that D-amino acids can be detrimental to the cell and exhibit synergy with β -lactam antibiotics (3, 52). A mixture of D-amino acids was shown to prevent biofilm formation (26) and modify cell wall (28). In addition, excessive D-amino acids in the cell if not effectively removed or utilized, can also be wrongly charged on the tRNA's and there by stall protein synthesis. We hypothesized that deletion of all the three D-amino acid dehydrogenases would weaken the cell or make it more resistant. We also hypothesized that the addition of D-amino acids to the triple mutant in combination with antibiotics would have a synergistic or antagonistic effect in inhibiting the growth of PAO1. True to our hypothesis, we did observe that the triple mutant had lowered tolerance for antibiotics compared to the wild-type PAO1. When the cells were exposed to 1X MIC of carbenicillin, presence of D-Phe and D-Pro resulted in cell growth in both the wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant. In the case of gentamicin and tetracycline, the presence of D-amino acids made the cells more susceptible to these antibiotics. Complete growth inhibition was observed even at 0.5X MIC when gentamicin was given in combination with any of the D-amino acids tested. In the case of

tetracycline, a significant growth reduction was noted at 0.5X and 0.25X MIC when given in combination with any of the D-amino acids tested. This synergy between gentamycin or tetracycline and D-amino acids was noted in both the wild-type and the $\Delta dguA$, $\Delta dauA$, and $\Delta dada$ triple mutant cells. However, no special synergistic or antagonistic effects were observed specifically in the $\Delta dguA$, $\Delta dauA$, and $\Delta dada$ triple mutant as compared to the wild-type PAO1 when D-amino acids were given in combination with antibiotics.

5 GENERAL CONCLUSION

P. aeruginosa, an opportunistic human pathogen, is also well known for its enormous metabolic capacity making it a model organism to study nutrient utilization genes and pathways. In addition, owing to its resistance to multiple antibiotics, the pursuit of new targets for potential antibiotic development has been of great interest in this organism. In this dissertation, we studied the lysine catabolic pathway to identify the missing players. We have shown that the *gcdGH* genes encoding glutaryl-CoA dehydrogenase and as putative acyl-CoA transferase, respectively, are essential for lysine catabolism. Transcription of this operon is activated by GcdR in the presence of glutarate and we showed the possible GcdR binding site on the *gcdH* promoter DNA. Though glutarate is an intermediate compound of L-lysine catabolism, the *gcdG* and *gcdH* mutants cannot grow on L-Pip, a D-lysine metabolite, suggesting that L-Pip is also degraded via glutarate. This is the first link indicating that L- and D-lysine catabolic pathways are connected. We further showed that PA2035 is induced by D-Lys and L-pip and mutants of this gene cannot grow on L-Pip. We believe that PA2035 encoding a TPP-dependent decarboxylase is a possible player in the conversion of 2-aminoadipate to glutarate.

There are multiple reasons that possibly contribute to lysine being a poor nutrient source in *P. aeruginosa* PAO1. Previous studies from our lab showed that lysine decarboxylase enzyme *ldcA*, the first enzyme in L-lys catabolism, is under the control of an arginine-responsive transcriptional regulator *argR*. In this study, we showed that lysine uptake and export are two other bottlenecks for growth on L-Lys. We found the *lysXE* operon encoding an uncharacterized protein and a putative lysine efflux protein is induced by exogenous L-Lys. This operon is under the transcriptional regulation of LysR and we have identified the LysR operator site on *lysX* promoter

DNA. Knockout mutants of *lysR*, *lysX*, and *lysE* were found to grow much better than the wild-type PAO1 on MMP agar with L-Lys as the sole source of C & N and had a much shorter lag-phase and generation time in liquid media. We believe that the shorter lag phase and generation time observed in *lysR*, *lysX*, and *lysE* mutants was due to the lack of a functional lysine exporter, resulting in a higher intracellular concentrations of L-Lys compared to the wild-type PAO1. In this study, we also showed that *lysP* mutant grew very poorly on L-lys compared to the wild type PAO1. Transcription of the *lysP* gene is positively regulated by *arusR* that responds to L-Lys and L-Arg and negatively regulated by *argR* that responds to L-Arg making this another bottleneck for lysine catabolism. *P. aeruginosa* is well equipped to grow more efficiently on Arg than on Lys, with the ArgR regulator in response to L-Arg as the major player to control the entire arginine network. The physiological significance of this observed nutritional preference for Arg over Lys in *Pseudomonas* was a very intriguing question for future investigation.

Previous studies from our lab showed that catabolic and anabolic dehydrogenases DauA and DauB are essential for D-to-L racemization of arginine which is a prerequisite for D-arginine utilization in *P. aeruginosa* PAO1. In addition to D-arginine, DauA can utilize D-Lys as a substrate to generate keto lysine or α -keto- ϵ -aminohexanoate which can further be degraded into L-Pip and so on. In this dissertation, we studied the substrate specificity of DauB to see if D-Lys can be racemized to L-Lys for catabolism. We also studied the enzyme kinetics of DauB. Using a reverse reaction to measure DauB activity, we found that DauB showed little or no activity against lysine or other L-amino acids as substrates as compared to that against L-Arg (taken as 100 %). This indicates that DauB is an arginine-specific dehydrogenase unlike its partner enzyme DauA eliminating the possibility of D-to-L racemization of Lysine for catabolism (there is no other lysine racemase in PAO1).

In bacteria, D-amino acids are formed by racemization from cognate L-enantiomers which is an energetically expensive process for the cell. *P. aeruginosa* PAO1 has three known D-amino acid dehydrogenases: *dadA*, *dauA*, and *dguA*. The *in vitro* substrate specificity of these three enzymes has been demonstrated by our lab previously. However, we do not know if these results are true and the only physiological functions of these enzymes. With the emerging understanding of the far more important functions of D-amino acids and some of these D-amino acid catabolizing genes in the bacterial world, it is intriguing to see if the D-amino acid dehydrogenase genes/enzymes also have a wider physiological implication. We hypothesized that the lack of *dadA*, *dauA*, and *dguA* genes might weaken the cell and make it more susceptible to antibiotics when given in combination with D-amino acids. We also tested the role of each of these dehydrogenases in the utilization of various D-amino acids *in vivo*. We found that the *in vivo* substrate specificity of the D-amino acid dehydrogenases is different from that reported using *in vitro* studies.

The $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple dehydrogenase mutant is weakened by an unknown mechanism resulting in increased susceptibility to carbenicillin and tetracycline. The mechanism by which the loss of *dauA*, *dadA* and *dguA* genes affects the cell is yet to be studied. This might pave the way for better strategies to combat *P. aeruginosa* infections. At concentrations below the MIC, synergy was observed between gentamicin or tetracycline and D-amino acids resulting in growth inhibition or reduction, in the wild-type PAO1 and the $\Delta dguA$, $\Delta dauA$, and $\Delta dadA$ triple mutant, respectively. However, no special synergistic or antagonistic effects were observed specifically in the $\Delta dguA$, $\Delta dauA$ and $\Delta dadA$ triple mutant as compared to the wild-type PAO1 when D-amino acids were given in combination with antibiotics.

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