Structure-function relationships of the lysine decarboxylase from Pseudomonas aeruginosa

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Structure-function relationships of the lysine decarboxylase LdcA from *Pseudomonas aeruginosa*

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Abstract

The lysine decarboxylase (LDC) belongs to a family of decameric PLP-dependent enzymes that catalyse the reaction transforming L-Lysine into cadaverine while consuming a proton. They have been extensively characterised in enterobacteria, where they have been shown to play a crucial role during acid, oxidative stress and antibiotic resistance.

Since mechanisms allowing bacteria to counter stress challenges are important for displaying full virulence, we wondered if the opportunistic bacterium *Pseudomonas aeruginosa* could be using LdcA to counter stress conditions that have already been described for enterobacteria. During my PhD, we addressed this question by using different but complementary.

First of all, we used promoter-gene fusions and western-blot analysis to determine the conditions in which *ldcA* was expressed and its product synthesized.

In parallel, we constructed an *ldcA* mutant and its complemented strain to understand whether LdcA was involved in acid and oxidative stress response. We used manual screenings and high-throughput technologies (Biolog) and we discovered that the cadaverine produced by LdcA is needed for full growth fitness when growing in minimal medium using L-glutamate as carbon source. Since slow growing phenotypes are linked to heightened bacterial persistence and because cadaverine has been shown to reduce the persisters population, we also examined if the presence of LdcA is modifying the amount of persisters during carbenicillin treatment.

Finally, by combining phylogenetic and structural analysis, we discovered that LdcA belongs to a different subgroup of bacterial LDCs. Sequence alignments show that key residues needed for binding ppGpp are not present in the predicted binding site which also suggests that the enzymatic activity is not inhibited by this molecule. These observations are coherent with the fact that LdcA seems to be close to Arginine Decarboxylase (ADC) from *E. coli* which does not bind ppGpp.

Our work shows that, in spite of the fact that LdcA catalyses the same enzymatic reaction and shares the same structural fold than enterobacterial lysine decarboxylases, it is not implicated in acid stress or oxidative stress responses. Its role is linked to physiological effects of cadaverine and to the relationship between L-lysine and L-Arginine catabolism.

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List of abbreviations

AAA+ - ATPases Associated with a Variety of Cellular Activities

AAT – aspartate aminotransferase

AC – adenylate cyclase

ADC – Arginine decarboxylase

ADP – Adenosine diphosphate

AHL – Acyl homoserine lactone

AHQ – 2-alkyl-4-quinolone

ANR – Anaerobic transcriptional regulator

AR – Alanine racemase

ASL – Airway surface liquid

ATP – Adenosine triphosphate

AUC – Area under the curve

BLASTP – Basic local alignment search tool protein

CAD - Cadaverine

CAMHB – Cation Adjusted Mueller Hinton Broth

cAMP – Cyclic adenosine monophosphate

CDD – Conserved domain database

c-di-GMP – Cyclic diguanylate

CDS – Coding DNA sequence

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CFU – Colony forming units

CLSI – Clinical & laboratory standards Institute

CRISPR – Clustered regularly interspaced short palindromic repeats

CTD – C terminal domain

CV – Column volume

DGC – Diguanylate cyclase

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleoside triphosphate

FNR – Fumarate and nitrate reductase

GDP – Guanosine diphosphate

GFP – Green fluorescent protein

GTP – Guanosine triphosphate

H+ - Proton

HHQ – 4-hydroxy-2-heptylquinoline

HMM – Hidden markov models

ICE – Integrative and conjugative models

ICU – Intensive care units

IQS – Integrated quorum sensing

IS – Insertion sequence

kb - Kilobase

kDa – Kilodaltons

KEGG – Kyoto encyclopedia of genes and genomes

LB – Lysogeny broth

LDAR – Lysine dependent acid stress response

LDC – Lysine decarboxylase

LPS - Lipopolysaccharide

MIC – Minimal inhibitory concentration

MMP – Minimal medium P

MOI – Multiplicity of infection

MUSCLE - Multiple sequence comparison by log-expectation

NADH – Nicotinamide adenine dinucleotide (reduced)

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced)

OD600nm – Optical density at 600 nanometers

ODC – Ornithine decarboxylase

OMP – Outer membrane proteins

OMV – Outer membrane vesicules

ONPG - ortho-nitrophenyl- β-D-galactopyranoside

ORF – Open reading frame

PBS/T – Phosphate buffer saline/ tween 20

PCR – Polymerase chain reaction

PDE - Phosphodiesterase

PIA – *Pseudomonas* isolation agar

PLP – pyridoxal 5' phosphate

Pmf – proton motive force

ppGpp - guanosine tetraphosphate

PQS – Pseudomonas quinolone system

PUT - Putrescine

QS – Quorum sensing

RGP – Regions of genome plasticity

RLU – Relative light units

RNA – Ribonucleic acid

RNAP – RNA polymerase

RPM – revolution per minute

ROS – Reactive oxygen species

RSAT – Regulatory sequence analysis tool

SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOD – Superoxide dismutase

SOE PCR – Splicing by overlap extension polymerase chain reaction

SPM - Spermidine

STEC – Shigatoxin producing *Escherichia coli*

T3SS – Type three secretion system

TA – Toxin antitoxin

WT – Wild type

Thesis presentation and objectives

The lysine decarboxylase (LDC) belongs to a family of decameric PLP-dependent enzymes that catalyze the reaction transforming L-Lysine into cadaverine while consuming a proton and producing a CO_2 molecule. They are part of the bacterial arsenal against stress conditions such as acid stress, oxidative stress and antibiotic treatment. In enterobacteria like *Escherichia coli*, two paralogs are present, LdcI and LdcC. LdcI takes part in acid stress response by buffering bacterial cytoplasm and the surrounding extracellular environment. LdcC, in its turn, is produced during stationary phase and also when bacteria face fluoroquinolone treatment. Cadaverine produced by LDCs is known to scavenge reactive oxygen species (ROS) and is capable of blocking outer membrane proteins, thus reducing the permeability of molecules responsible for acid and oxidative stresses.

The laboratory of Irina Gutsche (IBS) has become a leading group in analyzing and solving the structures of the lysine decarboxylases from *E. coli* by cryo-electron microscopy. In collaboration with Walid Houry lab (University of Toronto, Canada), they discovered that the activity of the LDCs from *E. coli* is regulated during the stringent response (nutrient starvation) to prevent intracellular L-Lysine depletion. Indeed, the stringent response signal molecule ppGpp can bind directly to LDCs and inhibit their enzymatic activity. Another milestone in the field of the Lysine decarboxylase was the discovery of a cage-like complex formed by LdcI and the AAA+ ATPase RavA. This peculiar complex prevents the inhibition by (p)ppGpp thereby allowing bacteria to face the challenge of both acid and nutrient stresses.

Since the role of the lysine decarboxylase has only been studied in enterobacteria such as *E. coli*, we wondered whether the lysine decarboxylase was also protecting the well-known opportunistic pathogen *Pseudomonas aeruginosa* from the stress conditions experienced in the host. For instance, *P. aeruginosa* is a serious health problem for patients affected by Cystic Fibrosis. In particular, one can wonder if under conditions encountered in the lungs of cystic fibrosis patients whose lungs' secretions were shown to be acidified and to become oxidative, LdcA is playing a role in promoting fitness of the bacterium.

To tackle our project with *P. aeruginosa*, we stablished a collaboration with Ina Attrée's lab (BiG, CEA), where they study the pathogenesis of *P. aeruginosa* and possess the proper equipment and expertise to manipulate this bacterium. With the expert advise of Dr. Sylvie

Elsen, who is also my thesis co-director, we constructed a project that would allow us to examine the role of LdcA of *P. aeruginosa* by using different approaches:

- First of all, we used promoter-gene fusions and western-blot analysis to determine the conditions in which *ldcA* was expressed and its product synthesized.
- In parallel, we constructed an *ldcA* mutant and its complemented strain to investigate whether LdcA was involved in acid and oxidative stress response, but also in growth fitness and persistence via production of cadaverine.
- Finally, by combining phylogenetic and structural analyses, we discovered that LdcA belongs to a different subgroup of bacterial LDCs.

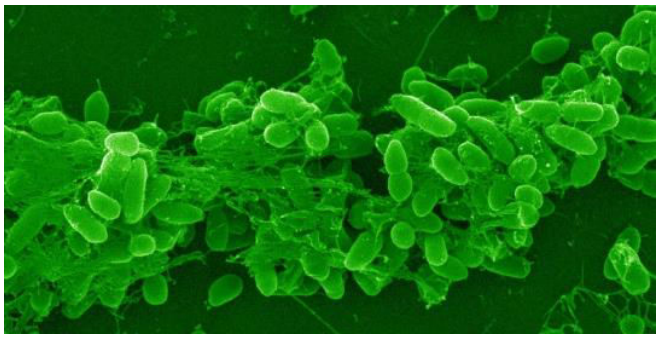
Before showing our results, I will start by introducing the general characteristics of the genome and physiology of *P. aeruginosa* and then present what we know about the lysine decarboxylases.

Part I: Introduction

Chapter I: Pseudomonas aeruginosa

Pseudomonas aeruginosa is a bacterium that belongs to *Pseudomonadaceae* which is a representative family of the *Gammaproteobacteria* class. It has been called "*Bacillus pyocyaneus*" for a longtime by physicians because it generates blue pus in the wounds of infected patients. It is a gram-negative non-sporulating bacillus, measuring 1- 5 µm long and 0.5 - 1 µm wide. It is mobile in liquid media and on solid surfaces and its motility depends on a polar monotrichous flagellum and type IV pili.[1] *P. aeruginosa* is a mesophilic bacterium that is capable of growing in temperatures ranging from 4°C to 42 °C, with an optimum growth temperature between 30°C and 37°C.[2,3] It is also a neutralophile and is found in aquatic and terrestrial environments that span a pH range from 4.5 to 9.5. More than 50 different organic and inorganic molecules can be degraded by the metabolism of *P. aeruginosa*. It uses efficiently O_2 as the principal electron acceptor but has also the capability of using nitrates for respiration. When oxygen and nitrates or nitrites are unavailable, the bacterium is capable of fermenting L-Arginine for growth and pyruvate for survival only.[3,4]

All these properties give *P. aeruginosa* the ability to thrive in most natural and man-made environments and explain why the bacterium can ultimately infect multiple hosts such as humans and other mammals, insects, nematodes, amebae and even plants[5,6].



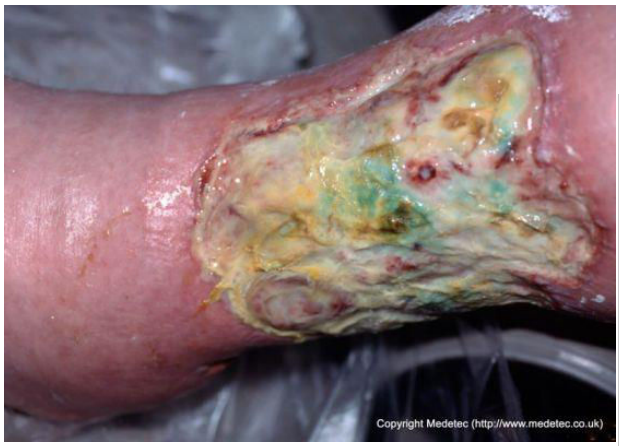


Figure 1. *Pseudomonas aeruginosa*. Up left: Electron microscope image of *P. aeruginosa* biofilm. **Up right:** Culture of *P. aeruginosa* on solid agar, the green fluorescent pigment pyoverdin gives the bacterium its characteristic color. **Left:** *P. aeruginosa* infection in burn wound patient. Images obtained from Pr. Filloux website and from microbeworld.org and medetek.co.uk

1. The genome of *Pseudomonas aeruginosa*

1.1. The genome of PAO1 strain

The genome of the strain PAO1 was the first one to be sequenced in the year 2000. As presented in **table 1**, PAO1 has a genome size of 6.3 mega base pairs (Mbp) with 5,684 predicted open reading frames (ORF)[7] (Pseudomonas.com). Around 40% of the genome consists of hypothetical and conserved hypothetical ORFs with unknown function[7,8]. The expression of the genome is coordinated by a complex regulatory network consisting at least of 690 genes (12% of total genome) encoding for sigma factors and anti-sigma factors, transcriptional regulators and two-component regulatory systems[9]. The regulatory network integrates and controls important biological process such as energy metabolism, cell division, biofilm formation, cell-to-cell communication, production of virulence factors and guarantees a rapid adaptation to environmental changes[10].

Strain	PAO1	PA7	PA14	LESB58
Genome Size (bp)	6264404	6588339	6537648	6601757
G+C content	66.6	66.5	66.3	66.3
protein coding genes	5684	6286	5892	5925
%coding	89	89	89	88
structural RNAs	77	75	72	81
Pseudogenes	5	8	-	34
Assigned function				
Translation, ribosomal structure and biogenesis	205	206	205	199
Transcription	516	530	537	501
Replication, recombination and repair	160	235	185	145
Cell cycle control, cell division, chromosome partitioning	34	37	35	34
Posttranslational modification, protein turnover, chaperones	200	215	210	201
Cell wall/membrane/envelope biogenesis	265	260	266	261
Cell motility and secretion	150	152	154	149
Inorganic ion transport and metabolism	376	355	377	313
Signal transduction mechanisms	337	346	345	337
Energy production and conversion	329	336	340	330
Carbohydrate transport and metabolism	252	250	249	196
Amino acid transport and metabolism	587	571	590	490
Nucleotide transport and metabolism	108	105	110	104
Coenzyme transport and metabolism	191	192	192	210
Lipid transport and metabolism	244	245	248	234
Secondary metabolites biosynthesis, transport and catabolism	205	198	212	171
General function prediction only	756	759	771	603
Function unknown	476	503	493	500

Table 1. Summary of the characteristics of the genome of 4 different reference strains of *P. aeruginosa*[11].

A disproportionate large number of outer membrane proteins (OMP) are encoded in *P. aeruginosa* genome compared to other species. 150 genes are predicted to be outer membrane proteins (OMP) involved in iron uptake, antibiotic export and secretion of virulence factors essential for pathogenicity[7]. Consistent with its environmental versatility, 300 cytoplasmic membrane transport systems are encoded and at least 200 appear to be involved in the transport of nutrients and other molecules such as dicarboxylates, sugars, fatty acids, alcohols, polyalcohols, glycols, aromatic compounds, amines and amino acids[7]. In comparison with *E. coli, P. aeruginosa* exhibits a limited number of genes encoding sugar transporters and uses the Enter-Doudoroff Pathway glycolytic pathway (Enter-Doudoroff Pathway) and an aerobic, oxidative metabolism[12]. A large number of genes involved in β-oxidation are present and ensure the versatile metabolic capacity of oxidizing a wide variety of organic compounds in a similar way as in *Mycobacterium tuberculosis*[7].

The *P. aeruginosa* genome appears to contain several undescribed drug efflux systems, predominantly of the Resistance-Nodule-Division (RND) and Major Facilitator Superfamily (MFS) families. The number of predicted drug efflux systems from the (MFS), small multi-drug resistance (SMR), ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) families is like that of other organisms such as *E. coli, Bacillus subtilis* and *M. tuberculosis.* However, PAO1 *P. aeruginosa* contains many more predicted AcrB/Mex-type RND multidrug efflux systems (10 genes) *than E. coli (4), B. subtilis (1)* and *M. tuberculosis (0)*[7].

An important number of virulence factors is also encoded in the genome of *P. aeruginosa* PAO1 such as toxins (exotoxin A), proteases (elastase, alkaline protease, protease IV), phospholipases, flagellum, pili, fimbriae and secretion systems (T1SS, T2SS, T3SS, T5SS, T6SS). Operons encoding the biosynthetic genes for virulence factors such as rhamnolipids, lipopolysaccharides, phenazines, siderophores and polysaccharides (alginates, pel, psl) are also present[13–26].

1.2. The pangenome of *P. aeruginosa*

Today high throughput sequencing technologies allowed us to access to the nucleotide sequences of more than 2200 genomes from different *P. aeruginosa* strains. Nevertheless, in the databases of the NCBI and PATRIC we found that less than 200 different genomes have been completely sequenced and annotated[2,7]. The analysis of the sequenced genomes has revealed that the pan-genome of *P. aeruginosa* can be divided in two different groups of genes:

1.2.1. The core genome

It encodes a set of metabolic and pathogenic factors shared by all *P. aeruginosa* strains, irrespective of origin. The core genome constitutes approximately 90% of the total genome and is highly conserved (>98% identity) from strain to strain. Today it is estimated to be composed of more than 4000 ORFS. Its G+C percentage is 67.1%[27–32]. Despite research efforts, there is limited knowledge about the function of more than 1000 genes in *P. aeruginosa* core genome **(figure 2).**

1.2.2. The accessory genome

It encompasses genes that are found in some *P. aeruginosa* strains but not others. In **figure 2,** we found a representation of *P. aeruginosa* pangenome in which we see both the accessory genome and the core genome. The accessory genome appears as segments that are not scattered randomly throughout the core genome but are inserted at specific sites, called Regions of Genome Plasticity (RGPs). The accessory genome can be grouped in four categories: (i) integrative and conjugative elements (ICEs), (ii) replacement islands, (iii) prophages and phage-like elements, and (iv) transposons, insertion sequences (ISs), and integrons. Horizontal gene transfer is by far the most important process contributing to its evolution. This is reflected by its G+C percentage of 62.4% (median). Up-to-date more than 10000 genes have been classified as part of the accessory genome, a number that should increase with the addition of newly sequenced *P. aeruginosa* strains[27–32].

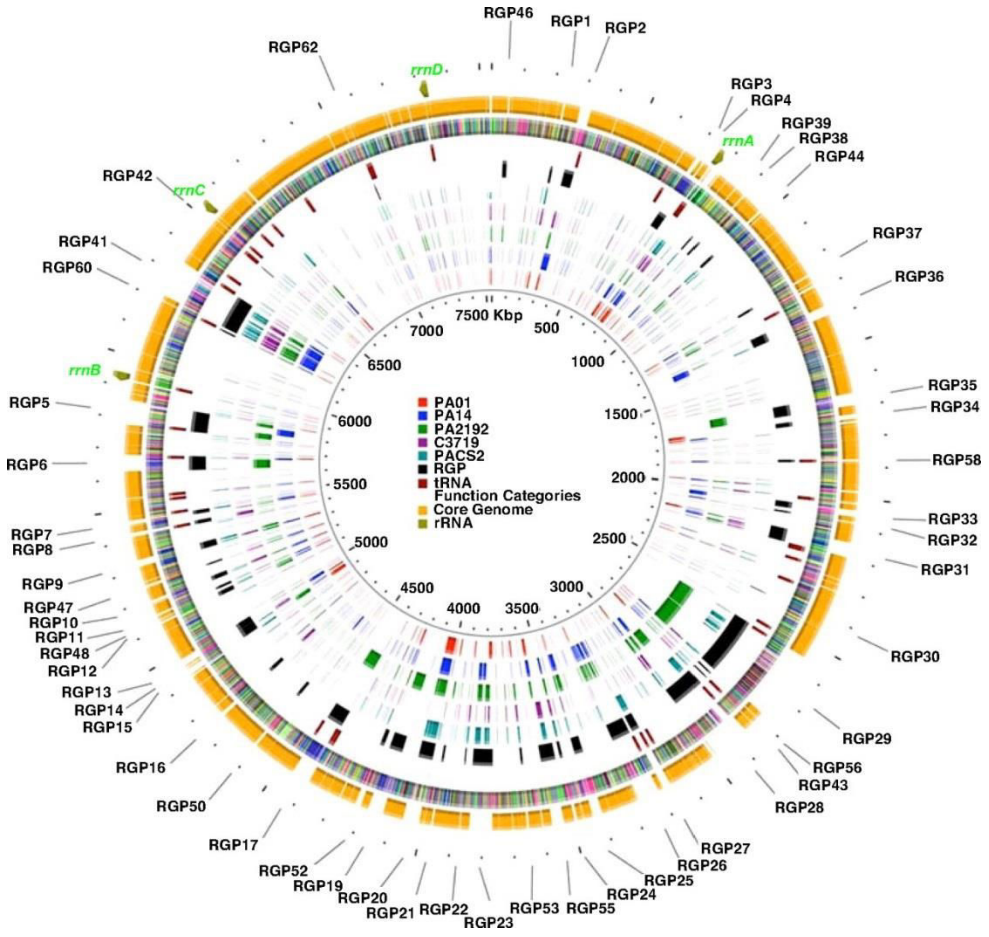


Figure 2. The pangenome of 5 strains of *P. aeruginosa*[27]. The pangenome is a compilation of all the genes from all the strains of a single species. In this figure, the core genes from the strain PA14 were used as matrix for putting the accessory genes from strains PA2192, C3719, PAO1 and PACS2. The golden circle indicates the core genome, while the second circle shows the totality of genes that have been annotated for all the strains back in 2008. The third circle indicates the position of tRNA and is generally used to identify insertion sites. The fourth circle, in black, indicates the RGPs which can be sites of insertions of common or unique genomic islands and bacteriophage genomes, or the result of deletions of segments of DNA in one or more strains. The accessory genomes from each strain are shown in different colours: PACS2 (turquoise), C3719 (violet), PA2192 (green), PA14 (blue) and PAO1 (red). The outer green arrows show the positions of rRNAs.

2. Clinical importance of *P. aeruginosa*

2.1. *P. aeruginosa* is an opportunistic pathogen

P. aeruginosa can cause both acute and chronic infections in its host. Acute *P. aeruginosa* infections are invasive and cytotoxic and frequently result in substantial tissue damage, systemic spread, sepsis, and mortality. The pathogenesis of acute infections relies upon the expression of many surface-exposed and secreted virulence factors, including: toxins, proteases (delivered by a type II secretion system; T2SS), type IV pili (Tfp), flagella and a type III secretion system (T3SS) that can inject a set of eukaryote specific effectors across the plasma membrane of target cells[33].

Chronic infections are minimally invasive and noncytotoxic and they involve the formation of biofilms that protect bacteria against assault by the host immune system and provide resistance to antibiotics[34,35]. Thus, chronic infections rarely result in systemic spread, but instead lead to unrelenting non-productive host infammation that contributes to the resulting morbidity and mortality[35].

P. aeruginosa is seldom a member of the normal microbial flora in humans. Clinical studies report that colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples[36]. However, when patients are hospitalized, colonization rates may exceed 50%, especially among patients in which the cutaneous or mucosal barriers have been damaged by mechanical ventilation, tracheotomy, catheters, surgery, or severe burns[36]. Immunocompromised patients have higher risks of colonization by this organism, and patients that have received antimicrobial therapy were also shown to suffer from increased colonization by *P. aeruginosa* [36].

Healthcare statistics indicate that *P. aeruginosa* has become the second leading cause of nosocomial pneumonia, the third most common cause of urinary tract infections, the fourth most frequently isolated pathogen in infections in surgical sites, and the seventh pathogen responsible for bloodstream infections and the leading cause of pneumonia among pediatric patients in the intensive care unit (ICU)[36].

P. aeruginosa presents a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections, making appropriate antibiotics are essential

for the restablishment of infected patients[36]. Unfortunately, selection of appropriate antibiotics is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection. Epidemiological outcome studies have shown that infections caused by drug-resistant *P. aeruginosa* are associated with significant increases in morbidity, mortality, need for surgical intervention, length of hospital stay and chronic care, and overall cost of infection treatment[36]. Healthcare statistics from ICUs around the world reveals that, in the periods between 2003-2007, the rates of antibiotic resistance among *P. aeruginosa* isolates show that : fluoroquinolone resistance is around 30% in US while more than 50% in the rest of the world ; piperacillin and piperacilline-tazobactam resistance concerns almost 20% in US while 50% in the world ; amikacin resistance is found in less than 10% in US but in 30% around the globe ; imipenem and meropenem resistance is seen in 25% in US isolates while almost 40% in world ones and finally cefepime resistance touches 10% of US isolates and shows an alarming 70% in isolates from the rest of the planet[36–38] . The virulence factors that enable *P. aeruginosa* to infect its host will be presented later in this chapter.

2.2. *P. aeruginosa* creates persistent infections in CF patients

Another clinical setting where *P. aeruginosa* has become an important health concern is Cystic Fibrosis (CF) in which the organism infects and colonizes patiens from the early childhood[39,40]. CF is a recessive inherited disorder caused by the presence of mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) protein, with an incidence of 1 in 2500 live births. CFTR functions as an ion channel that transports chloride ions across epithelial cell surfaces[41–43].

In CF airways, CFTR dysfunction modifies the composition and the properties of the airway surface liquid (ASL). ASL is a mucus layer which protects airway surfaces against irritants and infecting micro-organisms. The ASL becomes thin and viscous making the gel-forming mucins dry and compacted on top of the epithelial layers. The dehydration of mucin blocks the clearance of bacteria allowing them to colonize and grow[44,45] This default generates chronic inflammation generating a toxic pro-inflammatory local microenvironment, which damages the lung and the innate immunity, further facilitating infections. It is under this hostile context that *P. aeruginosa* must overcome challenges such

as osmotic stress, an acidified ASL, competition from other colonizers, nutritional inadequacy, antibiotics, acid and oxidative stresses, etc,in order to survive[45,46].

Figure 3, shows the initial process of colonization of the airways in CF patients and the establishment of early chronic lung infection. Briefly, patient's airways, both lungs and sinuses, are colonized by environmental *P. aeruginosa*. The sinus environment provides a protected niche were the bacteria can survive against waves of antibiotic treatment and immune response compared to the lung airways where they can be completely eradicated. The same survivors in the sinus are capable of intermittent colonization of the lung which can at first be controlled by antibiotic treatments. However, the cycles of intermittent colonization and eradication allows the progressive acquisition of mutations that increases the fitness and adaptability of bacteria. The genetically adapted strains can then migrate to the lower airways and generate chronic infections that are very difficult to eradicate.

The final outcome of the chronic infection is a constant but slow damage of the lung tissue until breathing is no longer possible. Under these dreadful conditions, a lung transplant is often the only long term treatment for these patients explaining why their lifetime is limited to around 40 years[47,48].

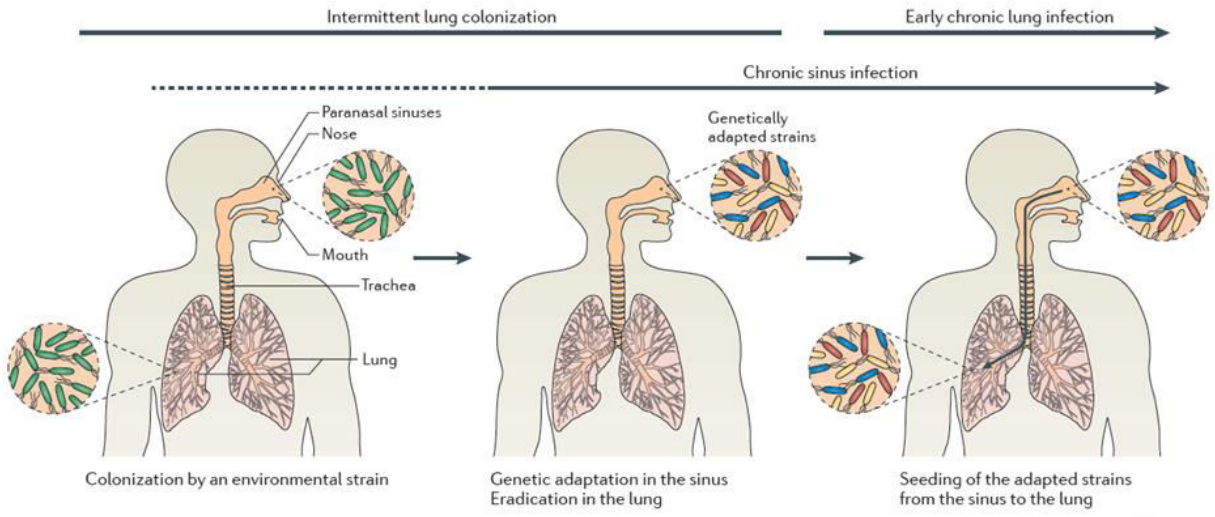


Figure 3. Colonization process of *P. aeruginosa* in CF lung. Environmentally acquired *P. aeruginosa* start invading airways of CF patients creating acute infections. Sinuses become the niche were the bacteria can survive antibiotic treatment and immune system. Bacteria from the sinuses can migrate and reinfect the lungs. The cycles of colonization-eradication allow the accumulation of mutations that gradually increase the fitness of the bacteria. Genetically adapted bacteria start to become more resilient and establish chronic infection in the patient[48].

One of the most important factors that enhance the survival of *P. aeruginosa* against the stress conditions in the CF lung is the sigma factor 22 also known as AlgU[47,48]. Activation of AlgU leads to a coordinated downregulation of central metabolism, motility and virulence, and a concurrent upregulation of genes affecting membrane permeability and efflux, and also genes that are involved in heat shock, osmotic and oxidative stress response. AlgU also is responsible for the production of alginate, which is a polysaccharide that is secreted to protect bacteria and that seems to be part of a general envelope stress response[47,48]. Overproduction of alginate has been linked to a phenomenon called mucoid conversion, which arises from mutations in the gene encoding the anti-sigma factor MucA that regulates the functioning of AlgU by sequestration. The consequence of this mutation is a constitutive activation of AlgU. However, it has been reported that the mucoid conversion is a reversible process because *mucA* seems to be a repetitive target for selective mutations during chronic pathogenesis[47,48].

3. Antibiotic Resistance

3.1. Intrinsic mechanisms of antibiotic resistance

Once a human host has developed a *P. aeruginosa* infection, the most important decision to save a patient's life is the administration of a proper antibiotic treatment. One of the biggest problems encountered by infected patients is that *P. aeruginosa* is a highly resistant organism against antibiotherapy. This is explained by the fact that *P. aeruginosa* possesses an arsenal of enzymes, efflux pumps and virulence mechanisms that allow the bacteria to resist and persist against treatments, and they are shown in **Figure 4**[7,36]**.** The expulsion of antibiotics from the cytoplasm to the outside of the cell is performed by using sophisticated efflux pumps that belong to 5 different families: Resistance-nodule-division (RND), major superfacilitator superfamily (MFS), small multi-drug resistance (SMR), ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE)[7,36]. Four families use the energy from *pmf* to expel antibiotics while one family, the ABC uses ATP, to pump and keep antibiotics far from their cellular targets. **Table 2** presents the best characterized families in *P. aeruginosa* and shows that the RND family is capable of pumping most of the antibiotics used by healthcare systems.

Other resistance mechanisms, depend on the modification of LPS mediated by the products of the *arnBCADTEF-ugd* and *pmrCAB* operons. The original lipid A is modified by the addition of 4-amino-4-deoxy-L-arabinose and phosphoethanolamine. This « alternative » LPS is responsible for generating resistance against colistin and polymyxin B[49–51].

Genes coding for efflux pumps	Expelled antibiotic	Reference
RND		
mexAB-oprM	Fluoroquinolones, β-lactams, β-lactamase inhibitors, tetracyclines, chloramphenicol macrolides, novobiocin, trimethoprim, sulfonamides	Lister et al., 2009
mexCD-oprJ	Fluoroquinolones, β-lactams, tetracyclines, chloramphenicol macrolides, novobiocin, trimethoprim, sulfonamides	
mexEF-oprN	Fluoroquinolones,chloramphenicol, trimethoprim	
mexXY	Fluoroquinolones, β-lactams, tetracycline, aminoglycosides, macrolides, chloramphenicol	
mexJK	Tetracycline, erythromycin	
mexGHI-opmD	Fluoroquinolones	
muxABC-OmpB	Fluoroquinolones, tetracycline, chloramphenicol, erythromycin	
mexPQ-opmE	Fluoroquinolones, tetracycline, chloramphenicol, macrolides	
mexMN	Chloramphenicol, thiamphenicol	
triABC	Triclosan	
muxABC-OmpB	Macrolides	Beceiro et al., 2013
MATE		
pmpM	Fluoroquinolones, benzalkonium chloride, ethidium bromide, acriflavine and tetraphenylphosphonium	He et al., 2004
ABC		
PA4456	Tetracycline	Chen et al., 2015

Table 2. Efflux pumps that have been characterized in *P. aeruginosa* with their corresponding expelled antibiotic and bibliographic reference[36,52–54].

Enzymatic mechanisms are also present, as it is the case for AmpC that possesses an extended spectrum of activities against β-lactams. This enzyme has also been detected inside outer membrane vesicules (OMVs) in biofilms. OMVs are liposome-like containers that act as molecular decoys to trap and neutralize all kinds of environmental aggressions such as antibiotics[55].

The strong selective preasure generated by the antibiotherapy of the infected patient favours the accumulation of mutations, ending up in the amplification of resistant phenotypes. Mutations on the DNA gyrase (*gyrA* and *gyrB*)[56] and the topoisomerase IV (*parC* and *parE*)[57] have been described to confer resistance to fluoroquinolones by modifying the binding site of the antibiotic.

Another important antibiotic resistance mechanism involves the porin OprD. OprD is a specialized porin which has a specific role in the uptake of positively charged amino acids such as lysine. Decrease in the expression of *oprD* and/or mutations that disrupt the translational production of a functional porin for the outer membrane are associated to a heightened antibiotic resistance against imipenems but not carbapenems[58]. Overexpression of efflux pumps by accumulation of mutations in the regulatory network controlling their expression is also a mechanism that generates multidrug-resistant phenotypes in *P. aeruginosa* strains[36].

3.2. Acquired mechanisms of antibiotic resistance

P. aeruginosa is also capable of acquiring resistance genes carried on mobile genetic elements that are obtained from other microorganisms in the environment. Acquired drug-resistance mechanisms include the resistance towards β-lactam antibiotics by the acquisition of genes coding for β-lactamases of the following types: class A serine β-lactamases; class A extended-spectrum β-lactamases; class A carbapenemases; class B metallo- β-lactamases, class D OXA-type enzymes. The resistance towards amiglycosides arises from the acquisition of enzymes modifying aminoglycosides such as phosphorylases, acetyltransferases and nucleotidyl transferases. The other mechanism relies on enzymes encoded by the genes *rmtA, rmtB, rmtC, rmtD* and *armA,* that modify the target 16S rRNA[36].

All these mechanisms explain the impressive ability of *P. aeruginosa* to develop antibiotic resistance. For this reason, treatments combining a variety of antibiotics are the basis for successful treatment against *P. aeruginosa*. Nevertheless, this is not always enough to avoid the appearance of drug resistant phenotypes. The great quantity of antibiotic resistance mechanisms and the genetic flexibility of *P. aeruginosa* demonstrate that this bacterium is a serious health concern and that it's possible that no effective antibiotic treatment will be available if the discovery of new antibiotic molecules and the understanding of resistance mechanisms don't keep up with the evolution of the microorganism.

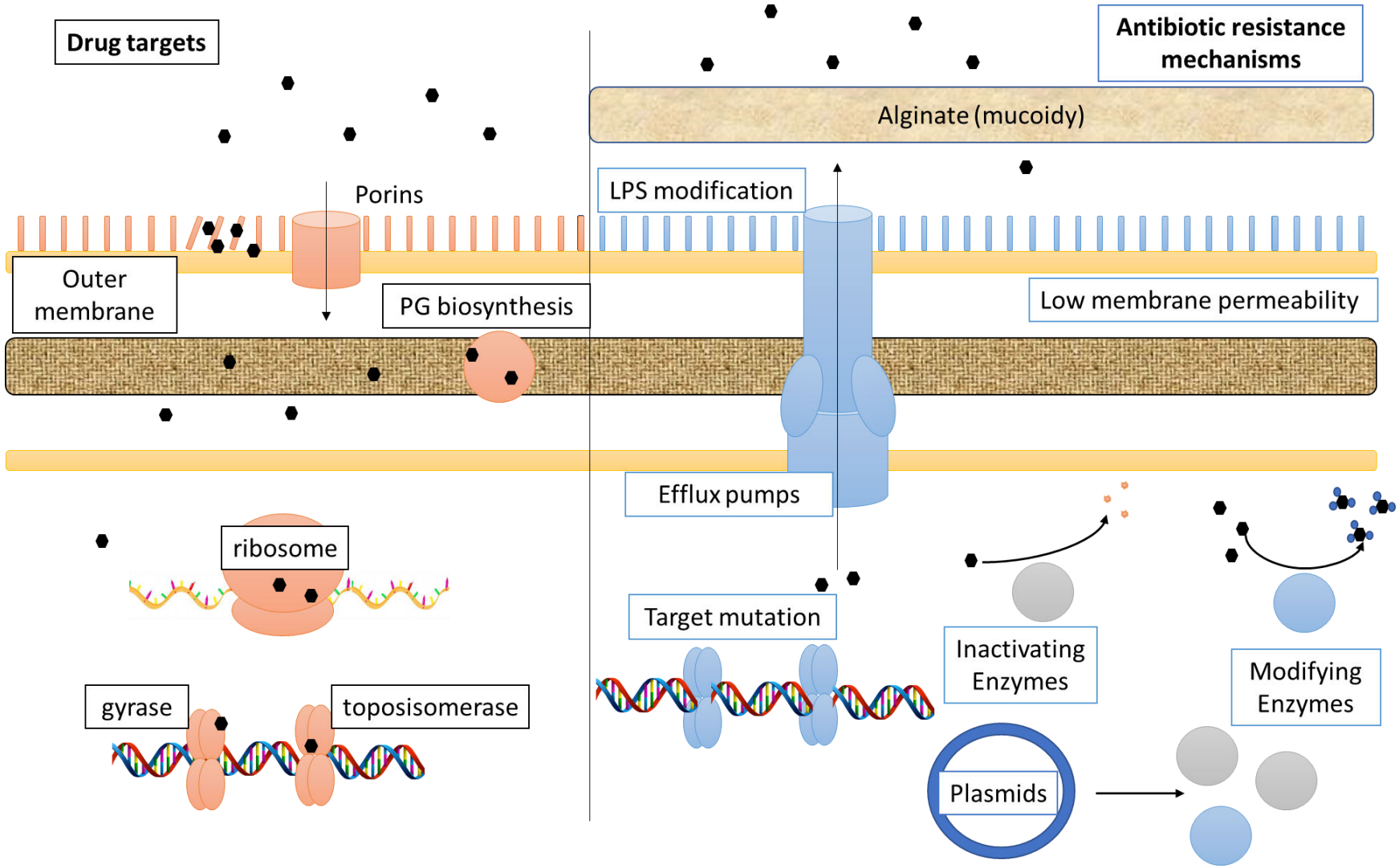

Drug targets
Antibiotic resistance mechanisms
Alginate (mucoidy)
Porins
LPS modification
Outer membrane
PG biosynthesis
Low membrane permeability
Efflux pumps
ribosome
Target mutation
Inactivating Enzymes
Modifying Enzymes
gyrase
toposisomerase
Plasmids

Figure 4. Drug targets identified in *P. aeruginosa* and the resistance mechanisms against antibiotics. Left: colistin and polymyxin B are responsible for the disorganization of LPS and the perforation of the outer membrane, β-lactams block peptidoglycan biosynthesis, aminoglycosides and tetracyclins and chloramphenicols interfere with the functioning of the ribosome, blocking protein synthesis, fluoroquinolones hinder the functioning of topoisomerases needed for DNA replication. **Right:** *P. aeruginosa* modifies the composition of LPS to prevent the binding of colistin and polymyxin B, mutations in topoisomerases impede the binding of fluoroquinolones to their targets, chromosomaly and plasmid encoded enzymes inactivate or modify antibotics, *pmf* and ATP-using efflux pumps drive out antibiotics from the cytoplasms, inhibition of porins (OprD) prevents the entrance of antibiotics, finally alginate production serves as a physical barrier for antibiotic diffusion.

3.3. Antibiotic persistence and the stringent response in *P. aeruginosa*

Persisters are one of the main reasons for recurrent and chronic infections of *P. aeruginosa*. They are known to withstand antibiotic treatments and spawn a new infecting population upon removal of antibiotic treatment. It has been described that persisters are abundant in *P. aeruginosa* biofilms, which is the hallmark of longterm infections particularly in CF[59,60].

Persisters are defined as subpopulations of cells occurring at very low frequency, which stochastically emerge in the presence of stress[61,62]. They show very slow growth and they are capable of surviving in stressful conditions where the viability of the majority of the population is severely impaired. Upon stress removal, persisters turn back to normal growth to propagate while regaining normal sensitivity to stress. Such persistence was suggested to be based on the heterogeneity of population by means of epigenetic mechanisms, not genetic mutations[63].

Even though, the molecular mechanisms determining the process of persister formation are not completely understood, various studies have provided evidences showing the link between stringent response and persistence.

Stringent response happens when bacteria like *E. coli* or *P. aerugionsa* encounter amino acid deprivation. This condition activates RelA and SpoT, which start synthezing ppGpp molecules. In association with the transcriptional regulator DksA (global regulator of metabolism), ppGpp interacts with RNA polymerase and inhibits the transcription of ribosomal RNA promoters. This inhibitory impact is concomitant with activation and upregulation of pathways for amino acid biosynthesis and the transcription of stress response genes[64–67]. Amato et al. (2013) found that stringent responses are linked to the

emergence of persisters by involving ppGpp based regulatory events[67]. **Figure 5** shows the current knowledge linking the stringent response and persistence in *P. aeruginosa*.

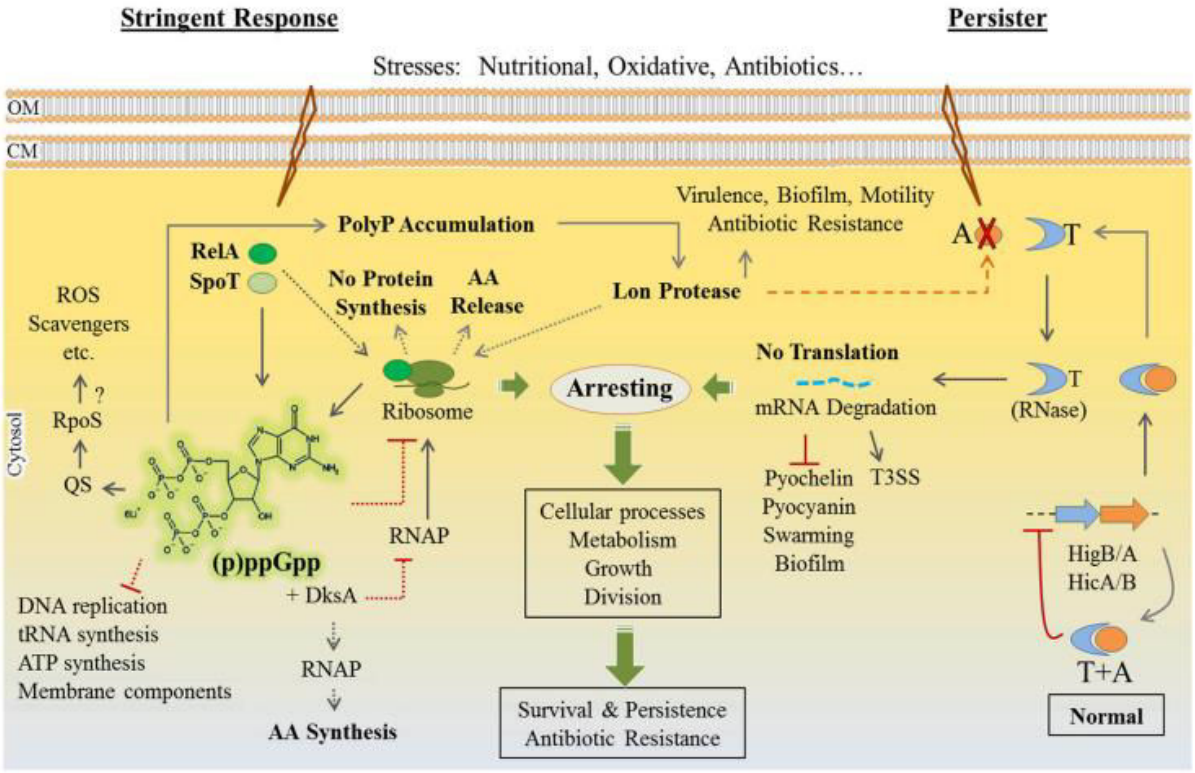


Figure 5. *P. aeruginosa* stringent response and persister formation. Stringent response is triggered by particular stresses such as amino acid and fatty acid starvation, iron/phosphate depletion and oxidative stress. The (p)ppGpp alarmone is a key determinant for stringent response and it is elevated by RelA/SpoT enzymes. Generally, (p)ppGpp elevation, PolyP (inorganic polyphosphate) and Lon protease complex interfere with normal biological processes in favour of bacterial survival *via* arrest of (?) metabolism, cell growth and cell division (dashed gray pathways are best understood for the *E. coli* model, but not or partially characterized in *P. aeruginosa).* In *E. coli*, (p)ppGpp signaling is linked to toxin (T)-antitoxin (A) system *via* activation of the Lon protease leading to the formation of persisters displaying dormant and antibiotic resistance phenotypes (dashed orange line). Generally, the TA complex is stable under normal conditions suppressing toxin activity and further expression of cognate genes. Upon antitoxin degradation, toxin becomes active to hinder biological processes. In the case of *P. aeruginosa* HigB/A and HicA/B, the toxin components, HigB or HicA,perform endoribonuclease (RNase) activity on mRNA molecules. In *P. aeruginosa*, the (p)ppGpp alarmone is linked to the production of ROS scavengers probably via QS or RpoS regulators and Lon activity is required for biofilm formation, motility, virulence and antibiotic resistance. Furthermore, the TA system(HigB/A) downregulates biofilm formation and virulence factor production while T3SS (type 3 secretion system) can be found upregulated. Although, the (p)ppGpp signaling, Lon protease activity and TA modules (i.e., HigB/A, HicA/B, and likely more complexes) are present in *P. aeruginosa*, their link to resistance to antibiotics and other stresses is poorly understood. AA, amino acids; QS, quorum sensing; RNAP, RNA polymerase. CM, cytoplasmic membrane; OM, outer membrane[68].

The persister state is typically based on the activity of genetically encoded toxin-antitoxin (TA) modules particularly in response to antibiotics. However, it is also proposed to be activated by RelA or SpoT and (p)ppGpp as it is the case *E. coli*[69].

Basically, the toxin element is a stable protein while the antitoxin appears as a protein or as a small RNA. Two mechanisms are described for the effect of small RNA antitoxins: i) they can inhibit the toxin translation by pairing the toxin mRNA, ii) they can inactivate the toxin by direct binding. When antitoxins are proteins they inhibit the activity of the toxin by protein-protein interactions.[70,71]. Under stressful conditions, the antitoxin releases the toxin which starts interfering with key cellular processes such as DNA replication, tRNA synthesis, membrane components and ATP generation. The inhibition of cellular proliferation leads to the formation of a dormant or persister cell.

Among the TA systems that are known in *P. aeruginosa* we find HigB/HigA and HicA/HicB[72,73]. Both systems present toxins having RNAse activity, that have a role in the regulation of virulence factors, biofilm formation and in the formation of persisters against ciprofloxacin[74].

Overall, the mechanisms of persister formation are a new emerging field that will be extremely important to understand and overcome the chronic infections of *P. aeruginosa*.

4. The Lifestyle of *P. aeruginosa*

The two different types of infection phenotypes observed in human disease, namely acute and chronic, have been associated to two different lifestyles of the bacterium: the planktonic and the biofilm modes of growth.

These two different lifestyles are coordinated by a complex network of regulatory factors that determine phenotypical traits that are responsible for the adaptation of the bacteria to their environment but also its pathogenicity and aggressiveness.

4.1. The planktonic lifestyle

In the planktonic lifestyle, individual or small groups of free living *P. aeruginosa* have the capacity to evade or defend against protozoan and metazoan predators, including higher metazoans such as the human host.[75]. To do this, *P. aeruginosa* relies in the expression of virulence factors that are shown in **figure 6**. Among them, the Type 3 Secretion System (T3SS) is a molecular machine capable of injecting toxins (ExoS/T: ADP ribosyltransferase and GTPase, ExoY: promiscuous nucleotidyl cyclase, ExoU: phospholipase) directly into eukaryotic cells; it is one of the most powerful weapons from *P. aeruginosa* which is crucial for acute infection[33,76,77]. The T3SS and its toxins allow *P. aeruginosa* to resist and defend against phagocytosis and predation in its environment. Another important mechanism to avoid predation and facilitate dispersal is motility. *P. aeruginosa* possesses a monotrichous flagellum necessary for swimming and a type IV pili that is used to twitch on solid surfaces[33]. Moreover, when *P. aeruginosa* reaches high numbers, other virulence factors such as Type 2 secretion system (T2SS) and its associated proteases are produced, and the bacterium synthesizes and secretes molecules such as surfactants and pigments[35]. The combination of these virulence factors associated to the planktonic lifestyle allow *P. aeruginosa* to generate acute infections that are invasive and cytotoxic and frequently results in substantial tissue damage, sepsis and mortality. However, it is important to highlight that virulence factors associated to the planktonic lifestyle have been detected during the sessile lifestyle, suggesting that both environmental cues and lifestyle are determinant for the expression of virulence factors[35].

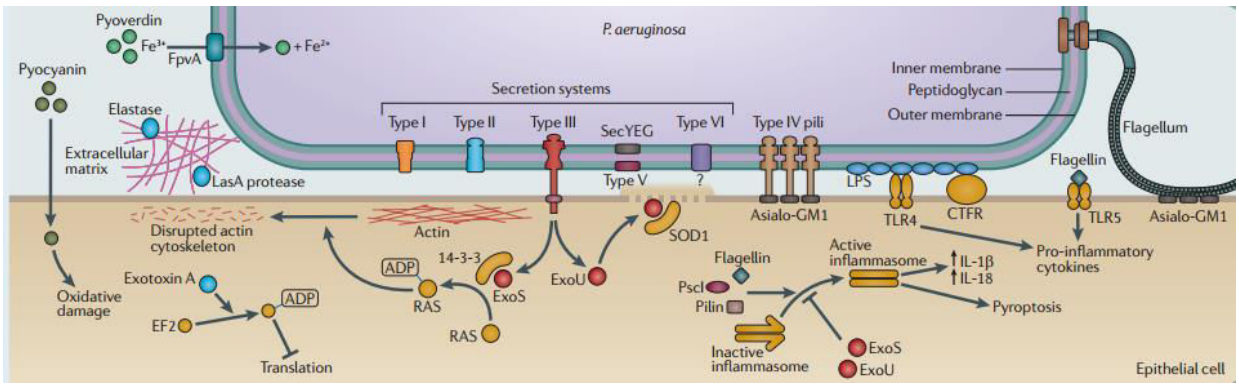


Figure 6. Virulence factors used by *P. aeruginosa* to infect epithelial cells during acute infection. Factors such as adhesins and secreted toxins, proteases, effector proteins and pigments that facilitate adhesion, modulate or disrupt host cell pathways and target the extracellular matrix.[76].

For decades, bacteriologists have studied the planktonic lifestyle in laboratory settings under controlled growth conditions. Even though laboratory cultures may seem far from the real environmental conditions, where *P. aeruginosa* switches between both planktonic and biofilm lifestyles, they have provided the framework to study gene regulation, metabolism, and virulence factors of this lifestyle[35].

4.1.1. The planktonic growth under laboratory conditions

When growing in laboratory conditions, *P. aeruginosa* presents 5 different phases that exhibit different physiological, behavioural and morphological properties in both minimal and rich media. In **figure 7,** a common growth curve is represented with its five phases.

4.1.1.1. The lag phase

It is defined by two subphases, the first in which bacteria begin to adapt to their environment by starting the expression of the different enzymes and complexes needed for growth, while the second subphase is characterized by an increase in the size of the bacteria, synthesis of different macromolecules needed for life and replication of DNA[78]. We observe higher activity of the sigma factor σ^{70} associated with the RNA polymerase coordinating the expression of housekeeping genes needed for cell growth. During this phase, the bacteria induce acquisition of metals and phosphate, synthesis of iron-sulfur clusters, protein repair systems, oxidative stress reponse enzymes and aerobic respiration[79].

4.1.1.2. The exponential phase

It is the period where bacteria are dividing intensively in the growth medium at a constant rate. The maximum growth rate during this phase follows Monod's law and depends on the amount of nutrients available in the medium[80]. As observed in the lag phase, expression of essential genes depends on the sigma factor σ^{70}. The physiology of the exponential phase involves multiple rounds of DNA synthesis, coupled with transcription and translation of essential macromolecules needed for growth. Moreover, metal acquisition and synthesis of iron-sulfur clusters are not as intense as during the lag phase, while protein repair systems and enzymes involved in respiration are upregulated[79,81]. Under laboratory conditions, the

main virulence factor of acute infection (T3SS) is not expressed but can nevertheless be induced by creating conditions reflecting calcium depletion[82,83]. The end of the exponential phase begins when nutrients become scarce and toxic metabolites accumulate too much, growth starts to decelerate and bacteria enter the stationary phase[78]. Through out growth, bacteria produce Quorum sensing (QS) molecules that allow the bacteria to sense their population density. The QS systems let bacteria to engage in complex social behaviour and coordinate processes such as biofilm formation, swarming motility but also the synthesis of virulence factors[84]. Depending on the initial concentration of the inoculum, the maximum growth rate and the duration of the exponential phase vary[80]. Under standard laboratory conditions in rich medium, *P. aeruginosa* is capable of doubling its population in more or less 30 minutes[2].

4.1.1.3. The stationary phase

When nutrients are not sufficiento to sustain growth, bacteria stop dividing and enter this phase. The transition to the stationary phase is governed by the sigma factor RpoS or σ^{38}, which controls the expression of more than 10% of the total genome. RpoS is also involved in the regulation of QS systems and the virulence factors associated with them[85]. It also controls the expression of enzymes that allow *P. aeruginosa* to be more resistant to hydrogen peroxide, high temperature, hyperosmolarity, low pH[86,87,85]. The lack of nutrients during this phase also elicits the stringent response which participates in the expression of virulence factors such as Pyocyanin, elastases, proteases and the T2SS, siderophores, twitching and swarming motility[88]. Moreover, stringent response makes *P. aeruginosa* more resistant to oxidative stress, osmotic stress, and heat shock.[88] Bacteria during the stationary phase present a sub-population of persister cells that are tolerant to antibiotiocs[89,66]. Depending on the composition of the medium (rich or minimum), the stationary phase can last for some hours and even for a few days.

4.1.1.4. The death phase

It occurs when nutrients are insufficient to maintain the number of cells during stationary phase; bacteria start to die very rapidly with a speed comparable to the exponential phase. The mechanisms that regulate cell death are unknown but in literature two explanations

arise: stochastic cell death or programmed cell death[90]. The dead cells provide to the surviving bacteria enough nutrients to survive for several months.

4.1.1.5. The late stationary phase

It is a highly dynamic phase were bacteria populations are at an equilibrium between dying and diving bacteria. An important accumulation of mutations during the stationary phase generates the Growth Advantage in Stationary Phase (GASP) phenotype. Arising mutant subpopulations exhibiting the GASP phenotype outcompete each other and create the dynamic process where bacterial death and division rates are balanced during months before cells stop growing and become nonviable[90–92].

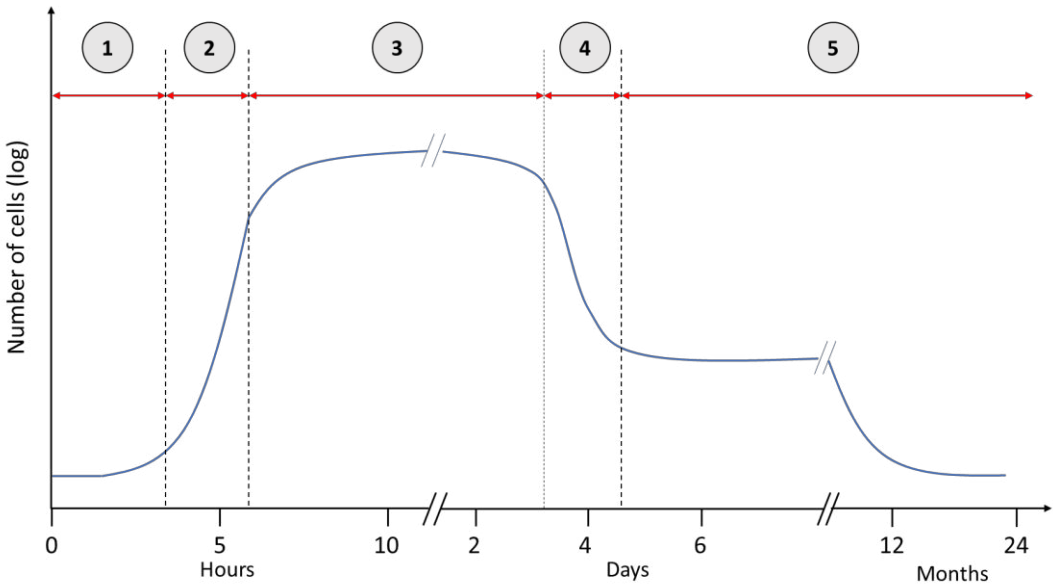


Figure 7. The five phases of the bacterial growth in planktonic lifestyle. Once bacteria are inoculated in fresh medium, there is a lag period (phase 1) followed by exponential growth (phase 2). After remaining at high density for a few days during the stationary phase (phase 3), bacteria enter death phase (phase 4). After the death of most cells, the survivors can be maintained under long-term stationary phase conditions (phase 5).

The transition between the planktonic phase and the biofilm is not a clear-cut process and it involves a substantial overlap and crosstalk between environmental detection systems. As shown in **figure 8**, there seems to be a phenotypical continuum in which the bacteria in planktonic phase shift progressively to the biofilm phase, and when the biofilm becomes mature bacteria reenter the planktonic lifestyle to colonize new environments. It has been suggested that the regulatory determinants for these two different lifestyles also participate in defining whether *P. aeruginosa* will generate an acute or chronic infection. Besides the

response to environmental cues, other processes such as mutant selection participate in the establishment of the lifestyle and pathogenicity[35].

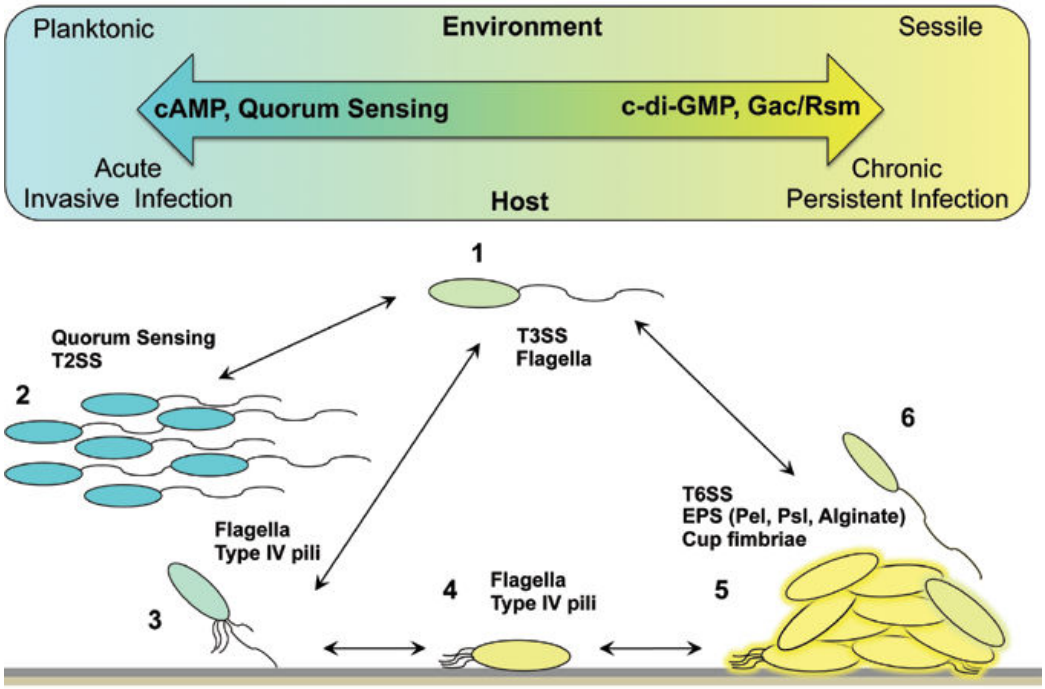


Figure 8. *P. aeruginosa* exhibits parallel lifestyle extremes in the environment and human host. Four global regulatory pathways (cAMP, Quorum Sensing, c-di-GMP and Gac/Rsm) create a phenotypic continuum that controls the transition between a planktonic and sessile lifestyle within the environment; an analogue process occurs in parallel during human infection in which the same pathways control the shift between acute and chronic infection. 1. In the environment *P. aeruginosa* can exist as a free living motile organism, 2. When population increases cells associate as a quorum, producing QS molecules and exhibiting social behaviours (toxin secretion), that promote nutrient acquisition and group survival. 3. *P. aeruginosa* attaches to solid surfaces via Type IV pili or flagellum. 4. Bacteria may exhibit surface motility to move towards nutrients. Microcolony formation starts upon solid surface attachment. 5. *P. aeruginosa* becomes sessile and starts to form a biofilm matrix that protects the bacteria from environmental fluctuations and provides a physical barrier against predators. 6. Unknown environmental signals or stochastic processes cause members of the sessile community to become motile, leave the biofilm, and return to a planktonic lifestyle[35].

4.2. Biofilm

In nature, most of the *P. aeruginosa* biomass is believed to exist in a structured and dynamic microbial community associated to a surface, also known as biofilm. In clinical terms, these biofilms have often been associated with chronic infections that are minimally invasive and noncytotoxic. The biofilm protects the bacteria against the assault of the host immune system while providing resistance to antibiotics, starvation, low pH and oxidative stress[93–95,36,96]. Moreover, they lead to unrelenting non-productive host inflammation that contributes to the resulting morbidity and mortality[97].

As shown in **figures 8 and 9**, the process of biofilm formation begins by the reversible adhesion of planktonic bacteria onto a surface suitable for growth, followed by irreversible attachment of bacteria, which thereafter form microcolonies in exopolysaccharide (EPS) matrix. Progressively, bacterial microcolonies expand and their confluences lead to a more structured phenotype with noncolonized space. Then, noncolonized spaces are filled with bacteria, which finally cover the entire surface. Meanwhile, the growth of three-dimensional communities is observed. The suprastructure of the biofilm induces changes in local environmental conditions such as nutrient starvation and low oxygen tension, that end up in the physiological and metabolical differenciation of cell populations within the biofilm. The cell-to-cell communication mechanisms involving *las*, *rhl*, PQS systems have been shown to be essential for creation of mature, differentiated biofilms in *P. aeruginosa* by regulating the production of different components of the biofilm matrix. Sigma factors such as RpoS are also needed for the expression of operons involved in polysaccharide synthesis[98]. Finally, once the biofilm becomes mature, an unknown environmental signal induces the bacteria to disperse from the sessile structure and re-enter the planktonic state to spread and colonize other surfaces.

4.2.1. The molecular components of the biofilm matrix

The biofilm architecture depends on the composition of the biofilm matrix, which consists of:

4.2.1.1. Polysaccharides

In the biofilm matrix we find aggregative polysaccharides such as Pel (pellicule, glucose rich polysaccharide[99]),Psl (Polysaccharide Synthesis Locus, pentasaccharide-repeating unit of D-mannose, D-glucose and L-rhamnose) and the protective polysaccharide alginate (1-4)-linked β-D-mannuronate and its C-5 epimer α-L-guluronate. Psl is the primary polysaccharide component of the biofilm matrix, it is used for cell-to-surface and cell-to-cell attachment[100]. Pel appears to have a redundant role with Psl and has also been shown to function in cell-to-cell attachment in biofilms[100]. Alginate plays important roles in structural stability, it also protects *P. aeruginosa* from dessication, hydrophobic agents but also against the consequences of inflammatory process. Indeed, the polysaccharide inhibits complement activation, decreasing phagocytosis by neutrophils and macrophage while scavenging free radicals from oxidative bursts[101,102]. Overall exopolysaccharides have been reported to provide protection from a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock, desiccation and oxidative stress[103–105].

4.2.1.2. Extracellular DNA (eDNA)

It comes from the autolysis of bacteria in the biofilm in a poorly understood process involving the QS system, the flagella and the type IV pili. eDNA plays a role in structuring the stalk of mushroom-shaped microcolonies.[106] By an unknown mechanism, eDNA induces genes involved in the modification of LPS, promoting antibiotic resistance but also is capable of trapping toxic charged molecules such as antibiotics[107]. Furthermore, it is also used as a source of energy during starvation.[106,108,107].

4.2.1.3. Matrix Proteins

The matrix proteins such as Fab (Functional Amyloid) in *Pseudomonas* forms amyloid fibers that promote biofilm robustness. Functional bacterial amyloid increases *P. aeruginosa* biofilm hydrophobicity and stiffness; while CupA, CupB and CupC fimbriae, lectins A and B, adhesin CdrA, mediate bacterial attachment during initial biofilm formation [109,110].

4.2.1.4. Surfactants

Rhamnolipids are amphipathic glycolipids that are important for the maturation of biofilms. They promote the formation of channels needed for the access of nutrients and oxygen

inside the biofilm. They are also involved in the detachment of cells from mushroom-like micro-colonies that will start the colonization of new environments[111].

4.2.1.5. Outer membrane vesicles (OMV)

OMVs interact with the eDNA helping the structuring of biofilm. OMVs can harbor different kinds of molecules involved in defense and virulence mechanisms but also for cell-to-cell communications as QS molecules, antibiotic and peptidoglycan degrading enzymes, hemolytic phospholipase C, alkaline phosphatase, pro-elastase, hemolysin, plasmids and genomic DNA facilitating gene transfer and drug-resistance[112–114].

4.2.1.6. Polyamines

Polyamines such as putrescine, spermidine and norspermidine, have been shown to be essential for the formation of biofilms in bacteria such as *E. coli, Yersinia pestis, Bacillus subtillis and Neisseria gonorrhoeae*[115,116]. The molecular mechanisms behind their function are still unknown but it has been suggested that they could be needed for the stabilization of the biofilm matrix by acting as counter-ions for eDNA and other anions[117–121].

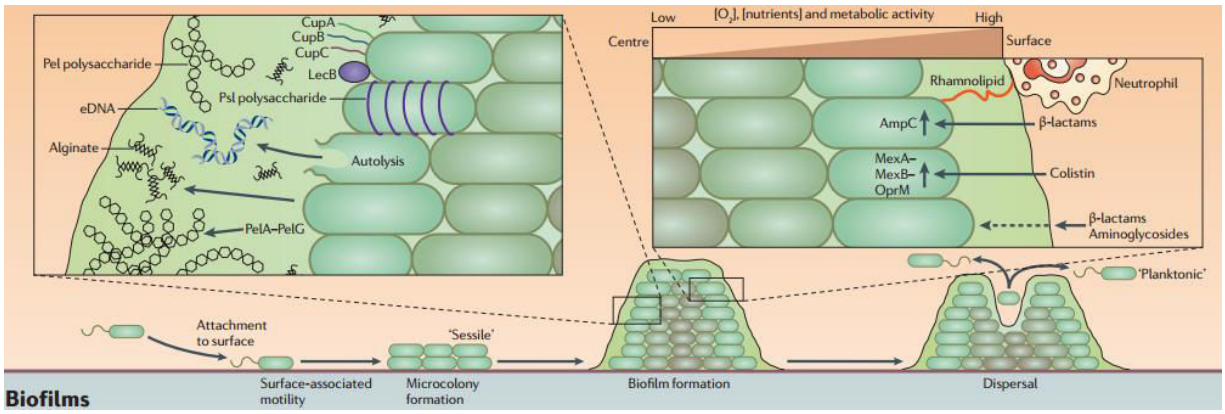


Figure 9. Schematics of the process of biofilm formation. Biofilm formation begins with the attachment of bacteria to a surface, then they associate into microcolonies by using twitching motility. Bacteria then start to synthesize the factors needed for the construction of the biofilm matrix. Biofilm architecture and components of biofilm matrix are illustrated in the left square. The maturation of the biofilm creates an oxygen and nutrient gradient that induces the formation of distinct bacterial subpopulations that vary in their susceptibility to antibiotics and stress resistance. Virulence factors, such as rhamnolipids, are secreted by bacteria allowing the formations of channels needed for the flow of nutrients in the matrix. They also participate in the necrosis of attacking neutrophils. The final step of biofilm maturation involves the release of planktonic bacteria that will start colonizing new environments. The steps of biofilm maturation shown here are based on *in vitro* studies in flow-cell chambers; the corresponding steps and biofilm structures that occur during *in vivo* infections are less clear[76].

4.3. The regulatory « switch » between planktonic and biofilm state

Four major signaling pathways are responsible for sensing and integrating the external stimuli that will determine the lifestyle that *P. aeruginosa* will adopt in response to the surrounding environmental conditions. They are : i) the GacS/Rsm, ii) cAMP/Vfr, iii) c-di-GMP and iv) Qs signaling pathways[35]. These interconnected signaling systems exert regulatory control at transcriptional, translational and posttranslational level that determines whether *P. aeruginosa* enters planktonic or sessile lifestyle, as illustrated in figure 8.

4.3.1. The Gac/Rsm signaling system

The main regulator of the switch between the planktonic and biofilm lifestyle is Gac/Rsm signaling cascade. The cascade involves the two-component regulatory system GacS/GacA which is in the spotlight of the regulation of biofilm formation, motility, antibiotic resistance and expression of virulence factors[46,98,122,123](**figure 10)**. After the detection of a still unknown signal, the histidine kinase GacS[124] autophosphorylates and the phosphate is transferred to its cognate response regulator GacA. Phosphorylated GacA is then able to bind to the promoters of *rsmY* and *rsmZ* and activate their transcription. RsmZ and RsmY are small regulatory RNAs that sequester the regulator RsmA, a protein that binds to different target RNAs[122,124].

RsmA is either capable of regulating expression negatively by blocking translation or positively by blocking RNA degradation by inhibiting endonuclease activity. These mechanisms allow RsmA to control directly and indirectly the expression of more than 500 genes. RsmA exerts an inverse control of factors involved in promoting motility and acute infection and those associated with biofilm formation and chronic infection[125].

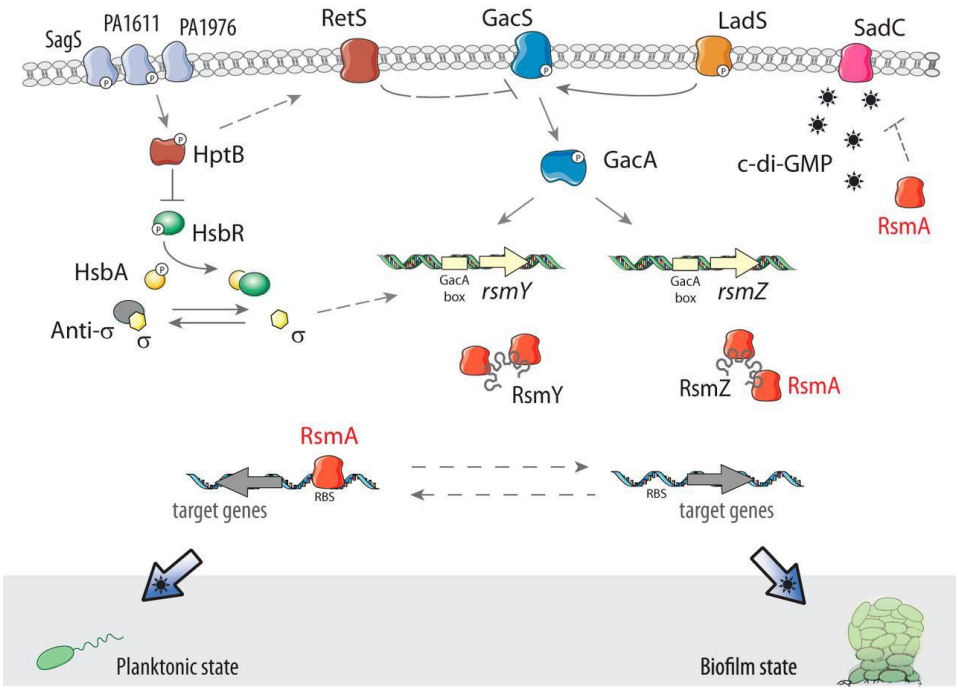


Figure 10. The Gac/Rsm cascade in *P. aeruginosa* is genetically linked to c-di-GMP through SadC. The GacS/GacA two-component system is promoting the expression of two small regulatory RNAs, RsmY and RsmZ, which sequester the post-transcriptional repressor RsmA. Titration of RsmA induces the production of sessile and biofilm determinants, whereas free RsmA leads to a planktonic and more virulent lifestyle. Several additional regulators modulate theGac/Rsm system, such as the two hybrid sensors RetS and LadS, as well as the histidine phosphotransfer protein HptB and other pathways. The elevated concentration of c-di-GMP in a hyperbiofilm-forming *retS* mutant was the first hint of the link between the Gac/Rsm and the c-di-GMP pathways. Later on, the molecular details of the link were elucidated: SadC, a DGC whose production is repressed by RsmA, is a central player for the Gac/Rsm regulation of biofilm formation. It appears therefore evident that the c-di-GMP signaling network and the Gsc/Rsm cascade are not independent to each other and that they are both instrumental for a proper development of the biofilm[126].

Several additional regulators modulate the activity of the Gac/Rsm system. Two hybrid sensors known as RetS and LadS are capable of interacting with GacS and modifing its function. RetS blocks the autophosphorylation of GacS and activates the expression of genes implicated in planktonic lifestyle and virulence factors important for acute infection such as T3SS, T2SS, lipase A, endotoxin A, Type IV pili[127]. The effect of RetS on GacS ends up in the inhibition of the expression of *psl* and *pel* operons necessary for biofilm formation and also the T6SS[46]. The sensor LadS is a calcium-responsive kinase that has the opposite role of RetS and favours the biofilm lifestyle[123,128].

The HptB/HsbR/HsbA is a signalling network that has also been shown to interact with Gac/Rsm system[129]. It involves HptB, a phosphorelay that activates HsbR, a response regulator, that is involved in the dephosphorylation of the anti-anti-sigma factor HsbA. HptB/HsbR/HsbA cascade has been linked to three additional hybrid sensors PA1611, PA1976, SagS, by the activation of HptB. The HptB/HsbR/HsbA system controls the expression of *rsmY* and *rmZ,* as it is the case for the Gac/Rsm cascade[126,129,130].

4.3.2. The c-di-GMP signaling pathway

The secondary messenger c-di-GMP appears to play a critical role in controlling chronic infection-related phenotypes and the biofilm lifestyle. The intracellular concentration of c-di-GMP is mediated by two different enzymes: diguanylate cyclases (DGC) that synthezise c-di-GMP from GTP and phosphodiesterases (PDE) that degrade c-di-GMP[131]. At least five diguanilate cyclases have been described to specifically control the transition from planktonic to surface-associated growth: WspR, SadC, RoeA, SiaD, and YfiN/TpbB. While, the diguanilate cyclases, GcbA and NicD, and the phosphodiesterases, DipA (Pch), RbdA, and NbdA, have been linked to biofilm dispersal[35,132]. The regulatory effects of c-di-GMP occur at multiple levels : i) allosteric regulation of enzyme activity or protein function; ii) regulation of gene expression by acting on transcription factors, iii) regulation of gene expression by interaction with riboswitches. Other signaling systems such as CdrA/B, FimX, RocA/R/S are needed to elicit the regulatory response of c-di-GMP concentration[133–135]. As a general rule, different studies have stablished that low levels of intracellular c-di-GMP are associated to planktonic mode of growth while high levels are associated to biofilm lifestyle.

4.3.3. The cAMP signaling pathway

The secondary messenger signaling molecule, cAMP, regulates multiple virulence factors involved in *P. aeruginosa* infection[136,137]. The concentration of cAMP is controled by the enzymatic activity of adenylate cyclases (AC) that synthetize cAMP, and phosphodiesterases (PDE) that degrade cAMP, in response to environmental cues. *P. aeruginosa* PAO1 genome encodes 3 ACs : CyaA, CyaB and the toxin ExoY ; and one PDE : CpdA[35,137,138]. CyaA is a cytosolic AC while CyaB is a membrane associated AC that has the highest enzymatic activity. The Chp chemosensory system modulates the enzymatic activity of CyaB, among other enzymes. However, the signals activating Chp are currently unknown[139]. Under laboratory

conditions we know that culture conditions of low calcium or high osmolarity influence cAMP levels. The main transcription factor associated to cAMP is Vfr[140,141]. cAMP/Vfr positively regulates the expression of genes associated with an acute virulence phenotype such as T2SS, T3SS and their toxins, Type IV pili. It activates the expression of *las* and *rhl* QS systems that also participate in the expression of more virulence factors[35]. Both *vfr* and *cpdA* are transcriptionally regulated by cAMP/Vfr creating a feedback loop to maintain cAMP homeostasis[35].

4.3.4. Quorum sensing signaling

QS is a signaling mechanism that bacteria use to regulate gene expression in a population density-dependent manner. This mechanism allows bacteria to coordinate their behaviour and influence their environment in ways they would not be able to adopt individually[142,143]. QS is based on production, release and detection of the concentration of hormone-like molecules (autoinducers) that will affect the expression of virulence determinants such as extracellular proteases, iron chelators, efflux pumps, motility and attachment, stress response, adaptative immunity (CRISPRs) and biofilm formation[144–151].

Today we known that production of the autoinducers not only depends on cell density but is also affected by many environmental factors such as host immune response and stress signaling, phosphate depletion, oxygen deprivation, starvation, lack of iron, etc[149,152–159].

The QS network of P. aeruginosa is organized in a multi-layered hierarchy consisting of at least four regulatory systems using three chemical families. **Figure 11** shows a simplified diagram that includes the most important factors involved in this regulatory network. Here we present a summary exposing the principal functions of each family of QS systems.

4.3.4.1. N-acyl homoserine lactones (AHL)

There are two canonical AHL QS signaling pathways in *P. aeruginosa*, the *las* and the *rhl* systems. Together they affect the expression of more than 10% of the genes found in the genome of *P. aeruginosa*[152]. The *lasI* (*PA1432*) and *rhlI* (*PA3476*) genes encode the N-3-oxododecanoylhomoserine lactone (3-oxo-C12-AHL) synthetase and N-butyrylhomoserine lactone (C4-AHL) synthetase, respectively[160,161]. The resulting AHLs then bind and activate

their cognate LuxR family regulators, LasR (PA1430) or RhlR (PA3477). LasR and RhlR multimerize in the presence of their cognate AHL which allows them to bind their target promoters[162,163]. Activated LasR/3-oxo-C12-AHL regulates the production of virulence factors including elastase, the LasA protease, alkaline protease, and exotoxin A[162,163]. Activated RhlR/C4-AHL induces the production of rhamnolipids, elastases LasA and LasB, hydrogen cyanide, pyocyanin, siderophores, and the cytotoxic lectins LecA and LecB[162–164]. Both systems participate in an intricate network that includes the other known QS systems (PQS and IQS). Another important system implicated in the regulation of *las rhl* regulon is the sigma factor RpoS which also regulates 800 genes involved in stationary phase[85].

4.3.4.2. 2-alkyl-4-quinolones (AHQs)

The *Pseudomonas* Quinolone Signal (PQS) system relies on two autoinducers: 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3,4-dihydroxyquinoline (PQS)[165]. HHQ is synthesized by the *pqsABCDE* operon and is converted into PQS by PqsH (FAD-dependent monoxygenase) in an oxygen-dependent reaction. Therefore, HHQ is the predominant AHQ autoinducer in anaerobic conditions and PQS the most important in aerobic conditions[166]. The secretion of AHQs involves the utilization of bio-surfactants such as rhamnolipids and OMVs because of their relatively insolubility in aqueous solutions[167]. One important chemical property of PQS is that it is capable of chelating iron. PQS acts as facilitator in iron acquision by *P. aeruginosa* siderophores pyoverdine and pyochelin[166,168]. The sequestering of iron by PQS has also been described as an important factor to outcompete other bacteria such as *Staphyloccus aureus*[169]. Both HHQ and PQS are co-inducers of PqsR (LysR family transcriptional regulator) and upregulate the expression of the *pqsABCDE* operon and virulence factors such as pyocyanin, elastase, LecA, rhamnolipids, twitching motility and biofilm formation[166]. QS is also needed for full virulence towards plants, nematodes and mice[18,170,171].

4.3.4.3. The IQS system

It constitutes the fourth and last QS system that has been identified in *P. aeruginosa* up-to-date. It integrates environmental stress cues in the QS network[149]. It relies on a new class of QS signal molecules, the 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde molecule, synthesized by the *ambBDCE* gene cluster. However, the regulator involved in the detection of IQS and the activation of IQS regulon is unknown. The system regulates the PQS and *rhl* systems, as

well as virulence factors such as pyocyanin, rhamnolipid and elastase. It is important under conditions of phosphate depletion and is capable of partially taking over the functions of the *las* system[84,172].

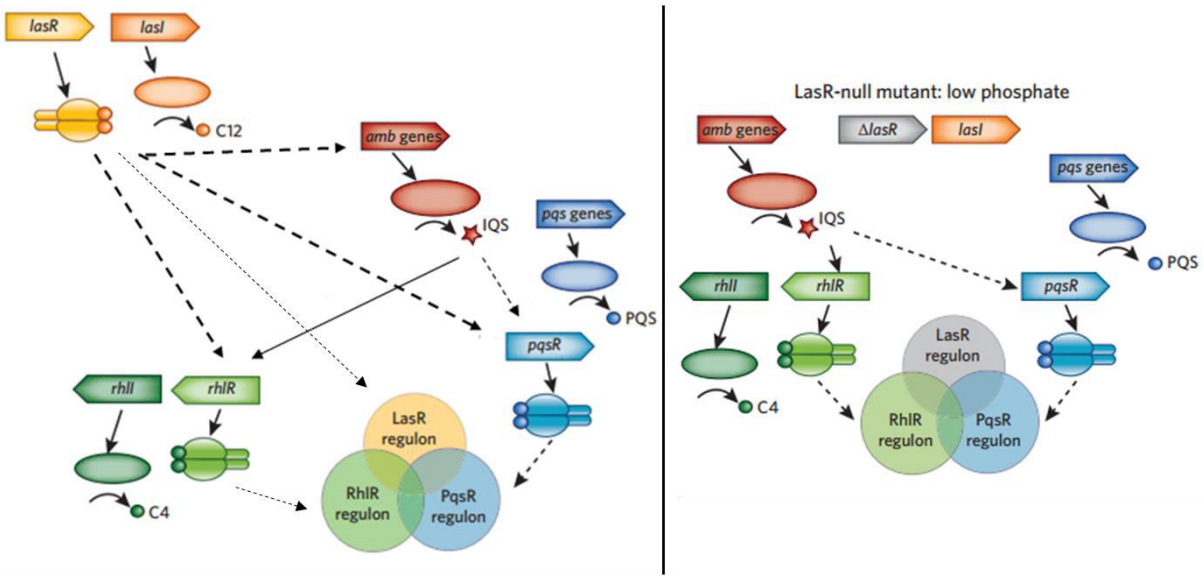


Figure 11. **Diagram of the QS circuitry in *P. aeruginosa***. Left panel, the integrated QS circuit consisting of overlapping genetic programs with three major transcription factors, LasR, RhlR and PqsR. LasR sits at the top of the hierarchical circuit, and when its signal, C12 (3-oxo-C12-AHL), reaches a concentration threshold, LasR activates transcription of a constellation of genes (the LasR regulon). The LasR regulon includes RhlR and PqsR, which, in response to their cognate signals C4 (C4-AHL) and PQS, activate regulons that overlap with the LasR regulon. LasR also activates the *ambBCDE* operon that synthesizes IQS and influences the expression of RhlR and PQS regulons. Thus LasR-null mutants (*ΔlasR*) do not activate genes in any of the four regulons. To right, when *P. aeruginosa* becomes LasR-null during CF chronic infections, the *ambBCDE* operon is activated by an alternate effector under phosphate-limited conditions, PhoB (not shown), and IQS is produced. IQS interacts with an unknown factor to activate RhlR and PqsR signaling. It circumvents LasR dependence. In this figure, functional systems are shown in colour, and inactive systems are shown in light gray. Solid lines indicate synthesis, and dashed lines represent positive regulation. C4, C4-AHL; C12, 3-oxo-C12-AHL; PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone[84,166,172].

5. The metabolism of *P. aeruginosa*

Scientist that study the virulence of *P. aeruginosa* usually focus on its weapons to describe its pathogenic potential. But the synthesis of these virulence factors requires a functional metabolism that furnishes the energy necessary for the lifestyle and virulence of the microorganism.

As we presented in the **section 1 of chapter I**, the genome of *P. aeruginosa* encodes a large number of transport systems and enzymes that allow the bacterium to assimilate many different kinds of molecules that can be used as substrates to produce energy explaining the extreme metabolic versatility of this bacterium[7,173,174]. When different kinds of carbon and nitrogen sources are encountered by the bacterium at the same time, their utilization is regulated in an efficient and ordered fashion through a mechanism called carbon catabolite repression. Preferentially chosen sources of carbon or nitrogen include short-chain fatty acids, amino acids and polyamines[174–178]. Sugars represent less preferred substrates for *P. aeruginosa* and are degraded via the Entner-Doudoroff pathway[12,179,180]. Growth on n-alkanes and (halogenated) aromatic compounds reflects the metabolic potential of *P. aeruginosa* to degrade complex xenobiotics[181,182].

5.1. The aerobic metabolism of *P. aeruginosa*

5.1.1. The electron transport chain in *P. aeruginosa*

The generation of energy in *P. aeruginosa* is based on a phenomenon known as respiration. During respiration, high energy organic molecules are degraded and release high energy electrons that are vehicled through redox reactions to an electron-transport chain composed of different kind of enzymes such as dehydrogenases and oxidases containing iron-sulfur clusters and iron or copper-containing heme groups[183]. The energetized enzymes in the electron-transport chain are capable of pumping protons from the cytoplasm to generate a powerful chemiosmotic gradient between both sides of the inner membrane. The high-energy hydrogen ion gradient is then used by the ATP synthase to channel protons back to the cytoplasm while using the potential energy to create ATP from the ADP and phosphate available in the cytoplasm (**Figure 12).**

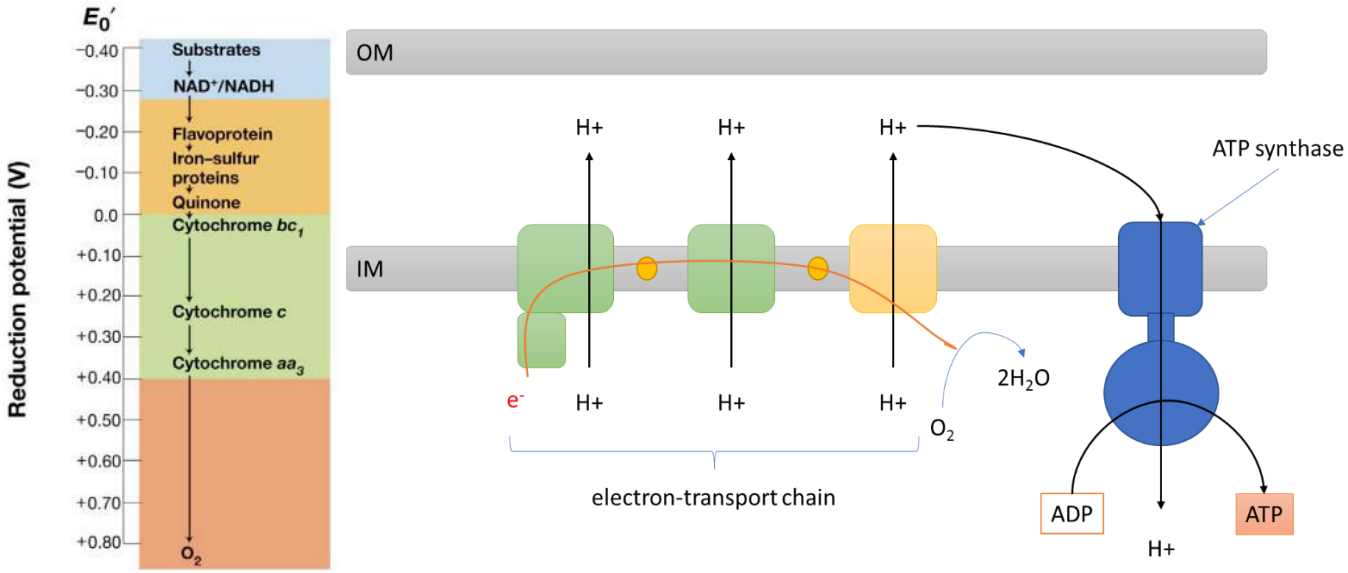


Figure 12. The electron chain of *P. aeruginosa*. Left: Reduction potential of the components of the electron transport chain of the mitochondria and bacterial cells. By breaking up the complete oxidation into a series of discrete steps, energy "recapture" is possible. **Right:** Diagram of oxidative phosphorylation process in *P. aeruginosa*. The production of ATP is coupled with the redox reactions of the electron-transport chain. Dehydrogenases are in green, ubiquinone is represented in golden circles, terminal oxidases are represented in yellow, and ATP synthase is in blue. Adapted from Molecular Biology of the Cell Biology, 4th Edition.

At least 17 respiratory dehydrogenases are predicted to be responsible for feeding electrons from respiratory substrates into the quinone pool, including three types of NADH dehydrogenases and a succinate dehydrogenase[147]. The activity of dehydrogenases is coupled to five terminal oxidases that catalyze the reduction of molecular oxygen to water but also participate in the creation of the *pmf* by pumping protons out of the cells[7,183–188]. Three of them are cytochrome *c* oxidases that receive electrons via the cytochrome bc_1 complex and *c*-type cytochromes. The other two are quinol oxidases that receive electrons directly from ubiquinone **(Figure 13).** These terminal oxidases are expected to have their specific affinity for oxygen, efficiency of proton-translocation, and resistance to various stresses such as cyanide and reactive nitrogen species.

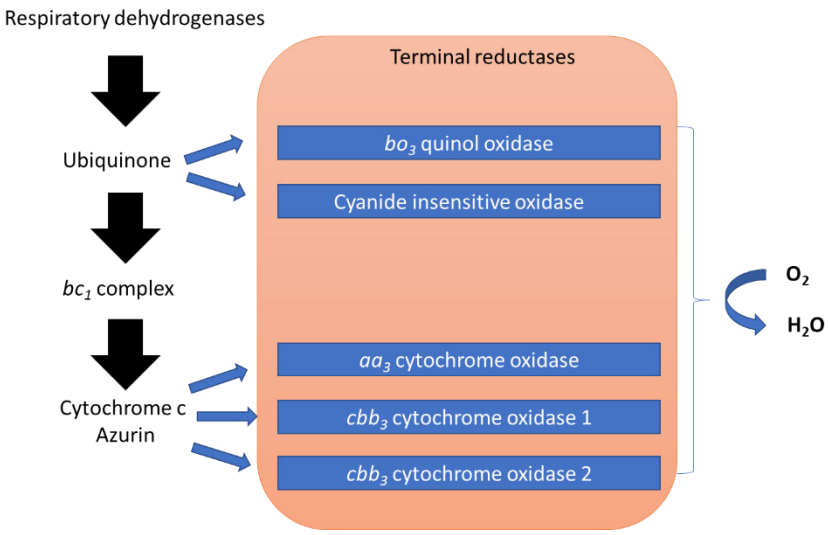


Figure 13. Diagram of the respiratory chain of *P. aeruginosa*. Electrons from respiratory dehydrogenases are passed through a series of redox reactions that end up in the final reduction of O_2 into H_2O. The energy released by the passage of electrons through the electron-transport chain is used for pumping of protons to the other side of membrane, a process that generates the *pmf*[183].

5.1.2. Regulation and functioning of the respiratory chain under oxygen conditions

The functioning of the respiratory chain is tightly regulated to adapt bacteria to the changing concentrations of nutrients and oxygen in the environment. The redox-responsive transcriptional regulator (ANR), the two-component system RoxSR, and the stationary phase sigma factor RpoS play dominant roles in the control of the multiple terminal oxidases in *P. aeruginosa*. **(Figure 14)**. ANR functions as a global regulator for anaerobic gene expression in response to oxygen depletion[189–191]. It is an analog of *E. coli* FNR (fumarate nitrate reductase regulator), which senses intracellular oxygen levels by an oxygen-sensitive $[4Fe–4S]^{2+}$ cluster bound to N-terminal Cys residues[192,193]. The transcriptional regulator of iron metabolism FUR also plays a role in inhibiting the expression of the *bo3* quinol oxidase when iron is abundant.

RoxSR is an analog of PrrBA/RegBA two component regulatory systems of purple photosynthetic bacteria, which activates expression of the photosynthesis genes under low oxygen conditions[194–196]. Even though the sensing signal of RoxSR of *P. aeruginosa* is still unclear, its similarity with RegBA of *Rhodobacter capsulatus* and *Rhodospirillum rubrum* suggests that it could be controlled by the redox status of ubiquinones.[197,198]. Other studies suggest that RoxSR senses the electron flow through *cbb3-1*[188].

RpoS is the main regulator of the stationary phase in *P. aeruginosa* and is important for survival under carbon starvation conditions.[199] It is needed for the expression of *aa3* but is less important in the regulation of *cio* (cyanide insensitive oxidase)[85,200,201]. Both oxidases are needed as the terminal oxygen acceptors and are characterized by low affinity to oxygen.

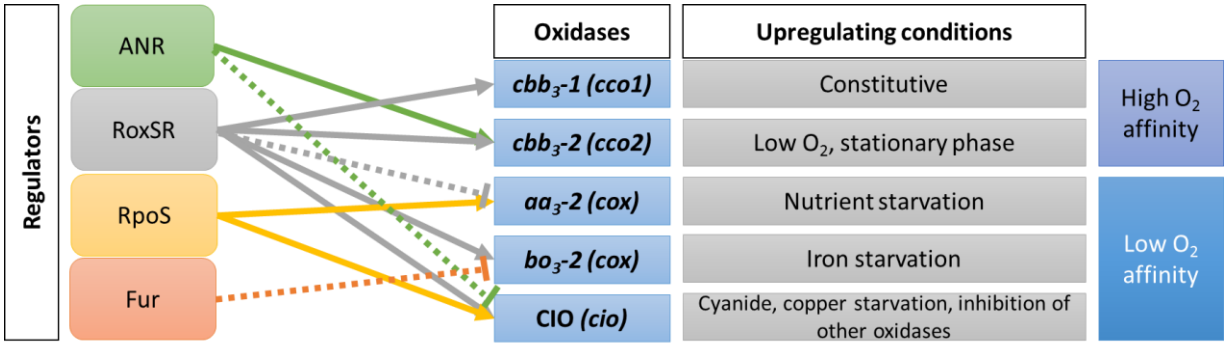


Figure 14. Schematic model of the regulatory network controlling the multiple terminal oxidases in *P. aeruginosa*. The four main regulators of of the terminal oxidases act upon the oxidases under specific conditions: ANR (Low O_2), RoxSR (Redox status), RpoS (stationary phase), Fur (Iron availability). Affinity for oxygen and upregulation conditions of the terminal oxidases are shown in the right columns. Activation is indicated by arrows. Inhibition is indicated by dotted lines[183].

Even when high concentration of oxygen is present in the growth medium, *P. aeruginosa* prefers microaerobic conditions for growth and for the formation of some of its virulence factors. It seems that *P. aeruginosa* creates the microaerobic conditions itself by using at least two mechanisms: by blocking the transfer of oxygen from the air to the medium and by the formation of a polysaccharide capsule on the cell surface. It has been suggested that the blocking of oxygen is advantageous for *P. aeruginosa* and allows it to survive under oxidative stress conditions encountered during the colonization of its host[202].

5.1.3. Oxidative stress response in *P. aeruginosa.*

In aerobic environments, *P. aeruginosa* produces energy by oxidative phosphorylation. This process involves the reduction of molecular oxygen O_2 into H_2O. During respiration, electron flow from the electron transport chain or cellular redox enzymes can end up in the reduction of O_2 in an uncontrolled manner, which leads to the successive production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$), within cells[203,204]. Besides the endogenous generation of ROS through normal aerobic metabolism, pathogenic bacteria are exposed to the oxidative burst caused by human phagocytes during the infection process. Furthermore, antiobiotic treatements interfere with core bacterial processess by binding to their cellular targets. The blocking of core functions ends up in the modification of redox state generating ROS and contributing to

the lethality of antibiotics[205]. Detoxification of ROS is provided by iron sequestration, free-radical-scavenging agents, DNA-binding proteins, DNA repair enzymes, and most importantly antioxidant enzymes, such as superoxide dismutases (SODs), catalases, and peroxidases[206]. The elaborate detoxification systems often require specific regulators for proper gene expression, constituting multiple regulons important in the adaptive response to multiple oxidative stresses.

The main regulator of the oxidative stress response is OxyR. OxyR acts as a H_2O_2 and $O_2^{\cdot -}$ sensor[161,207]. The detection mechanism of H_2O_2 in *P. aeruginosa* involves the formation of a disulfide bond between cysteines C199-C208. OxyR regulates the expression of genes coding ofr *oxyS* (small RNA), Fur (ferric uptake regulator), the catalase KatB, the major bifunctional catalase-peroxidase KatG, the alkyl hydroxyperoxide reductases AhpB, AhpC and AhpF, the Glutathione reductase (GorA), a non-specific DNA binding protein DPS, and the glutaredoxine 1 (GrxA)[208]**.** OxyR is also involved in regulating the expression of an uncharacterized pyoverdine reductase needed for the release and reduction of iron(III) from pyoverdine[207]. OxyR has also be shown to control the production of virulence factors such as rhamnolipids and pyocyanin[207].

Fur is a transcription factor that senses the redox state of the cell by detecting oxidation of iron. In *P. aeruginosa*, Fur is responsible for the regulation of genes important for the synthesis of siderophores such as pyoverdine and pyochelin and their receptors, metabolic genes *fumC* and *nuoA* coding for proteins harbouring iron-sulfur clusters for redox reactions and the superoxide dismutase SodA (Mn-SOD). The other superoxide dismutase SodB (Fe-SOD) is not controlled by Fur; instead it is regulated by *las* and *rhl* systems[209]. Under oxidative stress, Fur regulates the availability of free iron in the cell, to limit the possibility of the Fenton reaction that generates hydroxyl radicals ($HO^{\cdot}$). Fur is also responsable for the expression of virulence factors such as exotoxin A[209].

Another regulator factor of oxydative stress response is IscR, the main regulator of the biogenesis of sulfur-clusters. IscR contains two iron-sulfur (Fe-S) clusters and acts as its own repressor when it is oxidized. IscR affects the expression of the catalase KatA, but also the superoxide dismutases SodA and SodB[206].

OspR is a transcriptional regulator that acts as a redox sensor and regulates negatively a glutathione peroxidase important for oxidative stress resistance. The gluthatione peroxidase (GPx) is an enzyme that reduces H_2O_2 with the oxidation of glutathione. The detoxification by GPx depends on the GSH/GSSG ratio that is maintained by the intracellular production of NADPH[210]. It has been hypothesized that the GPx could work in coordination with the glucose-6-phosphate dehydrogenase that is needed for NADPH production and has been shown to be important against oxidative stress[211].

SoxR, an important transcription factor for oxidative stress in *E. coli,* does not work in the same way in *P. aeruginosa*[212]. SoxR regulon does not include genes involved in ROS detoxification. Moreover, *soxS*, a small RNA needed for regulating genes involved in stress reponse is absent from the genome of *P. aeruginosa*[212]. SoxR participates in the expression of the genes encoding the multidrug efflux pump MexGHI-OmpD involved in QS signal homeostasis[212].

RpoS is the main regulator of stationary phase in planktonic lifestyle, which has also been shown to participate in the expression of genes that enhance the fitness of *P. aeruginosa* during heat, osmotic and oxidative shocks[213]. RpoS is needed for the expression of catalases such as *katE* and *katG*. RpoS has also been shown to interact with the *las* and *rhl* systems to control the expression of the catalase KatA[213]. RpoS has also been shown to be essential for the production of alginate in the mucoid strain FRD1[213].

As we stated before in chapter I, 4.2.1., alginate is an important polysaccharide that protects *P. aeruginosa* from heat, osmotic and oxidative shock. Alginate is also linked to chronic infections in CF patients where isolates become mucoid or alginate overproducing. The main regulator of the synthesis of alginate is the alternative sigma factor AlgU[214]. AlgU is encoded in an operon containing four other genes, *mucA*, *mucB*, *mucC* and *mucD*, the products of which modulate its activity. Under uninduced conditions the activity of AlgU is sequestered by MucA and when stress conditions such as oxidative stress are sensed by the bacteria AlgU is released, allowing activation of AlgU-dependent promoters such as the alginate biosynthesis operon (*algD, alg8, alg44, algK, algE(alg76), algG, algX(alg60), algL, algI, algJ, algF, algA and algC*)[214]. This operon is known as the switch locus for alginate biosynthesis because mutations in this region are frequent among clinical alginate-

overproducing (mucoid) strains of *P. aeruginosa*[214]. The majority of these mutations inactivate the anti-sigma factor MucA, forcing the system into a permanent 'on' state[215].

Molecular ROS scavengers such as polyamines have also been shown to be important against oxidative stress. Polyamines are charged molecules that are associated with membrane LPS and nucleic acids. Spermidine, putrescine and cadaverine have been shown to protect outer membrane against antibiotics such as PolymixinB but also against oxygen radicals[216]. For instance, in *P. aeruginosa*, one study has already presented the scavenging properties of spermidine against ROS[216]. Even though, other polyamines such as putrescine and cadaverine have not been tested, there is evidence that this could also be the case as shown in other studies done in *E. coli* and *Vibrio vulnificus*[217,218].

Figure 15, resumes the main mechanisms involved in oxidative stress in *P. aeruginosa.*

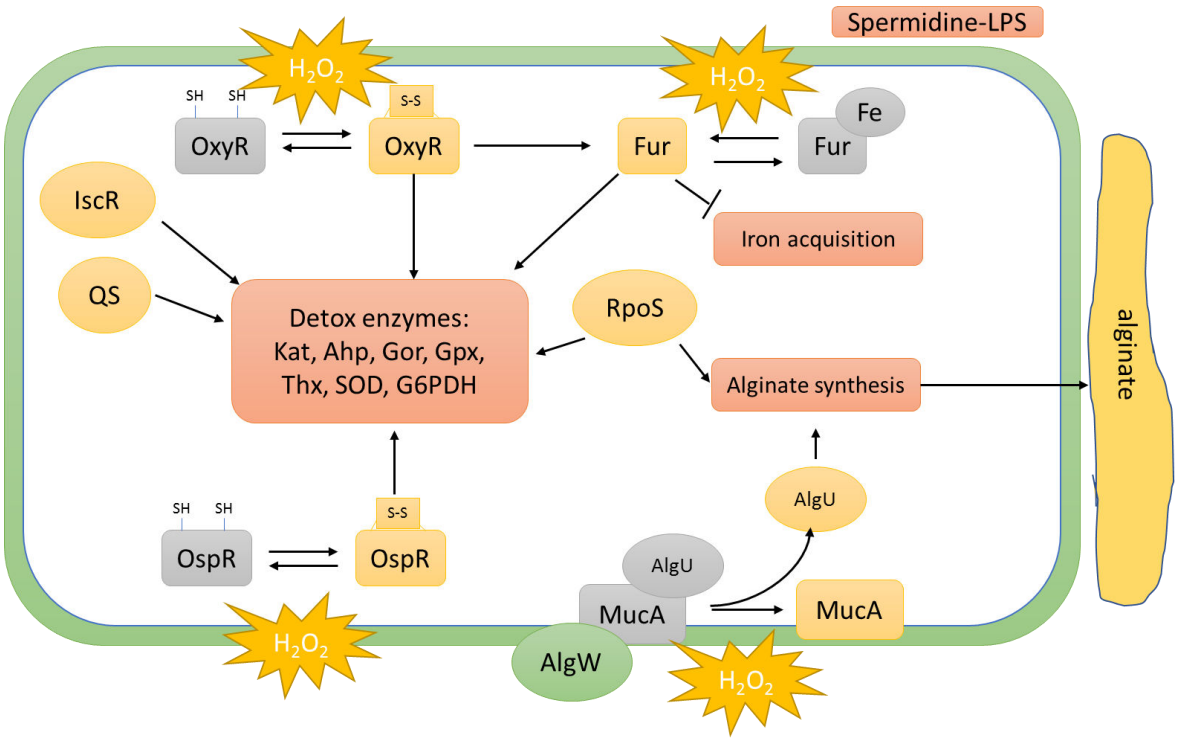


Figure 15. Diagram showing the principal regulatory, enzymatic and molecular scavengers involved in oxidative stress response in *P. aeruginosa.* OxyR, OspR, Fur and IscR are intracellular redox and H_2O_2 sensors that regulate the expression of enzymes such as catalase (Kat), the alkyl hydroxyperoxide reductases (Ahp), glutathione reductase (Gor), glutathione peroxidase (Gpx), thioredoxine(Thx), glutaredoxine (Grx), superoxide dismutase (SOD), and iron trapping and uptake mechanisms. AlgW is a redox sensor with protease activity, during oxidative stress AlgW degrades MucA (anti-sigma factors) and liberates AlgU (sigma factor). QS signaling and RpoS also participate in regulation of detoxification enzymes and alginate synthesis, linking oxidative stress resistance factors and virulence determinants. In *P. aeruginosa,* spermidine has been shown to bind to LPS, and to ROS scavenging.

5.2. The anaerobic metabolism

P. aeruginosa is a facultative anaerobic bacterium. In the absence of the preferred external electron acceptor oxygen, the bacterium can generate energy by anaerobic respiration through the complete reduction of nitrate or nitrite, as well as by fermentation of arginine or pyruvate[4,219,220]. Regulation of the anaerobic energy metabolism in *P. aeruginosa* is mediated by global transcriptional regulators ANR, DNR and the two-component regulatory system NarXL[221].

5.2.1. Denitrification in *P. aeruginosa*

In *P. aeruginosa*, the most efficient way to generate energy in the absence of oxygen is the reduction of nitrogen compounds via denitrification. During this respiration process, nitrate or nitrite serve as external electron acceptors and are reduced in several steps to nitric oxide, nitrous oxide and finally dinitrogen, as shown in **Figure 16**[222].

Nitrate reductase catalyzes the first step of denitrification which is the reduction of nitrate to nitrite. There are three types of nitrate reductases, Nar (cytoplasmic membrane), Nap (periplasm), and Nas (cytoplasm), and they all contain a molybdopterin guanine dinucleotide cofactor[7,223].

When nitrate is present and oxygen tension is low, the two-component system NarXL and the nitric oxide sensing regulator DNR activate the expression of the *narK1K2GHIJ* gene cluster[147]. NarK1K2 are involved in the transport of nitrate and nitrite, however only NarK2 seems to be essential for nitrate uptake and nitrite efflux while the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is catalyzed by dissimilatory nitrate reductase NarGHI [224,225], **Figure 16**. NarI is a membrane-bound *cytochrome b* which transfers electrons from the quinone pool across the membrane to cytoplasmic subunit NarH. NarH transfers the electrons via its $[4Fe\text{-}4S]^{2+}$ clusters to the molybdenum cofactor of NarG, which then reduces nitrate to nitrite. NarJ has no catalytic activity but is involved in the assembly of the nitrate reductase.

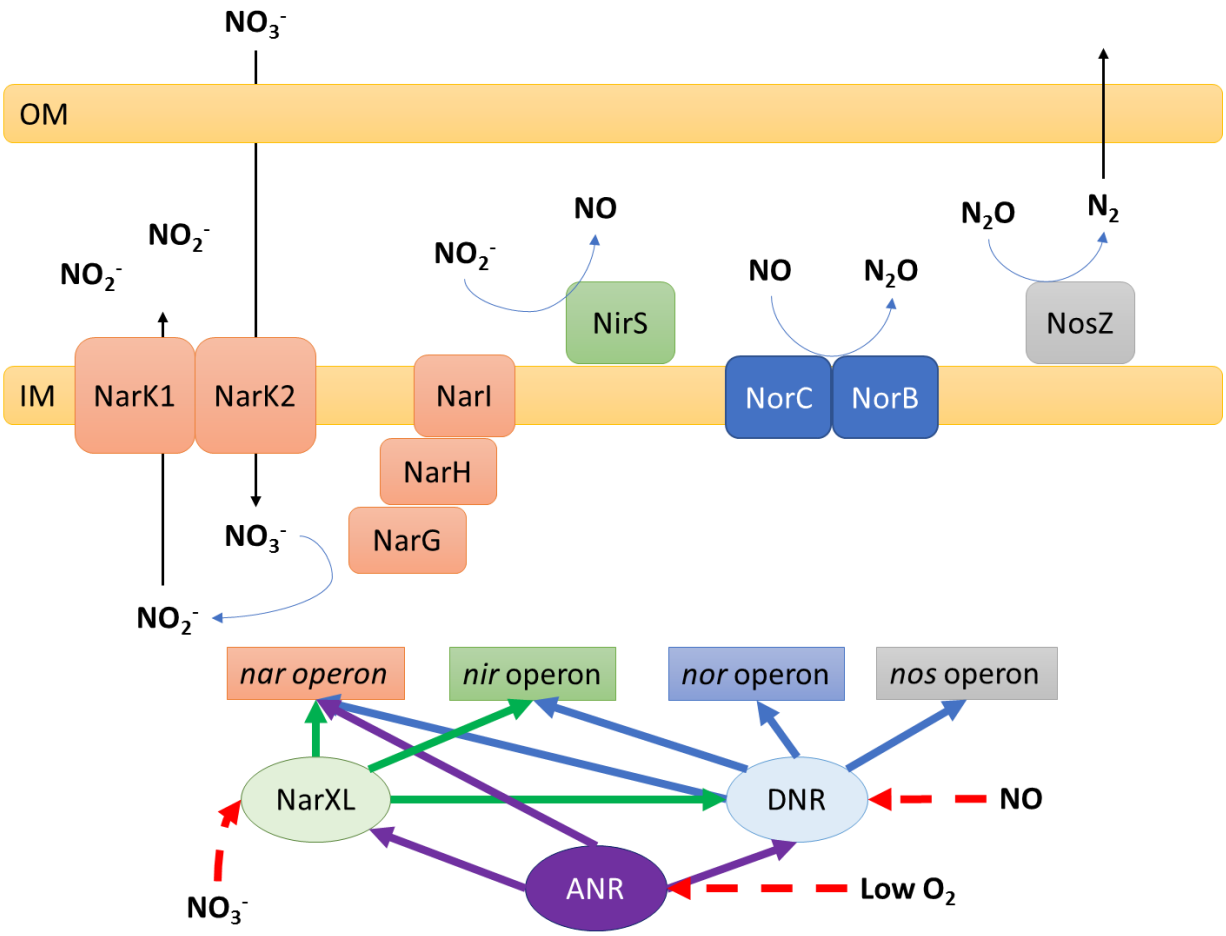


Figure 16. Reduction of nitrate to dinitrogen during *Pseudomonas aeruginosa* denitrification. Nitrate (NO_3^-) is converted to nitrite (NO_2^-) by membrane-bound nitrate reductase (NAR), nitrite is converted to nitric oxide (NO) by nitrite reductase (NIR), nitric oxide is converted to nitrous oxide (N_2O) by nitric oxide reductase (NOR), and nitrous oxide is converted to dinitrogen (N_2) by nitrous oxide reductase (NOS). In the lower part, we observe the regulation of the denitrification operons by NarXL, ANR, DNR and the inducing factors such as nitrate (NO_3^-), low O_2 tension and nitric oxide (NO). Modified from Arai *et al.*, 2011 and Zumft et al., 1997[183,222]. OM: Outer Mebrane, IM: Inner Membrane. Arrows show positive regulations. Dotted arrows show positive activations by nitrate, nitric oxide and low oxygen tensions.

A second dissimilatory nitrate reductase in *P. aeruginosa* is encoded by genes *napABCDEF*[147]. Expression of *napABCDEF* was shown to be independent of oxygen, nitrate or nitrite and it is repressed by NarL during anaerobic growth; however it is not known which signal induces expression of the operon[221,226]. The operon encodes proteins that form an enzymatic complex located in the periplasm containing a $[4Fe\text{-}4S]^{2+}$ cluster and a molybdenum cofactor. In contrast to NarGHI, NapABC is unable to translocate protons and does not play in the build up of the chemiosmotic gradient. However, it was proposed that NapABC has a role in sustaining the cellular redox balance, which might be important during transition from aerobic to anaerobic growth[225].

The third nitrate reductase in *P. aeruginosa* is encoded by *nasC.* This enzyme is present in the assimilatory pathway of nitrate and is used to synthesize ammonium needed for nitrogen metabolism[223].

The second step of denitrification is catalyzed by nitrite reductase, encoded by the *nirSMCFDLGHJEN* gene cluster, whose expression is regulated by nitric oxide sensing regulator Dnr[227]. NirS is a *cytochrome cd1* reductase which is located in the periplasm catalyzing the reduction of nitrite (NO_2^-)[228] Electrons are transfered to NirS by NirM (cytochrome *c551*), NirC (periplasmic *cytochrome c*), and by azurin.[229–231]NirFDLGHJE are involved in biosynthesis of *heme d1*, NirN is a periplasmic *cytochrome c* with currently unknown function[229–231].

The third step of the denitrification process is ensured by the products of the *norBCD* gene cluster. Their expression is induced by nitric oxide sensing regulator Dnr[232]. NorB (*cytochrome b*), and NorC (*cytochrome c*), catalyze the reduction of nitric oxide (NO) to nitrous oxide (N_2O)[227]. The function of NorD is currently unknown, but it has been shown to be essential for anaerobic growth with nitrate[227].

During the last step of *P. aeruginosa* denitrification, nitrous oxide (N_2O) is reduced to dinitrogen (N_2). This reaction is performed by the nitric oxide reductase NosZ, which is a periplasmic homodimer containing four copper centers. NosZ is encoded by the *nosRZDFYL* operon whose expression is also regulated by nitric oxide sensing regulator DNR[232]. NosR was shown to be a membrane-bound regulator essential for NosZ activity in *Pseudomonas stutzeri* and it was proposed that NosDFY participate in the insertion of copper into NosZ[232].

5.2.2. Anaerobic fermentation processes in *P. aeruginosa*

In the absence of external electron acceptors oxygen, nitrate or nitrite*, P. aeruginosa* is able to grow by fermentation of arginine, which generates ATP by substrate-level phosphorylation[220]. The arginine is metabolised via the arginine deiminase pathway (ADI), in which the molecule is converted into ornithine, carbon dioxide and ammonium, resulting in one molecule of ATP. The process of arginine fermentation begins with the desamination of arginine to citrulline by arginine deiminase ArcA, and the subsequent conversion of citrulline to ornithine and carbamoylphosphate by carbamoyltransferase ArcB. ArcD, an arginine/ornithine antiporter, is involved in exporting the generated ornithine from the

cell[233]. Carbamoylphosphate is converted to carbon dioxide and ammonium by carbamate kinase ArcC, during which one molecule ATP is generated[147,220].

Proteins required for the arginine deiminase pathway are encoded by the *arcDABC* gene cluster. Expression of *arcDABC* is induced by ANR when oxygen tension is low, but also when arginine is present in the growth medium[189,234].

Another regulator of the expression of *arcDABC* operon is the transcriptional factor ArgR. ArgR is the major arginine-responsive transcriptional regulator of the AraC/XylS family. It is part of the *aotJQMOP-argR* operon which encodes transporters for arginine and ornithine uptake. Expression of the operon is regulated positively by ArgR. Therefore expression of *argR* is thus activated when arginine is present in the medium or when arginine is accumulated intracellularly[235]. As shown in **figure 17,** ArgR is responsible for upregulating the expression of the major pathways of arginine degradation : the arginine deiminase (ADI) pathway, the arginine succinyltransferase (AST) pathway, and the arginine dehydrogenase (ADH) pathway but also the the lysine decarboxylase (LDC) pathway and the synthesis of arginine[236]. In the *arcDABC* promoter, the ArgR and ANR binding sites are adjacent, suggesting that ArgR and ANR proteins may physically interact with each other during the activation of the transcription[236,237]**.**

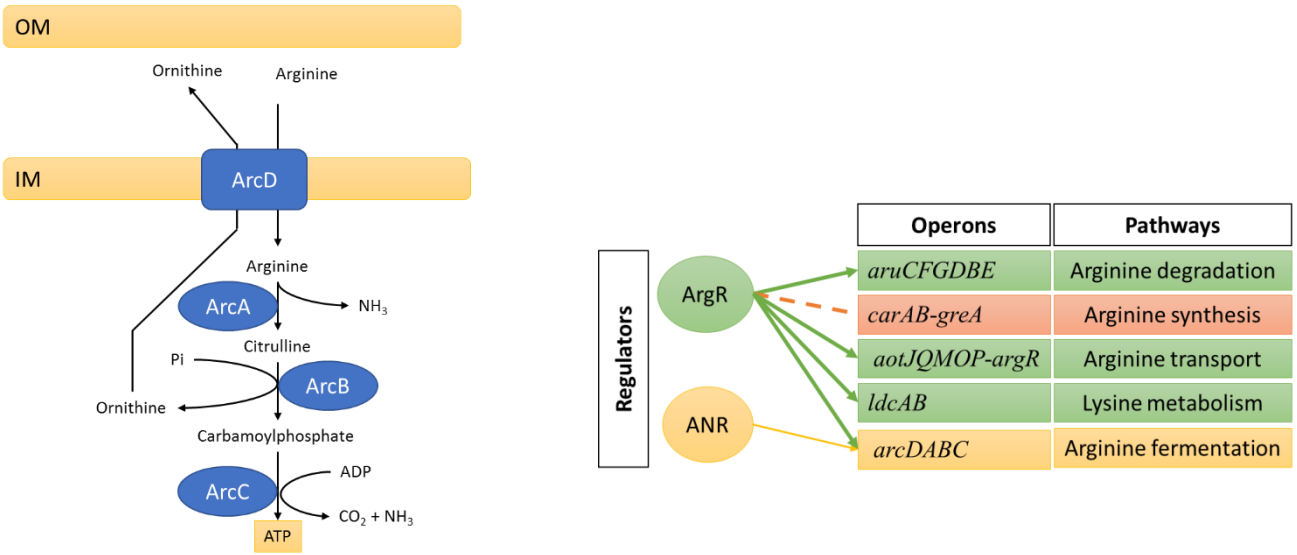


Figure 17. Schematic representation of arginine fermentation in *P. aeruginosa*[220]. Left: ArcD is an arginine-ornithine antiporter. ArcABC catalyze the conversion of arginine to ornithine, carbon dioxide and ammonia, with generation of one molecule ATP per molecule arginine. **Right:** ArgR and ANR are the main regulators of *arcDABC* operon involved in arginine fermentation. ArgR also upregulates other operons involved in arginine and lysine metabolism and represses the expression of genes involved in arginine synthesis[237,236].

5.2.3. Pyruvate fermentation in *P. aeruginosa*

When *P. aeruginosa* is uncapable of respiration because of a lack of electron acceptors, the bacteria can ferment pyruvate. This fermentative metabolism does not promote growth, but sustains long-term survival[4]. During pyruvate fermentation, the substrate is metabolized to succinate, acetate and lactate through different enzymatic reactions while producing ATP, as shown in **figure 18**. Even though regulatory mechanisms of this pathway are not completely understood, it is known that ANR controls the expression of the *ackA-pta* operon encoding phosphotransacetylase and acetate kinase. Meanwhile, the lactate dehydrogenase encoding *ldhA* gene seems to be expressed constitutively. Pyruvate fermentation is a pH dependent process and it is optimal between pH 6 to 7[4].

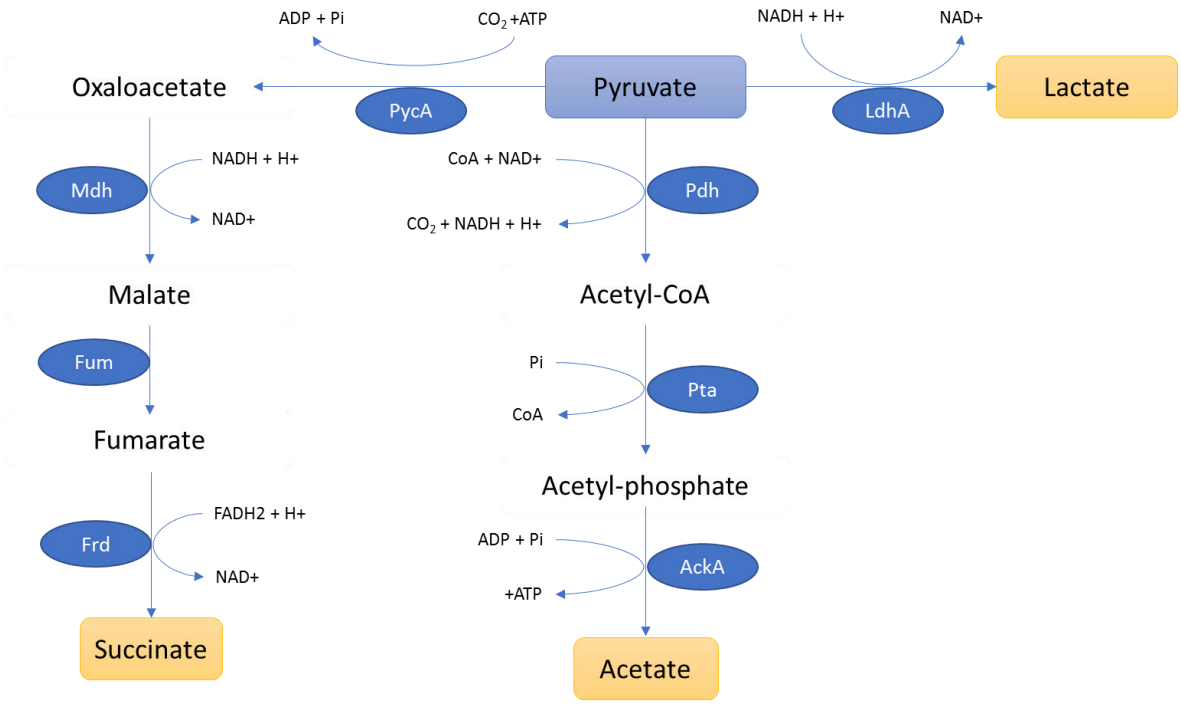


Figure 18. Schematic model of pyruvate fermentation in *P. aeruginosa*[4]. PycA (pyruvate carboxylase), Mdh (malate dehydrogenase), Fum (fumarase) and Frd (fumarate reductase) metabolize pyruvate to succinate. Pdh (pyruvate dehydrogenase), Pta (phosphotransacetylase) and AckA (acetate kinase) metabolize pyruvate to acetate. Pyruvate is also degraded into lactate by LdhA (lactate dehydrogenase) by consuming NADH.

Chapter II. The lysine decarboxylase

For more than 60 years, the lysine decarboxylase (LDC) is an enzyme that has been thoroughly studied and characterized in different species of enterobacteria. Today, we know that the buffering capabilities of the LDC confers a decisive advantage for foodborne pathogens that try to pass the extreme acid conditions of the host's stomach. The cadaverine synthetized by the LDC has also been shown to play a protecting role against the toxic effects of oxygen and during urinary tract infections.

In Irina Gutsche's lab, where we study the structural properties of the LDC, we wondered whether this enzyme could confer a fitness advantage for the opportunistic pathogen *P. aeruginosa*. This idea came from the fact that in CF patients, the lung secretions have been described to be an acidified due to aberrant ion transport and an oxidative environment caused by a problem in glutathione transport[238]. It is under this context where the protective role of LDCs become pertinent.

In literature, there are reports that *P. aeruginosa* genome encodes at least 2 lysine decarboxylases. The first one, the product of the gene *PA1818* with a confirmed LDC activity, is the closest one to the LDCs from enterobacteria considering sequence identity[235]. The second one, the product of the gene *PA4115*, has been shown to participate in cadaverine synthesis but has never been characterized biochemically[239].

In this chapter, I present a general overview of the current knowledge in the field of the Lysine decarboxylases.

1. The roles of the lysine decarboxylase in bacterial physiology

Lysine decarboxylases (LDC) are PLP-dependent enzymes that catalyse the decarboxylation of L-Lysine into cadaverine in a multistep reaction that consumes a proton and produces a CO_2 molecule that diffuses out of the cell. These enzymes have been studied for more than 70 years and they were at first noticed for their capacity to enhance the survival of enterobacteria during the passage of the gastro-intestinal tract. Today we know that there are two structural families of lysine decarboxylases: **the aspartate aminotransferase-fold family (AAT-fold family) LDCs**, with representatives that have been studied in the phylum of proteobacteria (Gram-negative bacteria) and the more widespread **alanine racemase-fold family (AR-fold family) LDCs,** with representatives that have been found in bacterial

kingdom (phylums of actinobacteria, bacteroidetes, firmicutes and proteobacteria) but also with homologues in eukaryotic kingdom (plants). In bacteria, these two families have been involved in different physiological processess such as acid stress response[240], oxidative stress response [218,241], growth fitness[242], adherence[243], virulence in biofilms[244], synthesis of siderophores[245], synthesis of peptidoglycan[246]. In plants they are involved in the metabolism of secondary metabolites such as alkaloids[247]. The involvement of LDCs in so many different biological processes partly resides in the versatility of functions that cadaverine can be used for.

During this thesis, we will focalize only in the LDCs of the AAT-fold family that have been involved in stress response.

1.1. The inducible Lysine decarboxylase LdcI

1.1.1. LdcI is part of the acid and oxidative stress response

The inducible lysine decarboxylase LdcI in *E. coli* is part of the lysine-dependent acid resistance system (LDAR) which is a three-component system composed of one pH sensor CadC and two effectors CadA (also known as LdcI) and CadB[248,249]. The system has been shown to be also present in *S. enterica* serovar Typhimurium*, V. cholerae and V. vulnificus* where it confers a significant advantage against acid stress and oxidative stress.

The earliest studies were performed in *E. coli* in which it was shown in 1940, that the system was capable of raising the low pH (4.5) of the growth medium[250]. Further studies confirmed that the LdcI conferred *E. coli* enhanced survival to mild acid stress, but also in medium containing weak acids produced from fermentation (acetic acid, propionic acid and butyric acid) and in conditions mimicking the small intestine (anaerobic growth, phosphate starvation, and the presence of formic, acetic, pyruvic, L-lactic, L-glutamic and L-aspartic acids)[251–255]. The system was also shown to provide some protection to extreme acid stress condition pH 2-2.5 [255], but other systems such as the inducible glutamic acid decarboxylases GadA and GadB or the inducible arginine decarboxylase AdiA are more important for survival under these conditions[256].

The LDAR system has also been shown to be important for acid stress response in the close bacterial species *S. enterica* and *V. cholerae* that need to survive the transitof the gastrointestinal tract in order to colonize their host. In *S. enterica* serovar Typhimurium, the LdcI protein is 79% identical to the homologue in *E. coli*. It allows the bacteria to raise

internal pH and to mount a robust acid stress response. Even though *Salmonella* also uses arginine decarboxylase to resist acid stress, LdcI is the only one that enhances growth at pH4.5[257]. In *V. cholera,* the presence of this system allows pre-adapted cells at pH 5.7 to survive a normally lethal acid challenge at pH 4.5 in the presence of an external source of L-lysine[258]. Even though LdcI from *V. cholarae* is not required for colonization and infection of the mouse intestine, acid adapted cells are at a competitive advantage compared to naive cells in a murine intestinal colonization assay .

Another effect of the Lysine decarboxylase activity is that cadaverine induces closure of outer membrane porins OmpC and OmpF, thereby contributing to bacterial protection from acid stress, but also from certain antibiotics, by reduction in membrane permeability in *E. coli*[240,259–262].

In experiments investigating the role of LdcI in polyamine deficient *E. coli*, it was shown that in addition to the neutralization of acid, the action of LdcI and CadB, the L-lysine-cadaverine antiporter, is able to generate proton motive force at low pH due to its non-electron neutral transfer. It was also observed that cells, grown at low pH in presence of lysine, had three-fold higher levels of cellular ATP that resulted from the boost of in *pmf* generated by the action of CadB[263].

The LdcI has also been shown to be involved in oxidative stress response of *V. vulnificus* when the bacterium faces treatment with superoxide generators such as methylviologen, plumbagin or menadione. The cadaverine produced by LdcI would be capable of scavengingthe radicals created by these molecules[241]. The ROS scavenging properties of cadaverine observed in *V. vulnificus* are also coherent with previous reports indicating that polyamines protect *E. coli* from paraquat and the toxic effects of oxygen[264,265].

The cadaverine produced by both LdcI and LdcC is necessary for detoxifying nitric oxide radicals (NOS, nitrosative stress) produced by immune system cells that pathogenic *E. coli* encounter during the course of urinary tract infection (UTI). This protective effect enhances the colonization of the bladder by uropathogenic *E. coli* (UPEC)[266,267]. A similar protective effect from nitrosative stress has been also reported in the pathogen *Salmonella enterica* serovar Typhimurium where LdcI protects the pathogen from acidified sodium nitrate, a curing agent in fermented foods[268]. **Figure 19**, resumes the different protective effects for LDC, among other roles that are found in the literature.

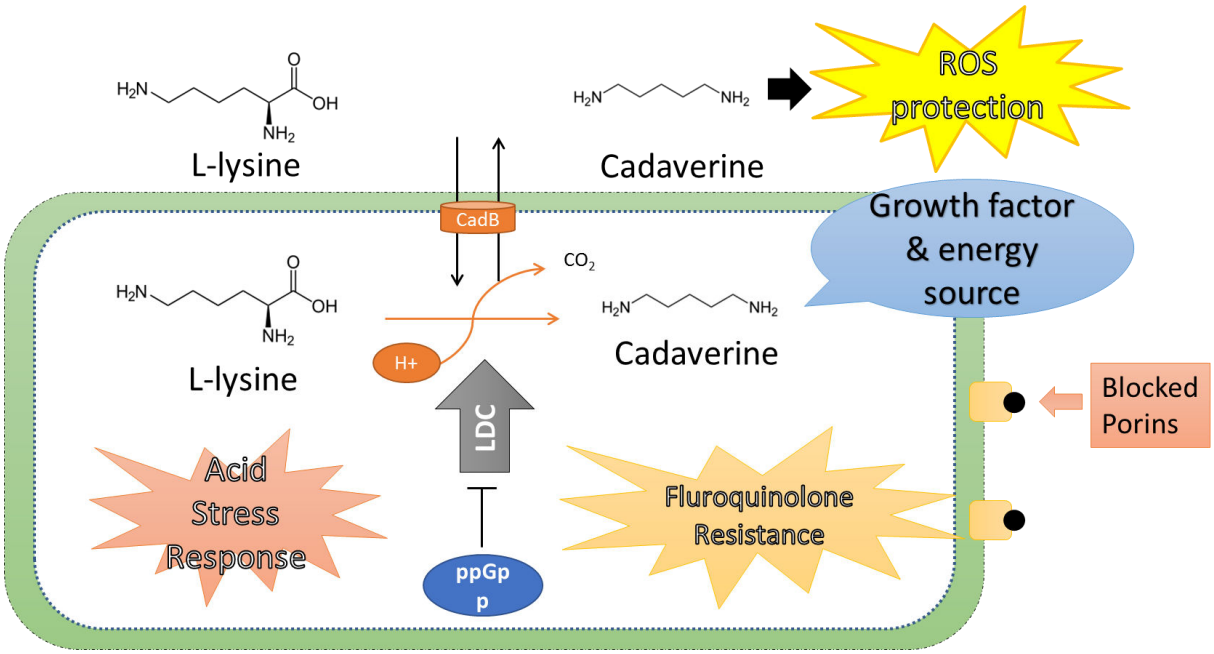


Figure 19. Diagram of the different roles of the lysine decarboxylase in bacteria. The proton consuming reaction of LDC allows the buffering of cytoplasm when acid stress exists. Cadaverine produced by LDC can be used as a growth factor and energy source. Cadaverine exchanged with fresh L-lysine by CadB is used for ROS protection but also to block porins (OmpC and OmpF), which in turn enhances antibiotic resistance.

1.1.2. LdcI affects virulence in pathogenic *E. coli* and *Shigella sp.*

The loss of LdcI in *Shigella* and enteropathogenic *E. coli* was described as a pathoadaptative mutation of the bacteria required for full pathogenesis[243]. One common trait among *Shigella* species is the loss of the functionality of LdcI. Depending on the *Shigella* species we can find the presence of insertion sequences (IS elements), phage-like insertions, or genetic rearrangements in the *cadBA* operon and mutations in the *cadC* gene[269]. The restoration of *cadA* expression in *Shigella flexneri* results in the attenuation of virulence and the inhibition of enterotoxin production. This inhibition has been explained as a side-effect of cadaverine which downregulates expression of the genes carried on the virulence plasmid in *S. flexneri*. Another effect is that cadaverine blocks the release of the bacteria from the phagosome to the cytoplasm by stabilizing the phospholipids of the phagosome[270–272].

In Shiga-toxin producing strains of *E. coli* (STEC O157:H7) in which *ldcI* is missing, it has been shown that the complementation with *ldcI* caused a defect in the adherence that affected the virulence of the strain *in vivo*[243].

1.1.3. Regulation of the inducible Lysine decarboxylase LdcI.

In *E. coli*, the *cadBA* operon is regulated by *cadC* which is a gene encoding a ToxR family receptor. CadC is a one-component signal transduction system whereby a single polypeptide is involved in signal sensing, signal transduction and transcription regulation[273]. CadC is a 512-amino acid protein characterized by three different domains: **N-terminal ToxR-like DNA binding domain** (1-158), **Transmembrane domain** (159 – 187) and **C-terminal sensing domain, composed of two subdomains,** an N-terminal mixed α/β subdomain and a C-terminal 11-membered α-helical bundle.

As shown in **figure 20**, CadC is capable of detecting three induction signals: low pH, excess lysine and anaerobiosis[251,252]. The detection of low pH conditions involves conserved negatively charged residues as well as disulfide bridges that change their oxidation state in response of external pH conditions[274]. Moreover, it appears that CadC is also capable of sensing changes in the oxidation state of the cellular quinone pool[275]. In normal or low pH conditions, LysP (lysine permease)[276] forms a complex with CadC that inhibits its regulatory activity. When Lysine is present in the growth medium during acid stress conditions, LysP releases active CadC which will be capable of inducing the expression of the *cadBA* operon by binding the Cad1 and Cad2 binding sites[252,277,278]. The mechanism by which CadC activates the transcription of the *cadBA* operon under anaerobic conditions is unknown but involves the two-component response system ArcAB (oxygen tension sensor)[251,279]

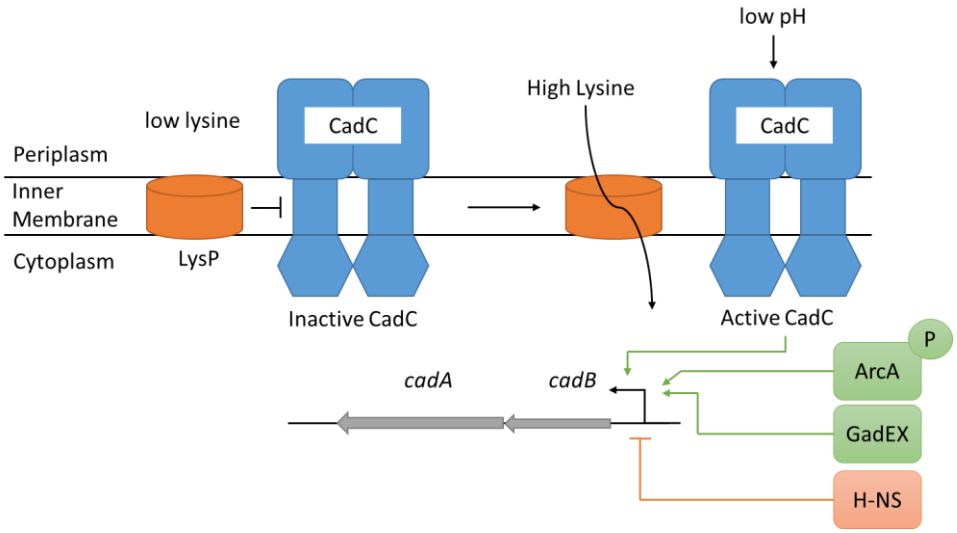


Figure 20. **Regulation of *cadBA* operon by CadC in *E.coli*.** Under low lysine concentrations, the inner membrane lysine permease LysP binds to the CadC transmembrane helix (CadCTM) and inhibits CadC activity[278]. CadC dimerizes via its periplasmic domain (CadCPD) and this domain is involved in activating CadC in response to low pH [274]. At low pH and in the presence of lysine, CadC is maximally activated and the CadC DNA-binding domain (CadCDBD) induces transcription from the *cadBA*

operon (green arrow). Expression of *cadBA* operon is also induced during anaerobic conditions by ArcA-P, and by acid conditions by GadEX[280,281]. H-NS inhibits transcription during aerobic growth[282].

1.1.4. Other factors regulating the LDAR system

Other factors besides CadC have been involved in the regulation of the *cadBA* operon. GadE and GadX, two regulators of the glutamic acid decarboxylase, are also able to up-regulate expression of the *cadBA* operon under low pH growth conditions in *E. coli*[280,281]. The DNA-binding protein HNS represses expression of *cadBA* during aerobic growth by changing the conformation of the promoter and blocking the binding of CadC to the Cad1 and Cad2 binding sites[282,283]. In *V. vulnificus,* the oxidative stress response regulator SoxR activates the expression of the *cadBA* operon by directly binding to the promoter. The effect was induced by superoxide generators such as Methylviologen, Plumbagin and Menadione but not by H_2O_2[241]. Moreover, the increase of cadaverine production by the induction of the *cadBA* operon could scavenge ROS generation by the superoxide generators[241].

1.2. LdcC from *E. coli*

The lysine decarboxylase LdcC is a close paralogue of LdcI that has pH optimum of 7.5[284]. Few studies about its role in the physiology of *E. coli* were conducted. It was shown to be responsible for the synthesis of cadaverine at neutral conditions in stationary phase. The cadaverine produced by LdcC has also been suggested to take part in oxidative stress resistance, helping *E. coli* to fight against nitrosative stress encounteredin UTIs. Initially LdcC was assumed to be an enzyme with constitutive expression but it has been shown that it is also inducible. The expression of *ldcC* is dependent on the sigma factor RpoS, it is growth phase dependent with a maximum level of expression in the stationary phase. LdcC has also been shown to be upregulated when *E. coli* is treated by fluoroquinolones[262].The overproduction of cadaverine under inducing conditions is thought to provokethe blocking of generalist outer membrane proteins involved in the diffusion of the antibiotic inside the cell[260].

1.3. LDC as a virulence factor

Eikenella corrodens is a commensal bacterial that can be found in our oral cavities. It is responsible for the formation of robust biofilms generating inflammation of the colonized

gums. It has been shown that lysine decarboxylase is a virulence factor in the biofilm that locally depletes L-Lysine. This Lysine depletion impairs the dental epithelial barrier to bacterial proinflammatory products. Recent research involving the inhibition of LDC in *E. corrodens* biofilm has led to strong reduction of biofilm formation and a reduced periodontal inflammation in young infected adults[244,285]. The knowledge acquired from the LDC from *E. corrodens* suggests that other LDCs could play a new role as a virulence factor in biofilms and opens new perspectives in biofilm eradication treatment.

1.4. The role of cadaverine in bacteria

As shown in **figure 21,** the decarboxylation of lysine generates the polyamine cadaverine. Polyamines are molecules that are characterized by two or more positively charged amino groups that are separated by a defined alkane scaffold. Their chemical properties allow them to counter-balance negative charges found in cells. For instance, they have been found to interact with nucleic acids (DNA, RNA), nucleotide triphosphates, phospholipids, lipopolysaccharides.[286] In *E. coli,* a set of genes known as the "polyamine modulon" has been described to have an important effect in cell growth and are regulated by polyamines. The binding of polyamines to the mRNA of the genes from the polyamine modulon enhances the performance of the ribosome stimulating the translation of the modulon. The products of the genes of the polyamines modulon includes OppA, a periplasmic substrate-binding protein of the oligopeptide uptake system; adenylate cyclase (Cya); RpoS sigma factor (σ^{38}), for transcription of genes expressed at the stationary phase; FecI sigma factor(σ^{18}), for transcription of the iron transport operon; Fis, a global regulator of transcription of some growthrelated genes including those for rRNA and some tRNAs; RF2, polypeptide releasing factor 2; RpoN (σ^{54}), for transcription of genes for nitrogen metabolism; Cra, a global transcription factor for a large number of genes involved in glycolysis and glyconeogenesis; and H-NS, a positive regulator of expression of genes involved in flagellin synthesis and ribosomal protein synthesis[286–291]. Even though, these mechanisms have been described for putrescine and spermidine, it is possible that cadaverine also plays a similar role because it shares the same chemical properties. It would be coherent to think that cadaverine would participate in cellular processes such as growth, translation, protein synthesis, membrane stability[286,289–293].

Cadaverine is also known to block porins in enterobacteria, which is important for preventing the entrance of antibiotics, as well as for acid and oxidative stress resistance[218,294,264]. Finally, cadaverine is also known to be used as a substrate for the synthesis of siderophores such as desferrioxamine and bisucaberin[245,295].

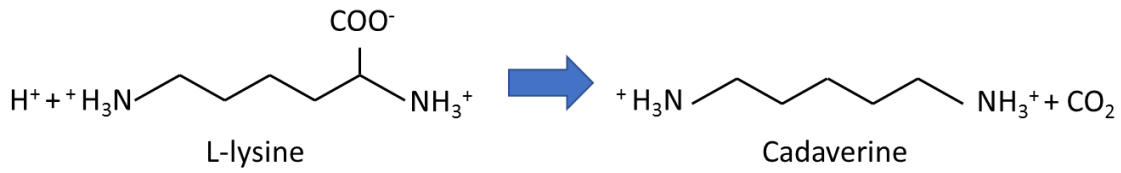


Figure 21. Decarboxylation reaction of Lysine. Lysine is decarboxylated in a pyridoxal 5' phosphate (PLP)-dependent reaction that consumes one proton from the medium while releasing one molecule of cadaverine and CO_2. At neutral conditions cadaverine is in equilibrium between charged and uncharged species: the charged ones are by far the most abundant at neutral pH conditions.

2. The structure characteristics of LDCs from AAT-fold family

Most studies of the lysine decarboxylase have been conducted in *E. coli.* This organism encodes two isoforms of the lysine decarboxylase that participate in different physiological functions, LdcI and LdcC, whose structures were solved by X-ray crystallography and by Electron Microscopy (EM)[284,296,297]. As shown in **figure 22**, each monomer of the Lysine decarboxylase exhibits three different domains:

2.1. N-terminal domain (wing domain)

It adopts a flavodoxin-like fold and belongs to the CheY-related SCOP-fold family also known as the receiver domain (REC) of the response regulator protein[298]. It is characterized by a five-stranded parallel β-sheet sandwiched between two sets of amphipathic α-helices. The wing domain is thought to originate from a fusion of a REC domain with an ancestral decarboxylase from the AAT family[120,284].

2.2. The core domain

It contains the catalytic pocket containing the cofactor needed for the decarboxylation reaction. It is made up of a linker region (residues 130–183) followed by two subdomains: a

pyridoxal 5'-phosphate (PLP) binding subdomain (PLP-SD) (residues 184–417) corresponding to the large domain of PLP-fold type I enzymes and subdomain 4 (SD4) (residues 418–563) [299]. The PLP-SD contains the conserved Lysine (K367 in LdcI) needed for the binding of the PLP cofactor. The completion of the active site requires the dimerization of the protein which is a common feature of the AAT-fold decarboxylases. The majority of the dimer interface is formed by the linker region and PLP-SD with smaller contributions from the remaining protein domains[284,300].

2.3. C-terminal domain

It is characterized by a predominantly α-helical outer surface and an inner surface that has two sets of β-sheets (residues 564–715). The CTD folds over the surface formed by PLP-SD and SD4 and forms part of the entry channel into the active site of the enzyme[284]. The CTD domain has been shown to be crucial for the interaction with the LdcI partner RavA (AAA+ ATPase)[297] . This interaction is specific to LdcI because RavA is not capable of binding the paralogue LdcC[254]. Structural analysis of LdcI and LdcC chimerasrevealed that the CTD contains a β-sheet which is the major determinant for the interaction with RavA[297]. Moreover, sequence and phylogenetic analysis of the β-sheet of the CTD shows that this structural element contains a conserved amino acid signature that discriminates between LdcI and LdcC. This discovery suggests evolutionary pressure on LdcI towards the formation of the cage-like complex with RavA, **figure 23**[297].

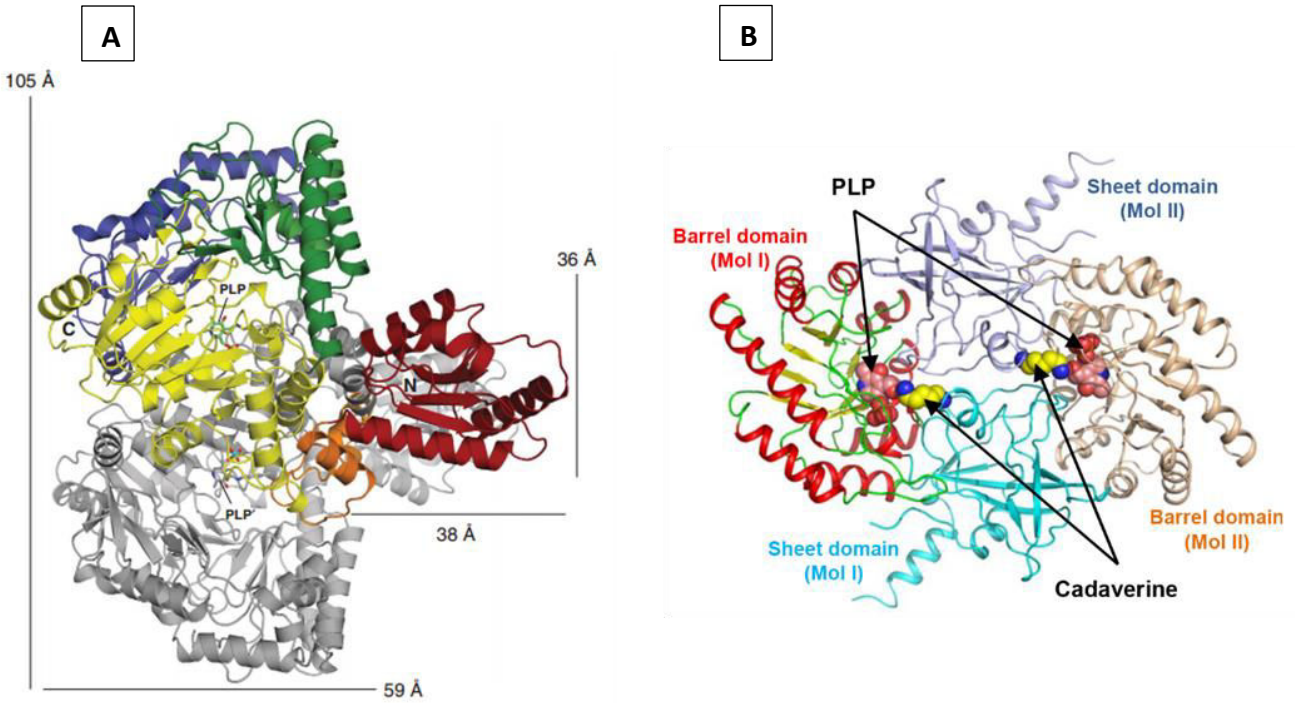


Figure 22. Lysine decarboxylases of the two different structural families. **A) Dimer of the lysine decarboxylase LdcI from *E. coli.*** N-terminal wing domain—red, linker region—orange, PLP-SD—yellow, SD4—green, and CTD—blue. The wing domain adopts a flavodoxin-like fold and belongs to the CheY-related SCOP-fold family, which is characterized by a five-stranded parallel β-sheet sandwiched between two sets of amphipathic α-helices. The linker region consists of a short helical bundle (α5 – η2 – α6 – α7) that extends outwards from the PLP-SD and is almost at right angles to the wing domain. The PLP-SD has a seven-stranded β-sheet core surrounded by three sets of α-helices. Following strand β14 of the β-sheet core is a short α-helix (α13) that contains the conserved lysine residue K367 that forms a covalent bond to the PLP co-factor. SD4 stacks on top of PLP-SD and has a central four-stranded antiparallel β-sheet core and the outer face has three prominent α-helices (α16, α17, and α18), where α16 and α18 are almost parallel and form part of the oligomerisation interface. The CTD has a predominantly α-helical outer surface and an inner surface that has two sets of β-sheets. The CTD folds over the surface formed by PLP-SD and SD4 and forms part of the entry channel into the active site of the enzyme[240]. **B) Dimeric structure of LDC from *Selenomonas ruminantium.*** The sheet domains are found in cyan and violet and the barrel domain in red and light orange. The bound PLP and cadaverine are shown as sphere models with salmon and yellow colors, respectively. The barrel domain (Leu27-Cys261) is composed of eight α-helices (α2-α9) and eight parallel β-strands (β2-β9) in an alternating pattern. The eight α-helices wrap the eight-stranded β-sheet core that serves as a cofactor binding site. The sheet domain (Met1-Ser26, Gly262-Val393) is formed by a seven-stranded anti-parallel β-sheet (β1, β10-β15) that is surrounded by three short α-helices. This domain contributes mainly to dimerization. The SrLDC homodimer is formed by a head-to-tail contact between the barrel domain of one monomer and the sheet domain of the other monomer[246].

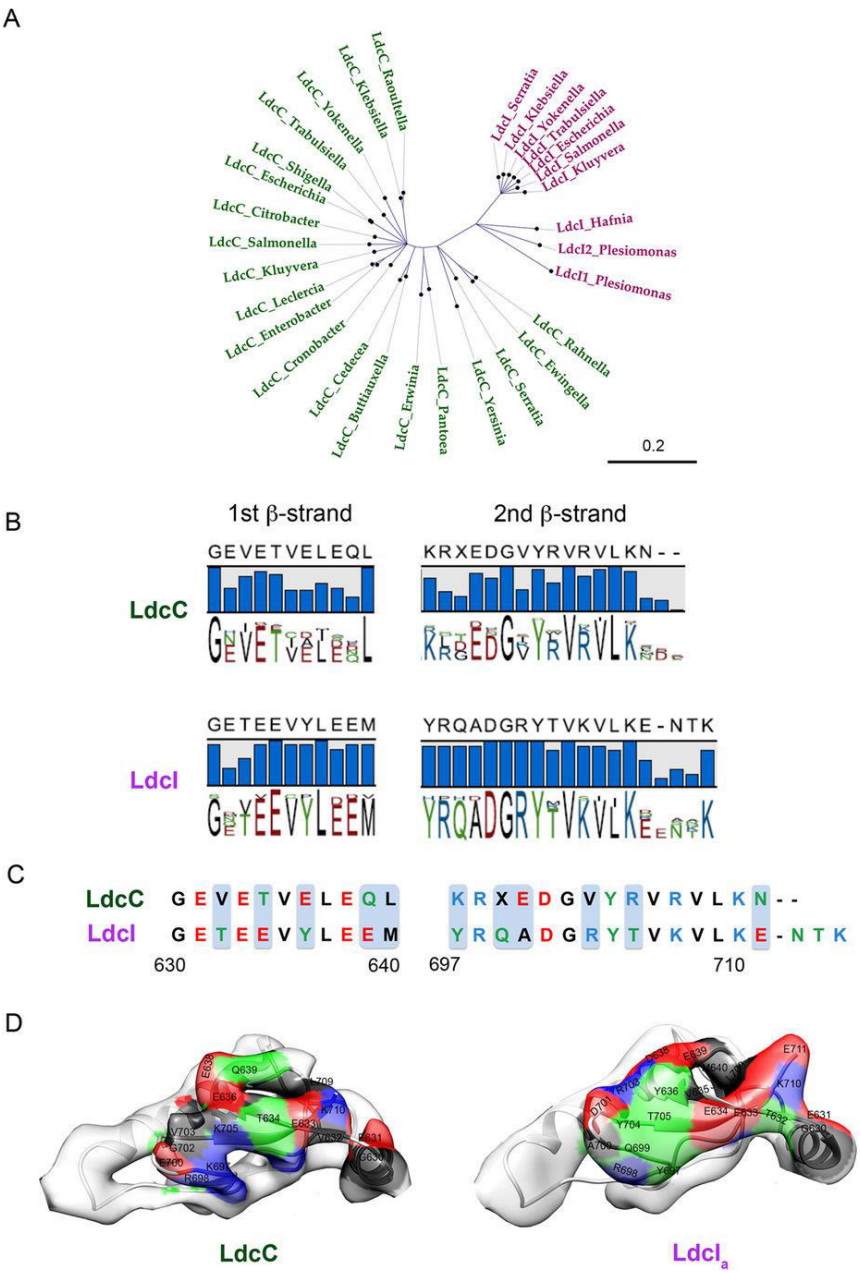


Figure 23. The C-terminal of LdcI is determinant for RavA binding. (A) Maximum likelihood tree with the "LdcC-like" and the "LdcI-like" groups highlighted in
green and pink, respectively. Only nodes with higher values than 95% are shown (500 replicates of the original dataset, see Methods for details). Scale bar indicates the average number of substitutions per site. (B) Analysis of consensus "LdcI-like" and "LdcC-like" sequences around the first and second C-terminal β-strands. The height of the bars and the letters representing the amino acids reflect the degree of conservation of each particular position in the alignment. Amino acids are colored according to a polarity color scheme with hydrophobic residues in black, hydrophilic in green, acidic in red and basic in blue. Numbering as in *E. coli*. (C) Signature sequences of LdcI and LdcC in the C-terminal β-sheet. Polarity differences are highlighted. (D) Position and nature of these differences at the surface of the respective cryoEM maps with the color code as in B[297].

2.4. The decameric structure of the Lysine decarboxylase

Structural characterization of LdcI and LdcC has revealed that dimers are capable of associating to form decamers with a pronounced D5 symmetry, as shown in **figure 24**[254,284,300]. Decamer formation is pH and protein concentration-dependent[284]. In the case of LdcI, the decamer is the predominant species at pH 6 and high enzyme concentrations, but the protein dissociates to form dimers at a higher pH (pH 8.0) and low salt concentrations. At low pH (pH 5.0), the decamers are capable of oligomerizing to form stacks of decamers[284]. Decamers of LdcC are much less sensitive to pH[284].

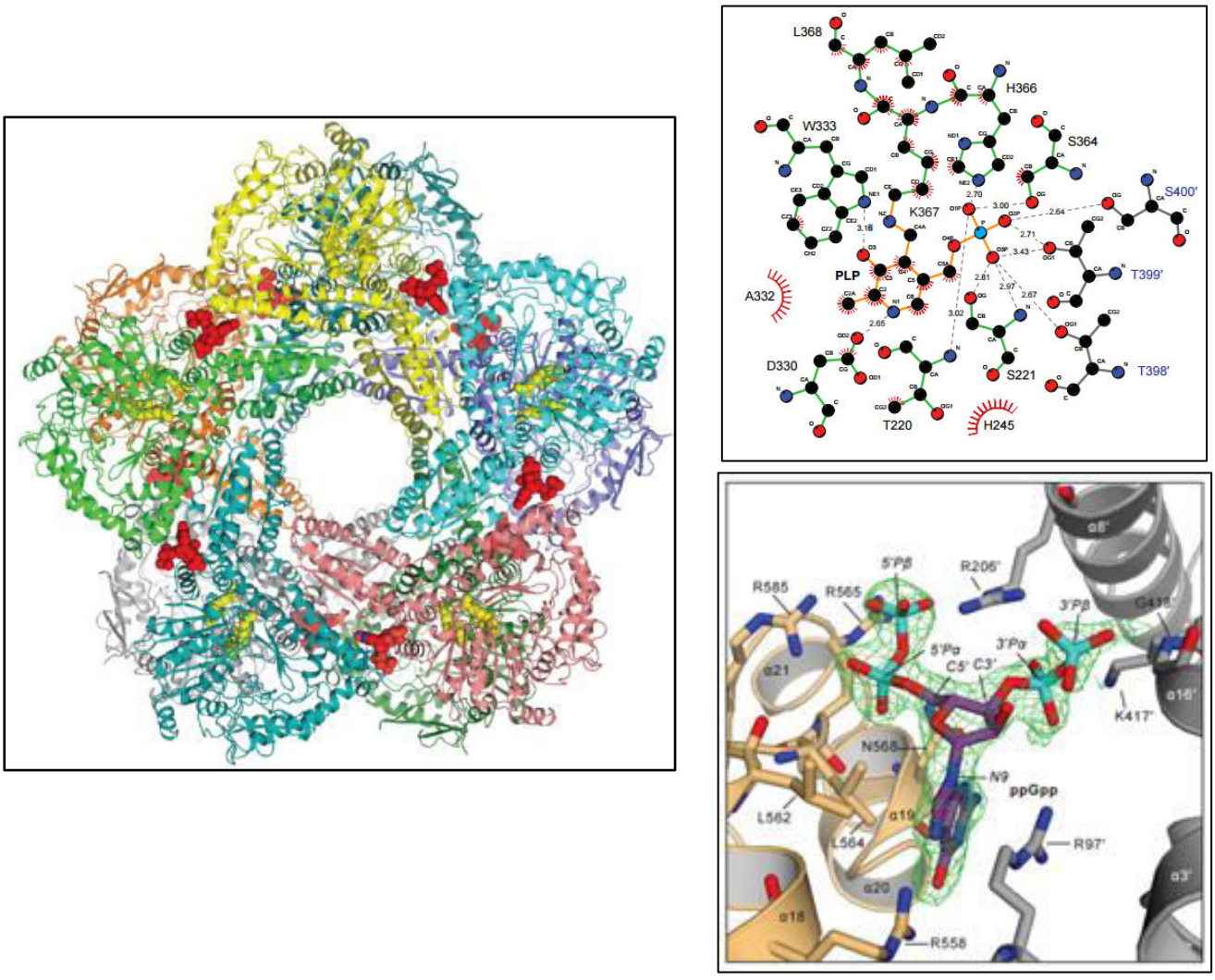


Figure 24.- **The decameric structure of the lysine decarboxylase LdcI from *E. coli* (left).** Each monomer is shown in a particular color, ppGpp residues are displayed in red. **Right, up :** Residues of the PLP Binding site and the important residues for stabilizing the PLP cofactor. **Right, down :** ppGpp binding site, the ppGpp is shown in the electron density while important residues belonging to LdcI are displayed as cartoon in beige or gray. Nitrogen are displayed in blue, oxygen atoms are in red, phosphate atoms are in cyan, the carbon atoms from the ppGpp molecule are shown in violet or black as shown in the upper figure[254,284,300].

During the process of X-ray crystallographic refinement of the LdcI structure, an unexpected electron density that didn't belong to the protein was found. After testing different small molecules, it was found that a total of 10 molecules of ppGpp, the alarmone which mediates the stringent response, were bound at a specific interface between neighboring protomers in the LdcI decamer. This led to the identification of a novel regulatory function for ppGpp, which inhibits the enzymatic activity of LdcI and LdcC under conditions of nutrient starvation[284]. Importantly, the inhibitory effect of ppGpp is prevented by the formation of the cage-like structure between LdcI and its partner RavA[284].

3. The enzymatic activity of LDCs

Enzymatic activity of the lysine decarboxylase has been characterized in different microorganisms such as *E. coli, P. aeruginosa, Burkholderia sp AIU 395*, *Hafnia alvei* and *Vibrio. parahemolyticus,*[235,284,301] as shown in **table 3.** But so far, the most complete analysis was done with LdcI and LdcC from *E. coli*, where the inhibition of ppGpp and other related nucleotides was assessed. In absence of the alarmone, LdcI activity exhibits Michaelis-Menten kinectis with a *Km* for L-lysine in the range of 420 µM and its optimum pH close to pH 5.7, while LdcC has a similar enzymatic activity but with a optimum pH close to 7.5[284]. Addition of ppGpp results in 10-fold inhibition of both LdcI and LdcC. The related nucleotides GTP and GDP were ineffective in both enzymes suggesting a high degree of specificity towards ppGpp. In the presence of ppGpp, the kinetics can be modelled as a simple inhibition mechanism with comparable kinetic parameters to apo-LdcI with Ki values in the nM to mM range. This suggests that LdcI will be effectively inhibited *in vivo* at the physiological concentrations of ppGpp (in the mM range, also see below) reached during the stringent response[302]. It was proposed that the physiological importance of this inhibition mechanism is to avoid the excessive consumption of L-Lysine during stringent response when this amino acid becomes limited. Biochemical studies of other LDCs from *Burkholderia sp., V. parahemolyticus, H. alvei and P. aeruginosa* show similar *Km* values to the ones obtained for LdcI and LdcC. We see also that there are two kinds of LDCs as far as their optimum pH is concerned: the acid adapted ones (LdcI *E. coli,* LDC *Burkholderia, V. parahemolyticus, Hafnia alvei*) the neutral – alkaline ones (LdcC *E. coli,* LdcA *P. aeruginosa)*. Nevertheless, the kinetic values from the other LDCs (not LdcI or LdcC) could be influenced by the inhibition of ppGpp which was not taken into account, and since these other bacteria

also produce ppGpp, it thus possible that their values are ppGpp-influenced and are not comparable to the ones with *E. coli.*

Protein	pH optimum	Km (mM)	Activity (µmol of cadaverine mg of LDC^{-1} min^{-1})	ppGpp inhibition	Reference
LdcI (*E. coli*)	5.2	0.42	234	Yes	Kanjee et al., 2011
LdcC (*E. coli*)	7.5	0.4	200	Yes	Kanjee et al., 2011
Ldc (*Burkholderia sp. AIU 395*)	6	0.87	120	Not tested	Sugawara et al., 2014
Ldc (*Vibrio parahemolyticus*)	5.5	3.2	340	Not tested	Yamamoto el al., 1991
Ldc (*Hafnia alvei*)	5.8	1.7	n.d.	Not tested	Yamamoto el al., 1991
LdcA (*Pseudomonas aeruginosa*)	8.5	0.87	2.2	Not tested	Chou et al., 2010

Table 3. Enzymatic activities of Lysine decarboxylases (AAT-fold) found in literature. From the table, two different types of LDCs appear according to their pH optimum, the acid ones (pH 5-6) and the neutral-alkaline ones (pH 7.5-8.5). The effect of ppGpp inhibition has only been tested in *E. coli*. N. d.: not determined.

4. The molecular partner of the Lysine decarboxylase

4.1. LdcI interacts with the MoxR AAA+ ATPase RavA

In Irina Gutsche's lab, where I did my PhD, it has been shown that the *E. coli* AAA+ ATPase RavA tightly interacted with LdcI but was not capable of binding its close homologue LdcC. RavA belongs to the MoxR family of AAA+ family of proteins (ATPases Associated with a Variety of Cellular Activities) that are a widely-distributed family of ATPases involved in numerous essential cellular processes including DNA replication, protein folding and degradation as well as operating as molecular motors[303,304].

Interaction between LdcI and RavA results in a complex cage-like architecture that is unique in bacteria. The complex is composed by 2 LdcI decamers interacting with 5 RavA hexamers with a nanomolar affinity[296]. The proteins form a complex with dihedral fivefold symmetry and a large central cavity of 3×10^6 Å^3. Enzymatic studies of the cage have shown that RavA prevented binding of LdcI to its potent inhibitor ppGpp by a yet unkown mechanism[240,297]. Thus, the LdcI–RavA complex maintains LdcI activity even if the bacterium experiences both acid stress and starvation, which is often the case when bacteria transit through the stomach before reaching the bowel where the pathogenesis typically occurs[305].

Literature about RavA has recently shown that it binds to respiratory complexes from the electron-transport chain in the membrane such as the NADH dehydrogenase (complex I) and

the Fumarate reductase (complex II)[306]. In addition, it has also been genetically linked to genes participating in the genesis of iron-sulfur clusters[307]. Since RavA belongs to a family of chaperones, my supervisor Irina Gutsche in collaboration with the laboratory of Frédéric Barras in Marseille, is working on the function of the LdcI-RavA complex and its potential involvement in the maturation of respiratory complexes[296,305].

Part II: Materials and Methods

1. Strains, plasmids and oligonucleotides

1.1. *P. aeruginosa* & *E. coli* strains

The tables present the strains that were used during my PhD project.

Strain	Comment	Reference
	P. aeruginosa	
PAO1	Wild type strain, cytotoxic.	Holloway *et al* .,1955
PAO1 *ΔldcA*	PAO1 strain containing a in-frame deletion in the *ldcA* ORF between amino acids 151-641 + TGA replaces codon 151. Gm^S.	This work
PAO1 *ΔldcA ldcA*	Complemented PAO1 *ΔldcA*. miniCTX *PldcA* (CDS) was used to insert an *ldcA* copy and its promoter in the *attB* site. pFlp2 plasmid was used to remove integrase and ATB resistance genes from miniCTX vector. GmS, TcS.	This work
PAO1 *ΔargR*	PAO1 strain containing a in-frame deletion in the *argR* ORF between amino acids 9-207 + TGA replaces codon 9. Cb^S.	This work
CHA	Mucoid CF isolate, cytotoxic.	Toussaint *et al.* , 1993
CHA *ΔrpoS*	CHA strain containing an in-frame deletion in *rpoS* ORF corresponding to amino acids 1-207. RpoS is the sigma 38 factor and is responsible for the regulation of genes in the stationary phase. Cb^S/Gm^S.	Shen *et al.* , 2006
CHA *ΔlasI-rhlI*	CHA strain containing an in-frame deletion of amino acids 1–155 of *lasI* ORF and an in-frame deletion of amino acids 20–201 of *rhlI* ORF. This CHA strain is unable to produce the inducers AHLs needed for the functioning of the *las/rhl* signaling system. Cb^S/Gm^S.	Shen *et al* ., 2006
CHA *ΔpqsA*	CHA strain containing and in-frame deletion in *pqsA* ORF of amino acids 41-410, + TGA replaces the 41e codon. This strain is unable to catalyze the first step in PQS synthesis pathway. Cb^S.	Ina Attrée Lab (Sylvie Elsen)
	Strains for expression studies	
PAO1::*lacZ*	PAO1 with insertion of *lacZ* in *attB* site. miniCTX *lacZ* was used for integration. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1::*gfp*	PAO1 with insertion of *gfp* in *attB* site. miniCTX *gfp* was used for integration. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1::*lux*	PAO1 with insertion of *luxCDABE* in *attB* site. miniCTX *lux* was used for integration. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1::PldcA-lacZ	PAO1 with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ*. miniCTX *PldcA -lacZ* was used for integration in the *attB* site. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1::PldcA-gfp	PAO1 with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ*. miniCTX *PldcA -gfp* was used for integration in the *attB* site. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1::PldcA-lux	PAO1 with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ*. miniCTX *PldcA -lux* was used for integration in the *attB* site. pFlp2 plasmid was used to remove genes from the vector. Cb^S, Tc^S.	This work
PAO1 *ΔargR* ::miniCTX-*lacZ*	PAO1 *ΔargR* with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ*. miniCTX *lacZ* was used for integration in the *attB* site. Tc^r.	This work
PAO1 *ΔargR* ::miniCTX-*PldcA-lacZ*	PAO1 *ΔargR* with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ*. miniCTX *PldcA -lacZ* was used for integration in the *attB* site. Tc^r.	This work

Strain	Comment	Reference
	P. aeruginosa	
CHA::miniCTX-*lacZ*	CHA with insertion of *lacZ* in *attB* site. miniCTX *lacZ* was used for integration. Tc^r	This work
CHA *ΔrpoS* ::miniCTX-*lacZ*	CHAΔrpoS with insertion of *lacZ* in *attB* site. miniCTX *lacZ* was used for integration.Tc^r	This work
CHA *ΔlasI-rhlI* ::miniCTX-*lacZ*	CHAΔlasI-rhlI with insertion of *lacZ* in *attB* site. miniCTX *lacZ* was used for integration. Tc^r	This work
CHA *ΔpqsA* ::miniCTX-*lacZ*	CHAΔpqsA with insertion of *lacZ* in attB site. miniCTX *lacZ* was used for integration. Tc^r	This work
CHA*::* miniCTX-*PldcA*-*lacZ*	CHA with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ.* miniCTX *PldcA -lacZ* was used for integration in the *attB* site . Tc^r	This work
CHA *ΔrpoS::* miniCTX-*PldcA-lacZ*	CHA *ΔrpoS* with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ.* miniCTX *PldcA -lacZ* was used for integration in the *attB* site. Tc^r	This work
CHA *ΔlasI-rhlI* ::miniCTX *PldcA-lacZ*	CHA *ΔlasI -rhlI* with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ.* miniCTX *PldcA -lacZ* was used for integration in the *attB* site. Tc^r	This work
CHA *ΔpqsA::* miniCTX-*PldcA-lacZ*	CHA *ΔpqsA* with a transcriptional fusion betwen *ldcA* promoter (486bp before ATG) and the reporter *lacZ.* miniCTX *PldcA -lacZ* was used for integration in the *attB* site. Tc^r	This work
	E. coli	
DH5α	*E. coli* used for cloning. Genotype: *fhuA2 lacΔU169 phoA glnV44 Φ80' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
Top10	*E. coli* used for cloning. Genotype:mcrA, *Δ(mrr-hsdRMS-mcrBC), Phi80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(*Sm^r*), endA1, nupG*	Invitrogen
BL21(DE3) Rosetta 2	*E. coli* used for protein overexpression. Genotype:F- *ompT hsdS* B(rB- mB-) *gal dcm* (DE3) pRARE2 (Cam^r)	Invitrogen

Table 4. List of strains used during the study. $Tc^{r/s}$: tetracyclin resistance/sensibility, $Gm^{r/s}$: Gentamicin resistance/sensibility, $Cb^{r/s}$: carbenicillin resistance/sensibility.

1.2. Oligonucleotides

The oligonucleotides used during the thesis are cited in table 5.

Name	Sequences	Description
Mut-LdcA-F1	5' GGGGATCCGACACCGTTGCCGGCGAACG-3' (BamHI)	Amplification of the fragment from the 54 to 453 bp of *ldcA*
Mut-LdcA-R1	5'-CGCCGTTGTAGCGCTTGCTCAGAATGGCGGCAGCAGCCCGG-3'	
Mut-LdcA-F2	5'-GCAAGCGCTACAACGGCGTGG-3'	Amplification of the fragment from the 1843 to 2244 bp of *ldcA*
Mut-LdcA-R2	5'-GGAAGCTTGCATTCAACGGTATAGCAGCGC-3'(HindIII)	
LdcA-F°	5'-GCAAGTCTCTACCCTGAGCC-3'	Primers for mutant verification
LdcA-R°	5'-CAGCGCGCTGCAGGTCCAG-3'	
Prom-LdcA F	5'-CTCGAGCGTGCTTCGAACGACTTGC-3'(XhoI)	Amplification of 533bp fragment containing the promoter region of *ldcA.*
Prom-LdcA R	5'-GAATTCTGTTGATCCCGAGCAGCAGG -3'(EcoRI)	
LdcA-comp-F1	5'-CCGCTAGCGTT CGCCCGCCTGCTGCTC-3' (NheI)	Amplification of *ldcA* gene
LdcA-Comp-R1	5'-CCAGTACTAGTACACCCCTTAGCCTGTCGGC-3' (SpeI/ScaI)	
Mut-argR-F1	5'-CCCTCGAGGATATCCTACCCGGCGCACATGAGCG-3' (XhoI/EcoRV)	Amplification of the fragment from the -364 to 27bp of the ATG of *argR* gene
Mut-argR-R1	5'-GGTCAGCTTCGGATGGCTGGATCAACCGATGCGTTGGGGTTGGG-3'	
Mut-argR-F2	5'-TGATCCAGCCATCCGAAGCTGACC-3'	Amplification of the fragment from the 621 to 1006bp of the ATG of *argR* gene
Mut-argR-R2	5'-CCACTAGTGATATCGTCGCAAAGCCCGGCTGAAAC-3' (SpeI/EcoRV)	
Mut-argR-F0	5'-GAGCACTACCTGGCCAAGGT-3'	Primers for mutant verification
Mut-argR-R0	5'-GTCGCGAACTCGGCGACTTC-'3	

Table 5. List of oligonucleotides used during the study. Restriction sites are underligned.

1.3. Plasmids

The list of plasmids that were used and constructed for the thesis are cited in the table 6.

Plasmids	AB	Comments	Origin
Mini-CTX-*lacz*	Tc^r	Vector capable of chromosomic insertion by the site *attB* from *P. aeruginosa* and that allows a transcriptional fusion with the β-galactosidase gene; *tet, Ω-FRT-attP, lacZ, ori, oriT, int*	Schweizer,2001
Mini-CTX-*gfp*	Tc^r	Vector capable of chromosomic insertion by the site *attB* from *P. aeruginosa* and that allows a transcriptional fusion with the *gfp* gene; *tet, Ω-FRT-attP, lacZ, ori, oriT, int*	Schweizer,2001
Mini-CTX-*lux*	Tc^r	Vector capable of chromosomic insertion by the site *attB* from *P. aeruginosa* and that allows a transcriptional fusion with the *luxCDABE* operon; *tet, Ω-FRT-attP, lacZ, ori, oriT, int*	Calvo *et al.* 2000
MiniCTX-PLdcA-*lacZ*	Tc^r	MiniCTX1-*lacZ* , insertion of *ldcA* promoter (-492bp upstream of ATG), cloning by XhoI - EcoRI	This work
MiniCTX-PLdcA-*gfp*	Tc^r	MiniCTX1-*gfp* , insertion of *ldcA* promoter (-492bp upstream of ATG), cloning by XhoI - EcoRI	This work
MiniCTX-PLdcA-*lux*	Tc^r	MiniCTX1-*lux* , insertion of *ldcA* promoter (-492bp upstream of ATG), cloning by XhoI - EcoRI	This work
pET-29B-*ldcA*	Ap^r	pET-29B, insertion of *ldcA* gene by using NdeI- BglII restriction enzyme sites	This work
pEXG2- *ΔldcA*	Gm^r	Suicide plasmid containing a mutant *ldcA* allele. Fragment was cloned from pTOPO-mut-ldcA by using BamHI - Hind III restriction enzyme sites	This work
pEX100T-*ΔargR*	Ap^r /Cb^r	Suicide plasmid containing a mutant *argR* allele. Fragment was cloned from pTOPO-mut-argR by using EcoRV- EcoRV restriction enzyme sites	This work
MiniCTX-*pldcA* (CDS)	Tc^r	Mini-CTX, insertion of the *ldcA* gene with is own promoter (2778 bp fragment), cloning by XhoI, SpeI	This work
pCR-Blunt IITOPO	Km^r	Cloning vector . It allows the insertion of PCR fragments with high efficiency	Invitrogen
pTOPO-mut-ldcA	Km^r	Contains a mutant LdcA allele constructed by SOE PCR by assembling fragment 1 (54-453) + fragment 2 (1843-2244) amplified from PAO1 genome. BamHI - HindIII have been added at each end for cloning purposes	This work
pTOPO-PldcA	Km^r	Contains a *ldcA* promoter 533bp fragment (from -486bp up to +16bp from ATG of *ldcA* gene)	This work
pRK2013	Km^r	ColE1 mob+, tra+ (RK2) mobile « helper » plasmid	Ditta *et al.*, 1980
pFLP2	Ap^r /Cb^r	Plasmid encoding *flp* recombinase	Hoang *et al.*,1998

Table 6. List of plasmids used and constructed for the thesis. Tc^r: tetracyclin resistance, Ap^r: ampicillin resistance, Gm^r: Gentamicin resistance, Km^r: kanamycine resistance, Cb^r: carbenicillin resistance

2. Microbiology and cell culture

2.1. Growth media

Lysogeny Broth (LB medium)

- Tryptone……..10 g/L
- Yeast extract……5 g/L
- NaCl……10g/L
- Distilled Water…….up to 1 L

The medium is buffered at pH 7 (25°C) and sterilized by autoclave. LB agar is prepared by adding 15g/L of agar. The LB medium is the preferred medium to amplify bacterial populations for molecular biology techniques, for overnight pre-cultures.

***Pseudomonas* Isolation Agar (PIA medium)**

- PIA powder (Difco laboratories) ……..45g/L
- Glycerol……...20mL
- Distilled Water……up to 1L

The medium is sterilized by autoclave. The PIA medium contains the antibiotic Irgasan, which allows the selection of *P. aeruginosa*. It is particulary important for triparental mating where *P. aeruginosa* is mixed with *E. coli*.

Cation-adjusted Mueller Hinton Broth (CAMHB)

- Acid hydrolysate of casein…….17.5 g/L
- Beef extract……3 g/L
- Starch……..1.5 g/L
- Distilled Water……up to 1L

The medium is buffered at pH 7.4 (25°C) and sterilized by autoclave. The CAMHB medium was used for testing the antiobiotic resistance of *P. aeruginosa.*

Minimal medium P (MMP)

- **MMP Salts**
    - Na_2HPO_4……..30mM
    - KH_2PO_4……..14mM

    - $(NH_4)_2SO_4$……20mM
- $MgSO_4$……1mM
- $FeSO_4$……4µM
- PLP……0.4µM
- **Carbon sources (pH7.4)**
    - Glucose/L-Glutamate/L-Lysine/L-Arginine……..20mM
- Autoclaved distilled water……up to 1L

MMP Salts are prepared in water at 5X concentration and then filtered (0.2 µm), they are buffered at pH7 and autoclaved. The $MgSO_4$, the carbon sources are prepared at 1M and filtered (0.2µM). The $FeSO_4$ is prepared at a stock concentration of 4mM, to solubilize the iron solution we use H_2SO_4 at a final concentration of 0.1M. All solutions are then mixed at the final concentration as shown above.

M9 Minimal Medium

M9 Salts

- Na_2HPO_4……33.7 mM
- KH_2PO_4……22.0 mM
- NaCl……8.55 mM
- NH_4Cl……9.35 mM
- $MgSO_4$ ……1 mM
- $CaCl_2$……0.3 mM
- Carbon sources (pH 7.4)……20 mM
- Autoclaved distilled water……up to 1L

M9 Salts are prepared at 5X stock solution, it is then filtered (0.2 µm), buffered at pH7 and autoclaved, the other salts and carbon sources are filtered (0.2µM) and distillated water is added up to 1L. The preparation protocol is the same as MMP.

M63 Medium

M63 salts

- $(NH_4)_2SO_4$……15 mM
- KH_2PO_4……100 mM

- $FeSO_4$……..1.8 µM
- $MgSO_4$……..1 mM
- Carbon sources (pH 7.4)……20 mM
- Autoclaved distilled water……up to 1L

M63 Salts are prepared at 5X stock solution, it is then filtered (0.2 µm), buffered at pH7 and autoclaved, the other salts and carbon sources are filtered (0.2µM) . The preparation protocol is the same as MMP.

2.2. Antibiotics

The following antibiotics were used for my project. The higher concentrations of antibiotic in solid media were used for the CHA strain of *P. aeruginosa,* while the lower concentrations were used for the PAO1 strain.

	E. coli		***P. aeruginosa***	
Antibiotic	**Liquid**	**Solid**	**Liquid**	**Solid**
Ampicillin	100 µg/mL	100 µg/mL	-	-
Kanamycin	25 µg/mL	25 µg/mL	-	-
Tetracyclin	10 µg/mL	10 µg/mL	-	200 µg/mL
Gentamicin	50 µg/mL	50 µg/mL	200 µg/mL	200 to 400 µg/mL
Carbenicillin	-	-	300 µg/mL	300 to 500 µg/mL

Table 7. The antibiotics used for the thesis project. Antibiotic solutions are sterilized using 0.2µM filters. Tetracyclin has been prepared in 50% ethanol.

2.3. Culture of *P. aeruginosa*

P. aeruginosa is an opportunistic pathogen that has been classified as a class 2 pathogen, which are biological agents that can cause illness in humans and health workers, its dissemination capabilities in the community is very low, there are efficient treatments and prophylaxis.

2.3.1. PAO1

The common reference strain is *P. aeruginosa* PAO1, a spontaneous chloramphenicol-resistant mutant of the original PAO strain (earlier called "*P. aeruginosa* strain 1") that had been isolated in 1954 from a wound in Melbourne, Australia[308].

We have cultured PAO1 in erlenmeyers at 37°C at 300 RPM in LB medium, CAMHB medium, MMP, M9 and M63 medium. We also followed successfully the culture of the bacteria in 96-well plates at 37°C at 250 RPM. For anaerobic cultures, we designed an experimental set up in LB medium + 100mM KNO_3 in which the medium filled up hermetically closed falcons. Under this condition, the bacteria were put at 37°C without agitation and exhibited a generation time (0.75 h^{-1}) which is comparable with other reported generation time under anaerobic conditions (0.7h^{-1}). Bacteria were also culture in LB agar or PIA medium at 37°C.

2.3.2. CHA

The lab strain of *P. aeruginosa* CHA (Toussaint et al., 1993) was isolated at CHU of Grenoble, from a bronchopulmonary lavage from a CF patient. CHA presents a mucoid phenotype and possesses a T3SS (ExoS, ExoT, ExoY) that is active *in vivo* and *in vitro*. Its antibiotic resistance phenotype is more resistant to gentamicin and carbenicillin compared to PAO1, it has not acquired resistance to carboxypenicillins, aminosides, tetracyclin.

2.4. *E. coli* culture

E. coli has been cultured in LB medium at 37°C at 300RPM. For overproduction of proteins, cultured conditions varied and temperatures were as low as 18°C at 300RPM. *E. coli* was also cultured in LB agar at 37°C.

2.5. Follow up of cell growth and density.

The growth of bacteria was followed by measuring optical density at 600 nm (OD_{600nm}) by using the lab spectrophotometer (Eppendorf®). Bacterial growth was also followed by measuring at 595nm (OD_{595nm}) with a 96-well plate reader Multiskan EX THERMO®.

To follow the number of bacteria in growth medium, we counted colony forming units that were obtained by serial dilutions. Dilutions where done by taking 40µl of bacterial suspension and mixed with 160 µl of PBS, a volume of 30 µl of bacteria are then dropped in LB agar plates. The plates are put at 37°C degrees overnight, the next day CFU are counted

and only drops containing between 100 to5 colonies are taken into account for calculations. We obtained that the equivalence for 1 OD_{600nm} was 6.9 ± 0.3 x 10^8 CFU/mL for PAO1 strain.

2.6. Strain conservation

E. coli and *P. aeruginosa* strains were conserved at -80°C in culture medium with a final concentration of 40% Glycerol (Nunc Cryotubes®, Sigma Aldrich). *P. aeruginosa* grown in LB agar was inoculated in in tubes with microbeads (PROTECT® Bacterial Preservers, Technical service consultant Limited, UK) that were conserved at -80°C.

3. Molecular Biology Techniques

3.1. Polymerase chain reaction

PCR reactions were done in an Eppendorf® Mastercycler from our lab. PCR reaction started by denaturation of the matrix at 94°C for 2 to 5 minutes, this step was followed by 30 synthesis cycles that end in a final elongation step at 68°C or 72°C (depending on the polymerase) for 10 minutes.

A synthesis cycle is constituted by 3 steps

1. DNA denaturation which last 30 seconds
2. Annealing of the primers on the matrix at Ta: Tm -5°C for 30 seconds.

We defined the Ta (annealing temperature) as Tm – (4°C or 5°C). The Tm (fusion temperature) was calculed with the formula: Tm = 4°C(G+C) + 2°C(A+T).

3. Elongation by DNA polymerase. The duration and temperature of the reaction depends on the polymerase that is used (GC clontech, Phusion, Q5).

The usual reaction mix contains:

- DNA matrix……10 to 100 ng
- Primer concentration (forward and reverse)……0.1 µM
- dNTP (deoxytrinucleotide mix)……0.2 mM each one
- Reaction Buffer……as indicated
- Polymerase……as indicated
- GC melt (GC Clontech only)……as indicated
- Double distilled water……up to 20 or 50 µL

GC melt, DMSO up to 4% or $MgCl_2$ are additives that can be added to optimize the PCR reaction. For colony PCR, one colony was resuspended in sterile water 100µL. The cell suspension was heated at 95°C for 10 minutes to lyse the cells. The suspension was diluted 1/10 and 0.5µL of the suspension was used as DNA matrix.

To facilitate cloning, we added restriction site at the endof our primers. In this case, we use two different annealing temperatures. The first temperature is calculated without talking into account the restriction sites that were added, only 5 reaction cycles are done for this step. Afterwards we do 25 reaction cycle with annealing temperature corresponding to the full-length primer.

The GC clontech kit was used to amplify *P. aeruginosa* DNA for verifications, while the Phusion kit was used for amplying DNA for mutagenesis and complementation plasmids because it has a higher fidelity than the first one.

3.2. SOE PCR (Splicing by Overlap Extension- Polymerase Chain Reaction) technique

The SOE-PCR technique allows to fusion two DNA fragment by PCR byusing restriction enzymes, which is extremely useful to create truncated alleles needed for mutagenesis. As shown in **figure 25**, each fragment is amplified in separate reaction tubes, the 3' primer for the amplification of the fragment A has a complementary sequence with the 5' primer of fragment B. After the first amplification, we mix and hybridize both fragments A and B so the DNA polymerase can proceed with elongation, at the end we will obtain a fusion of both DNA fragments. When the first fusion is accomplished we can add the primer 5'A and 3'B for a normal PCR reaction, to amplify the full length fragment the fusioned DNA in high yields.

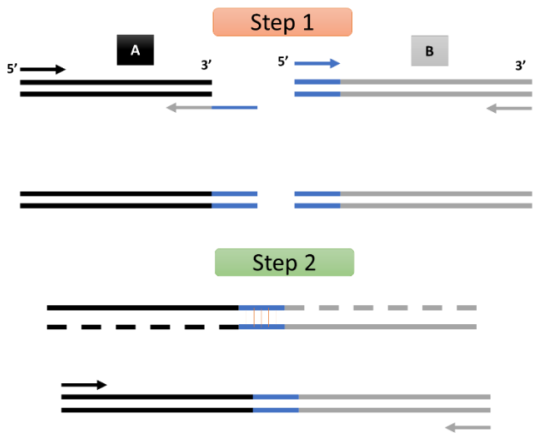


Figure 25. Diagram of the SOE-PCR technique

4. Mutagenesis and complementation

4.1. Triparental mating

Triparental mating is a form of Bacterial conjugation where a conjugative plasmid present in one bacterial strain assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third bacterial strain. Plasmids are introduced into bacteria for such purposes as transformation, cloning, or transposon mutagenesis.

The protocol we used consisted in using an overnight culture of *P. aeruginosa* is incubated for 2 h at 42 ° C in a water bath. In parallel, 30 µl of overnight cultures of the suicide plasmid-containing *E. coli* strain and the *E. coli* helper strain with pRK2013 plasmid are mixed and drop off on a dry LB agar plate. The plate is placed in an oven for 2 hours at 37 °C. Then 30µl of *P. aeruginosa* culture is added on top of *the E. coli* drop and incubated for additional 4 to 6 hours at 37 ° C. The mixed colony is resuspended in an Eppendorf containing 300µl of LB Eppendorf. 100 µl of the suspension is spread on PIA plates with the proper antibitioc. Clones (transconjugants) resistant to tetracycline will be able to grow on these plates.

This method was used for mutagenesis with suicide vector *pEXG2-mut-ldcA*, for complementation with miniCTX *pldcA(CDS)* and for the chromosomal insertion of the vectors constructed transcriptional fusion such as, miniCTX *lacZ*, miniCTX *gfp,* miniCTX *lux,* miniCTX *PldcA-lacZ*, miniCTX *PldcA-gfp*, miniCTX *PldcA-lux*.

4.2. Allelic exchange by using pEXG2 suicide plasmid

To investigate the role of LdcA in *P. aeruginosa* we created mutants and their complemented strains in PAO1. The mutants were obtained by performing allelic exchange between the wild type *ldcA* gene and a truncated one that had a deletion in the catalytic site containing PLP-binding domain of the protein. We created the pEXG2-mut-ldcA plasmid by using the SOE-PCR technique to create a mutant allele. The mutant *ldcA* had an internal deletion in the central region of the gene which contains the catalytic site and the conserved Lysine393 that binds PLP need for decarboxylation. The mutant *ldcA* also added a stop codon 400bp after the ATG to avoid the synthesis of a big truncated protein with undesirable deleterious effects on cell fitness. The advantage of doing allelic exchange with this truncated gene, is that we minimize the possibility of having polar effects on the expression of the *ldcB* gene which is found in operon downstream of *ldcA*.

As shown in **figure 26**, the mutagenesis involved 2 recombinations. The first one, involved the integration of the suicide plasmid in the chromosome, in one of the regions that are homologous between the truncated gene and the wild type gene. These recombinants are selected by using PIA plates with the proper antibiotic pressure.

When antibiotic pressure is lifted a second recombination event happens between the homologue regions between the truncated and the wild type gene of the chromosome. From this event, we can obtain a clone with either the truncated gene or the wild type gene. To select the bacteria that successfully recombinated for a second time, a counter-selection of bacteria is performed in a growth media without antibiotic and with 5-10% saccharose. The counterselection is based in the activity of the gene *sacB*, that encodes a levansucrase that generates toxic polymers that kill cells. The *ldcA* mutant candidates are checked for gentamicin resistance and then they are verified by PCR. By using the primers LdcA-F° and LdcA-R°, we could see the mutant allele as a 1Kb amplification fragment, while the wild type would appear as a 2.5 Kb amplification fragment. Finally, mutants were also verified by checking their capacity of growing in MMP containing 20mM of L-Lysine.

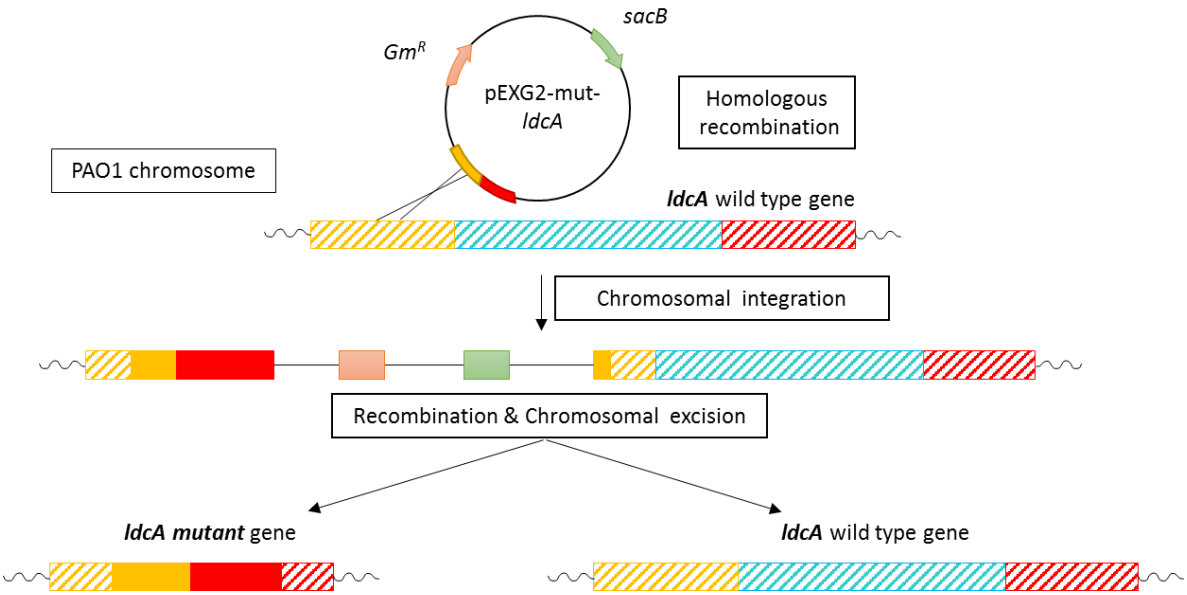


Figure 26. Schematic presentation of the mechanism of mutagenesis of *ldcA* using suicide plasmid *pEXG2-mut-ldcA*. PAO1 strains are conjugated with *pEXG2-mut-ldcA*, plasmid recombines with *ldcA* wild type and integrates in the chromosome and is kept by antibiotic pressure, the strains are then streaked in new media without antibiotics and with sucrose, this way we counter-selected strains that could be either wild type or *ldcA* mutant strain.

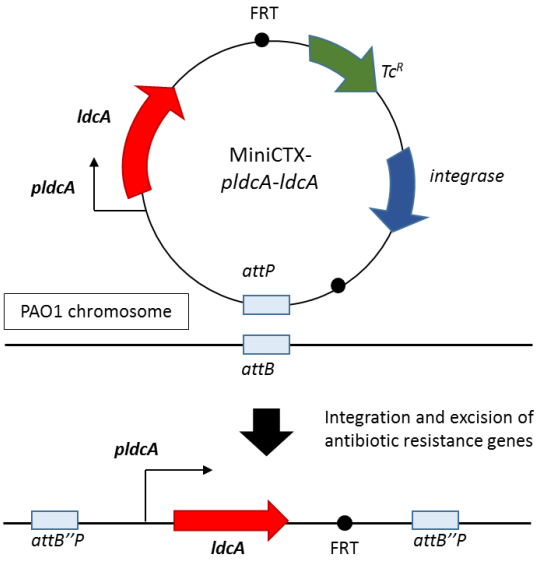


Figure 27. Complementation of *ldcA* mutants with integrative plasmid miniCTX-*pldcA-ldcA*. PAO1 *ΔldcA* mutants are complemetend with the plasmid miniCTX-*pldcA-ldcA* that is integrated into the *attB* site, due to the integrase of the CTX phage. Both the backbone of the miniCTX plasmid containing the integrase and *Tc*^{r} genes can be excised by using FLP recombinase that recognize FRT sites. By this method of complementation only one copy of *ldcA* gene expressed by its own promoter is integrated into the PAO1 chromosome.

4.3. Complementation by using miniCTX *PldcA*(CDS).

A complementation miniCTX plasmid containing the sequence of the open reading frame of *ldcA* with 489bp of the region upstream of the ATG as the promoter. This construction was done to complement the mutant strains with a *ldcA* expressed at wild type levels.

The complementation miniCTX *PldcA*(CDS) was introduced in the strain PAO1*ΔldcA* by using the technique of triparental mating, as shown in **figure27**. The miniCTX contains the elements that allow the integration of our complementation allele in *attB* site, such as the *attP* site and the integrase. The complemented candidates that were tetracyclin resistant were then verified by using PCR and by observing their capacity to grow on minimal medium MMP containing 20mM L-Lysine after 24H.

To get rid of the backbone of the miniCTXgenes *tet* (tetracyclin resistance) and *int* (integrase) from the miniCTX, we transformed our complemented strains with the plasmid pFLP2 (carbenicillin resistance) that expresses the pFLP recombinase and *sacB*. The Cb resistant clones are spread on PIA + 5% sucrose plates to select strains that have lost the pFLP2 plasmid with *sacB*. The *sacB*$^{-}$ clones are then selected for their sensitivity for tetracycline, carbenicillin and for their capacity to grow on MMP 20mM L-Lysine.

The same method was used to integrate the miniCTX that had the promoter-reporter gene fusions used to examine the activity of *ldcA* promoter. In the same way pFLP2 plasmid was used to get rid of the miniCTX genes.

4.4. Chemically competent *P. aeruginosa*

One milliliter of overnight culture is taken and centrifuged for 30 seconds at 13000g at room temperature. The supernatant is discarded and the cell pellet is re-suspended in 1ml of 0,1 M $MgCl_2$ solution at 4°C. The solution is centrifuged at 13000g for 30 seconds. Afterwards, the cell pellet is resuspended in a 1ml of a cold TG-salts solution (75mM $CaCl_2$, 6 $MgCl_2$, 15% Glycerol(w/v)) and incubated for 10 minutes at 4°C. After the incubation time, the solution is centrifuged one last time at 13000g for 30 seconds. The cell pellet is resuspended in 200µl of ice-cold TG-salts and cells are ready to transform.

4.5. Transformation of *P. aeruginosa*

We take 100 µl of the bacterial suspension that are mixed with 200ng of plasmid DNA. The DNA-cell mixture is incubated on ice for 15 minutes. The mixture is then heat-shocked at 37°C for 2 minutes, and 500 µl LB broth were immediately added. The tubes were incubated at 37°C for 1 h in a shaking incubator. Finally, 200-µL aliquot of each cell suspension was plated on LB agar containing the appropriate antibiotic.

5. Biochemistry

5.1. Expression of *ldcA* in *P. aeruginosa* PAO1 and CHA.

The expression of *ldcA* was studied in both the strains PAO1 and CHA, by inserting promoter-reporter fusions of the *ldcA* promoter region (from -489 to + 16 with respect of the ATG) with the *lacZ, gfp or luxCDABC* operon.

5.2. Measurement of β-galactosidase activity

500 µl of bacterial suspension are permeabilized by adding 20 µl of 0.1% SDS and 20 µl of $CHCl_3$ and then vortexed for 1 min. Volumes of 25 µl to 100 µl are collected and mixed with of Z buffer (0.06M Na_2HPO_4, NaH_2PO_4 0.04 M KCl 0.01M, 1 mM $MgSO_4$, 0.04 M β-mercapto-ethanol, pH 7) in order to have 1ml of mixture reaction. The reaction is initiated at 28 ° C by the addition of 0.2 ml of ONPG (ortho-nitrophenyl- β-D-galactopyranoside) at 4 mg/ml. When the color is yellow enough, the reaction is stopped by adding 0.5 ml of 1M Na_2C0_3 (pH

10). Cellular debris are separated by sedimentation, and the OD420nm of the supernatant is read with a spectophometer. A solution containing the reaction buffer with ONPG is used as blank for the readouts. The promoter activity is expressed in Miller Units using the formula:

$(1000 \times OD420\ nm) / (time_{minutes} \times Volume_{ml} \times OD6_{00nm})$

Where $Volume_{ml}$ is the volume of the bacterial suspension, $time_{minutes}$ corresponds to the reaction time and the OD600nm is the measurement of the turbidity of the bacterial suspension which is proportional to the number of bacteria. This protocol is based from the original protocol published in Experiments in molecular genetics by Jeffrey H. Miller, 1972.

5.3. Measurement *ldcA* expression by luminescence and fluorescence

The analysis of the transcriptional activity of the *ldcA* was carried out by measuring the bioluminescence emitted by the bacteria possessing the *luxCDABE* operon. We constructed the luminescence of the strains PAO1::*PldcA-lux* and a PAO1::*lux* control without a promoter. The strains were cultured in LB medium and MMP medium in Nunc® MicroWell 96 well optical bottom plates at 37°C at 250RPM. Luminiscence was measured every hour in a Fluoroskan Ascent™ FL Microplate Fluorometer and Luminometer while at the same time OD_{595nm} was measured in a Multiskan™ FC Microplate Photometer. The relative luminescence units (RLU) were normalized by the OD_{595nm}, to calculate the transcriptional activity of the promoter.

The same experimental setting was used to measure the transcriptional activity of the *ldcA* promoter fusioned with the *gfp.* The strains PAO1::*PldcA-gfp* and PAO1::*gfp* were cultured in the same way as the *lux* strains. The Fluoroskan was configured with an excitation filter (485/20 nm) and an emission filter (530/25 nm) to follow up the fluorescence of bacteria every hour, at the same time we followed the growth by measuring the turbidity at OD_{595nm}. The fluorescence units were normalized by OD_{595nm} to obtain the *ldcA* expression.

5.4. Separation of proteins by SDS-PAGE and Western Blotting

5.4.1. Preparation of cytosolic extracts of *P. aeruginosa*

To visualize the synthesis of LdcA by *P. aeruginosa*, 100ml of bacterial cell culture were followed throughout the day. Samples were collected at different phases of cell growth (exponential phase, late exponential, early stationary phase, and stationary phases), then

they were centrifuged and concentrated to an equivalent OD600nm of 10. The buffer used for resuspending the pellet is composed of 20 mM Tris/HCl, 500mM NaCl, 10 mM EDTA pH 8 and complete™, EDTA-free Protease Inhibitor Cocktail. The suspension is sonicated at 4°C for 3 times 1 minute (200 joules) or until the suspension gets clear. Lysed cells are ultracentrifuged at 80 000g with a TLA 120.2 (Beckman) rotor at 4°C for 30minutes. The supernatant containing the cytosolic extract is mixed with a 4X Laemmli buffer (277.8 mM Tris-HCl, pH 6.8, 44.4% glycerol (v/v), 4.4% SDS, 0.02% bromophenol blue, 355mM of 2-mercaptoethanol).

The mixture of supernatant and Laemmli buffer is denatured at 95°C for ten minutes. The proteins become denatured and disulfure bridges are disrupted, this process allows the separation of protein by their mass and not by their structure.

5.4.2. Denaturing Polyacrylamide gel electrophoresis (SDS-PAGE)

Denatured cytosolic extracts of PAO1 in the Laemmli buffer are separated by SDS-PAGE. The gels used in this technique typically consist of acrylamide, bisacrylamide, the denaturant SDS and a buffer with an adjusted pH. The gel is composed of two different parts: the stacking gel and the resolving gel. The composition of the gels is exposed in **table 8** for the stacking gel and in **table 9** for the resolving gel.

Concentration	6%	8%	10%	12%	15%
Volume	10ml	10ml	10ml	10ml	10ml
H_2O	5.3	4.6	4.0	3.3	2.3
30% acrylamide/bis	2.0	2.7	3.3	4.0	5.0
1.5 M Tris (pH8.8)	2.5	2.5	2.5	2.5	2.5
10% SDS	0.1	0.1	0.1	0.1	0.1
10% AP	0.1	0.1	0.1	0.1	0.1
TEMED	0.008	0.006	0.004	0.004	0.004

Table 8. Composition of resolving gel of SDS-PAGE.

Volume	1 mL	2 mL	5 mL	6 mL
H_2O	0.68	1.4	3.4	4.1
30% acrylamide/bis	0.17	0.33	0.83	1.0
1.0 M Tris (pH6.8)	0.13	0.25	0.63	0.75
10% SDS	0.01	0.02	0.05	0.06
10% AP	0.01	0.02	0.05	0.06
TEMED	0.001	0.002	0.005	0.006

Table 9. Composition of the stacking gel of SDS-PAGE (5% polyacrylamide).

The electrophoresis is performed in a buffer composed of TrisHCl 25 mM pH 8, glycine 200 mM, SDS 0,1% at 200V at room temperature. The bromophenol blue present in the Laemmli buffer allows to follow the migration. Once the migration is performed then we proceeded to stain the gel by using a Coomasie blue solution (2g/l of Coomassie R250, ethanol 50%, acetic acid 10%). To see the proteins from the gel, a first step of Coomasie blue coloring is needed, afterwards the gel needs to be decolored in a solution composed of acetic acid/ethanol/water (10/30/60).

6. Western Blotting

6.1. Purification of LdcA for antibody production

The *pET29b-ldcA* plasmid was constructed to overexpress recombinant His-tagged LdcA in BL21(DE3) *E. coli* in LB medium + 34µg/ml of Kanamycin at 20°C, 300RPM. Next day bacteria were pelleted at 5000g at 4°C and then resuspendent in **Buffer A: 25 mM Tris-HCL (pH 7.5), 0.3 M NaCl, 5% glycerol, 1mM DDT + complete (Roche)** and lysed by using a microfluidizer MP-110. Lysed cells were centrifuged at 40000g and the supernant was passed through a Ni-NTA column. The nickel column was washed with 10 column volumes (CV) of **Buffer A** and then by using 10 CVs of **Wash buffer** (Buffer A + 50mM imidazole). The protein was eluted by using **Buffer A +** 300mM of Imidazole and aliquots from every purification step were denatured and revealed by SDS-PAGE.The purest aliquots were pooled and concentrated, then they were purified further by a subsequent size-exclusion chromatography (SEC) step using a Superdex 200 10/30GL column in a buffer composed of **25 mM Tris-HCL (pH 7.5), 0.3 M NaCl, 5% glycerol, 1mM DDT, 0.1 mM PLP**. The elution fractions that contained pure LdcA were then analysed by negative stain electron microscopy and showed the presence of decamers.

6.2. Immunisation protocol for obtaining LdcA antibodies from rabbits.

Three miligrams of purified LdcA were used for immunization of 2 NZW rabbits with a 42-day protocol. Four injections of LdcA were administered (Day 0, 7, 14 ,27) and rabbit serum was extracted on day 0 (control) and at day 27. Finally, whole serum was obtained from the animals at day 42. The polyclonal antibodies from the serum where diluted 1/1000 in PBST + 0.5% skim milk and then tested against purified protein. The antiserum proved to be very sensitivy since it could detect 10ng of purified LdcA protein and thus interesting for Western Blotting.

6.3. Immunodetection of LdcA in purified samples or in cytosolic extracts

In order to dected LdcA protein by Western blotting, the proteins separated by SDS-PAGE Were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes.

First the gels and membranes were equilibrated for a few minutes in the transfer buffer (48 mM Tris, 39 mM glycine, 20% ethanol, 0.03% SDS, pH 8). A “sandwich” is done by stacking Wattman paper, the membrane and the polyacrylamide gel onto the transfer system (Invitrogen). The transfer takes place for 1h30 at 20V and 100mA. When the transfer is finished, the membrane is washed by using PBST (PBS + 0.1% Tween 20) and then the membrane is incubated for 1 hour in a solution of PBST + 5% Skim Milk, to saturate unspecific binding sites. The membrane is then washed 3 times with PBST and it is incubated with the anti-LdcA primary antibody. The primary antibody (anti-LdcA from rabbit) is diluted in PBSt + 0.5% Skim milk (1/1000). The incubation takes place for onehour and then the membrane is washed 3 times with PBST. The secondary antibody is an anti-rabbit IgG from mice coupled with the HRP (Horse Radish Peroxydase) and is diluted at 1/50000 in PBST + 0.5% Skim milk. After one hour of incubation, the secondary antibody is washed 3 times with PBST to avoid any excess signal. In a similar way, we used a commercial anti-RpoA antibody (RpoA is RNA polymerase protein that is expressed constitutively in *P. aeruginosa*), as a charge control to see whether the cytosolic extracts had the same amount of protein.

The reveal is done by a chemoluminiscent reaction using the Luminata Classico Western HRP substrate. The luminescent signal from the membrane is measured in a Chemidoc Imaging System.

7. Microbial physiology

7.1. Biolog

7.1.1. Evaluation of pH susceptibility of *P. aeruginosa* by using BIOLOG™.

The fitess of *P. aeruginosa* to different pH was analysed using the Phenotype MicroArray technology (Biolog inc). All fluids, reagents and PM Panels were supplied by Biolog and used according to the manufacturer's instructions. Briefly, bacteria were cultured for 16 h on LuriaBertani agar plates at 37°C. Cells were harvested with a sterile cotton swab and suspended in 10 ml of inoculating fluid (IF-0), and the cell density was adjusted to 85% transmittance (T) on a Biolog turbidimeter. The minimal media inoculating fluid (IF-0a) contained 100 mM NaCl, 30 mM triethanolamine-HCl (pH 7.1), 2.0 mM NaH_2PO_4, 0.25 mM Na_2SO_4, 0.05 mM $MgCl_2$, 1.0 mM KCl, 1.0 mM ferric chloride, and 0.01% tetrazolium violet. Before the addition to PM microtiter plates, bacterial suspensions were further diluted into 12 ml of IF-0a (per plate) in the relevant inoculating fluid. The carbon source for the experiments was 20mM Glucose and 5mM Ammonium sulfate was added as nitrogen source. Substrate utilization was measured via the reduction of a tetrazolium dye forming a purple formazan (supplied by Biolog) and is indicative of active cellular respiration at 37°C. Formazan formation was monitored at 15 min intervals for 30 h. Kinetic data were analyzed with OmniLog-PM software. Each experiment was performed at least twice per strain. The PM10 plate was used to test the presence of amino acid decarboxylases involved in acid stress response and to test the presence of amino acid deaminases involved in alkaline stress response.

Plate	Molecule	Description	Molecule	Description
PM10	pH 4.5 + L-Alanine	Decarboxylase assay	pH 9.5 + L-Alanine	Deaminase assay
	pH 4.5 + L-Arginine		pH 9.5 + L-Arginine	
	pH 4.5 + L-Asparagine		pH 9.5 + L-Asparagine	
	pH 4.5 + L-Aspartic acid		pH 9.5 + L-Aspartic acid	
	pH 4.5 + L-Glutamic acid		pH 9.5 + L-Glutamic acid	
	pH 4.5 + L-Glutamine		pH 9.5 + L-Glutamine	
	pH 4.5 + Glycine		pH 9.5 + Glycine	
	pH 4.5 + L-Histidine		pH 9.5 + L-Histidine	
	pH 4.5 + L-Isoleucine		pH 9.5 + L-Isoleucine	
	pH 4.5 + L-Leucine		pH 9.5 + L-Leucine	
	pH 4.5 + L-Lysine		pH 9.5 + L-Lysine	
	pH 4.5 + L-Methionine		pH 9.5 + L-Methionine	
	pH 4.5 + L-Phenylalanine		pH 9.5 + L-Phenylalanine	
	pH 4.5 + L-Proline		pH 9.5 + L-Proline	
	pH 4.5 + L-Serine		pH 9.5 + L-Serine	
	pH 4.5 + L-Threonine		pH 9.5 + L-Threonine	
	pH 4.5 + L-Tryptophan		pH 9.5 + L-Tryptophan	
	pH 4.5 + L-Tyrosine		pH 9.5 + L-Tyrosine	
	pH 4.5 + L-Valine		pH 9.5 + L-Valine	
	pH 4.5 + Hydroxy-L-Proline		pH 9.5 + Hydroxy-L-Proline	
	pH 4.5 + L-Ornithine		pH 9.5 + L-Ornithine	
	pH 4.5 + L-Homoarginine		pH 9.5 + L-Homoarginine	
	pH 4.5 + L-Homoserine		pH 9.5 + L-Homoserine	
	pH 4.5 + Anthranilic acid		pH 9.5 + Anthranilic acid	
	pH 4.5 + L-Norleucine		pH 9.5 + L-Norleucine	
	pH 4.5 + L-Norvaline		pH 9.5 + L-Norvaline	
	pH 4.5 + a-Amino-N-Butyric acid		pH 9.5 + Agmatine	
	pH 4.5 + p-Aminobenzoate		pH 9.5 + Cadaverine	
	pH 4.5 + L-Cysteic acid		pH 9.5 + Putrescine	
	pH 4.5 + D-Lysine		pH 9.5 + Histamine	
	pH 4.5 + 5-Hydroxy-L-Lysine		pH 9.5 + b-Phenylethylamine	
	pH 4.5 + 5-Hydroxy-L-Tryptophan		pH 9.5 + Tyramine	
	pH 4.5 + D,L-Diamino-a,e-Pimelic acid		pH 9.5 + Creatine	
	pH 4.5 + Trimethylamine-N-Oxide		pH 9.5 + Trimethylamine-N-Oxide	
	pH 4.5 + Urea		pH 9.5 + Urea	

Table 10. Testing conditions in plate PM10. Left column: List of conditions for testing decarboxylases involved in acid stress response in *P. aeruginosa.* We can find different compounds including amino acids, intermediates in amino acid biosynthesis, carboxylic acids and hydroxy-amino acids, electron acceptor: TMAO and Urea. **Right column: List of conditions for testing deiminases and other enzymes involved in alkaline stress response in *P. aeruginosa***.

7.1.2. Evaluation of antibiotic resistance of *P. aeruginosa* by using BIOLOG™.

PM11C and PM15B plates were used to determine the antibiotic resistance of the bacteria. In addition to a unique substrate (antibiotics), each well of the panels also contains the needed minimal medium components and a specific dye. The strain was grown overnight at 37°C on LB agar medium, and then cells were picked up with a sterile cotton swab and transferred into a sterile, capped tube containing 20 ml of the inoculation fluid (IF-0, Biolog Inc.). We added 1mM L-Arginine to induce the expression of the *ldcA* for the antibiotic resistance test. Cell density was adjusted to 81% transmittance on the Biolog turbidimeter. The PM11 and PM15 plates were inoculated

with the cell suspension (100 µL/well), and then incubated at 30°C for 48 h in the Omnilog Incubater/Reader (Biolog Inc., Hayward, USA).

Plate	Molecule	Description
PM11C	Amikacin	wall, lactam
	Chlortetracycline	protein synthesis, 30S ribosomal subunit, tetracycline
	Lincomycin	protein synthesis, 50S ribosomal subunit, lincosamide
	Amoxicillin	wall, lactam
	Cloxacillin	wall, lactam
	Lomefloxacin	DNA topoisomerase
	Bleomycin	DNA damage, oxidation
	Colistin	membrane, cyclic peptide
	Minocycline	protein synthesis, 30S ribosomal subunit, tetracycline
	Capreomycin	protein synthesis
	Demeclocyline	protein synthesis, 30S ribosomal subunit, tetracycline
	Nafcillin	wall, lactam
	Cefazolin	wall, cephalosporin
	Enoxacin	DNA topoisomerase
	Nalidixic acid	DNA topoisomerase
	Chloramphenicol	protein synthesis, amphenicol
	Erythromycin	protein synthesis, 50S ribosomal subunit, macrolide
	Neomycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
	Ceftriaxone	wall, cephalosporin
	Gentamicin	protein synthesis, 30S ribosomal subunit, aminoglycoside
	Potassium tellurite	toxic anion
	Cephalothin	wall, cephalosporin
	Kanamycin	protein synthesis, 30S ribosomal subunit, aminoglycoside
	Ofloxacin	DNA topoisomerase

Table 11. Testing conditions in plate PM11C.

Plate	Molecule	Description
PM15B	Procaine	wall, cephalosporin
	Guanidine hydrochloride	membrane, chaotropic agent
	Cefmetazole	wall, cephalosporin
	D-Cycloserine	wall
	EDTA	chelator, hydrophilic
	5,7-Dichloro-8-hydroxy-quinaldine	chelator, lipophilic
	5,7-Dichloro-8-hydroxyquinoline	chelator, lipophilic
	Fusidic acid	protein synthesis, elongation factor
	1,10-Phenanthroline	chelator, lipophilic
	Phleomycin	DNA damage, oxidation
	Domiphen bromide	membrane, detergent, cationic, fungicide
	Nordihydroguaiaretic acid	lipoxygenase, fungicide
	Alexidine	membrane, electron transport, biguanide
	Nitrofurazone	nitro compound, oxidizing agent, DNA damage
	Methyl viologen	oxidizing agent
	3,4-Dimethoxybenzyl alcohol	oxidizing agent, free radical-peroxidase substrate
	Oleandomycin	protein synthesis, 50S ribosomal subunit, macrolide
	Puromycin	protein synthesis, 30S ribosomal subunit, premature chanin termination
	CCCP	respiration, ionophore, H+
	Sodium azide	respiration, uncoupler
	Menadione	respiration, uncoupler
	2-Nitroimidazole	nitro compound, oxidizing agent, ribonucleotide DP reductase inhibitor
	Hydroxyurea	ribonucleotide DP reductase inhibitor, antifolate (inhibits thymine and methionine synthesis)
	Zinc chloride	toxic cation

Table 12. Testing conditions in PM15B.

7.2. Testing of antibiotics susceptibility by the CLSI guidelides.

MIC testing was performed using the broth macrodilution method in accordance with CLSI guidelines[309]. Briefly, antibiotics were added to 1 ml of CAMHB, and 2-fold serial dilutions were performed. Logarithmic-phase cultures of *P. aeruginosa* were diluted and inoculated into each tube to a final concentration of 1 x 10^6 CFU/ml. Cell cultures were then incubated at 37°C for 24 h. For MIC testing in the presence of cadaverine, it was added at specific concentrations to each tube prior to incubation for 24 h. The MIC was defined as the lowest concentration of a given antibiotic that inhibited the growth of the organism within 24 h.

7.3. Assay of bacterial persistence to antibiotics.

To determine the number of persister cells of P. aeruginosa, PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA* strains were grown in 10 ml CAMHB in 125-ml flasks to mid-logarithmic phase (0.3-0.6 OD_{600nm}) and were challenged with 8X the MIC of carbenicillin (500µg/ml) for 6 h at 37°C, a condition tested in literature[239]. A 100 µl aliquot was removed, and 10-fold serial dilutions were performed in 1X phosphate buffered saline (PBS). 30µl aliquots of the diluted samples were plated onto LB agar plates and were incubated overnight for determination of viability. The number of persisters is assessed by CFU counting and by comparing to the initial number of cells before antibiotic addition.

7.4. T3SS-dependent cytotoxicity against macrophages

One day before infection, 3 to $5x10^5$ macrophage (J774) in 500 µl of DMEM are put into a 24 well-plate and incubated overnight at 37°C, 5% CO_2. The DMEM growth medium is exchanged 3 hours before infection. The infection is being done at a MOI (Multiplicity of Infection: number of bacteria per macrophage) of 1 and 5 using culture of *P. aeruginosa* at OD_{600nm} of 1 ($6x1O^8$ bacteria/ml) that was grown under non-inducing T3SS conditions. 30 µl of the supernatant are being taken every 60 minutes, during 4 hours. The cytotoxicity is estimated by measuring the lactate dehydrogenase activity of the taken sample using a « Cytoxicity detection kit » (Roche Diagnostics). The reactions are developed in a dark environment and the measurement is obtained by reading the OD480nm with a Multiskan EX THERMO®. The negative control corresponds to the measurement of the macrophage supernatant without any bacterial infection (basal level of LDH from macrophages found in solution). The positive controle corresponds to the total lysis of macrophages that is obtained by adding 300µl of Triton X-100 2% solution. The cytoxicity values are calculated by substracting the negative control to each infection sample and by normalizing the values using the value of the positive control.

8. Bioinformatics

8.1. Promoter analysis.

The region that we decided to use for expression and bioinformatics analysis started at -489 bp and finished at + 12bp of the ATG of *ldcA* open reading frame. The analysis was carried

out in programs such as RSAT and PRODORIC used for the location of patterns and conserved sequences. The sequence used is the following:

GCTTCGAACGACTTGCCGCTGCGACGGTGGGTCGCTGCATATCGACCGACGGCGATCACGCTG
TCGCCGCCGTCGATCAGGCGCTCGATCTCGACCCTGAAGCCCTCCCAGTCCCGTTGCAAGGCCT
GGAACACACCGGCGCCGATGGCGGCCGCGCCGACATAGGTGCCGGCCAGCGGGAAACCGGCC
ATTTCCGTCCATTCGGTGCGTTCGTCCATGTCCGCCAGCATTCCGGCGAGGTCCCCACGGCTAG
AGGCCGCATAGTGATCGCTGACGATCCGATAAGCGCTGCGCATCGTTGTCGCTCCGTAGCAGGG
CAGAGAGGTCCATGCTCCTCGCTGGCGGGAGCGGAGGCAAGCGCGGCGCGCCGACTTGTGTTG
CCGCGACCTTTTCTTACAAGGGCAAGCCGCCGGGGGTTACGCGCGTGCCGGCAAGTCTCTACC
CTGAGCCGACCGGCGGGGTTCGCCCGCCTGCTGCTCGGGATCAACA**ATG**TATAAAGACCTCAAA
TTTCCCGTCCTCATCGTCCATCGCGA

In red and bold is the ATG start codon of the *ldcA gene*.

Transcription factor	Sequence	Comments	Reference
RpoN (Sigma54 or NtrA)	TGGCAC-N_5-TTGCA TGGC-N_9-GC TGGC-N_9-GC	Binding site for the Sigma factor 54 (-24/-12 elements) needed for initiation of RNA synthesis.	Heurlier et al. 2003
RpoD (Sigma 70)	TTGaCc-N_{17}-TAtAAT	Binding site for the Sigma factor 70 (-35/-10 elements) needed for initiation of RNA synthesis. Used for the	Potvin et al.,
RpoS (Sigma 38)	CTATACT	Binding site for the Sigma factor 38 (-35/-10 elements) needed for initiation of RNA synthesis. Needed for the transcription of genes involved in stationary phase.	Schuster et al., 2014
IHF	WATCAA-N_4-TTR	The integration host factor (IHF) is a DNA-binding and -bending protein with roles in local DNA structural organization and transcriptional regulation in Gram-negative bacteria.	Mohr et al. 1992
ArgR	TGTCGC-N_6-GNAA	The consensus ArgR binding site consists of two half-sites in a direct-repeat arrangement with the consensus sequence TGTCGCN_6-GNAA. In most cases, the second halfsite sequences are relatively more conserved than the first half-site sequences.	Lu et al., 2004
LasR (las-box)	NNCT-N_{12}-AGNN	Lux family regulator	Whiteley et al., 1999
Vfr	ANWWTGNGAWNYAGWTCACAT A-N_3-TGNGA-N_3-AGNTCACA	Homolog to the CRP (cAMP receptor protein) from E. coli. involved in catabolite repression. Involved in the expression of genes involved in acute virulence, quorum sensing, flagellum biogenesis, iron acquisition, enterotoxin production and the heat shock response.	Kanack et al. 2006

Table 13. Sequences of transcription factor binding sites that were searched in *ldcA* promoter region by RSAT. A: adenine, T: Thymine, G: Guanine, C: Cytosine, N: any nucleotide, W: weak bond (A or T), S: strong bond: (G or C), R: Purine, Y: Pyrimidine. Nucleotides with small caps are poorly conserved.

8.2. Genetic environment of Lysine decarboxylases in *Enterobacteria* and *P. aeruginosa.*

Out of a non-exhaustive list of 50 species of Enterobacteriaceae, 22 were found to contain genes annotated as *ldcI* or *ldcC*. An analysis using MUSCLE (EMBL-EBI) with default parameters showed that these genes share more than 65% identity. To verify annotation of these genes, we compared their genetic environment with that of *E. coli ldcI* and *ldcC*. Indeed, in *E. coli* the *ldcI* gene is in operon with the *cadB* gene encoding a lysine-cadaverine

antiporter, whereas the *ldcC* gene is present between the *accA* gene (encoding an acetyl-CoA carboxylase alpha subunit carboxyltransferase) and the *yaeR* gene (coding for an unknown protein belonging to the family of Glyoxalase/Dioxygenase/Bleomycin resistance proteins). Compared with this genetic environment, the annotation of several *ldcI* and *ldcC* genes in enterobacteria was found to be inconsistent. For example, several strains contain genes annotated as *ldcC* in the genetic background of *ldcI* and viceversa, as in the case of Salmonella enterica and *Trabulsiella guamensi*. Furthermore, the gene with an "*ldcC*-like" environment was found to be annotated as *cadA* in particular strains *of Citrobacter freundii, Cronobacter sakazakii, Enterobacter cloacae subsp. Cloaca, Erwinia amylovora, Pantoea agglomerans, Rahnella aquatilis, Shigella dysenteriae, and Yersinia enterocolitica subsp. enterocolitica*, whereas in *Hafnia alvei, Kluyvera ascorbata, and Serratia marcescens subsp. marcescens,* the gene with an "*ldcI*-like" environment was found to be annotated as *ldcC*. In addition, as far as the genetic environment is concerned, *Plesiomonas* appears to have two ldc genes with the organization of the *E. coli ldcI* (operon *cadBA*). Consequently, we corrected for gene annotation consistent with the genetic environment and made multiple sequence alignments using version 8.0.1 of the CLC Genomics Workbench software. A phylogenetic tree was generated based on Maximum Likelihood and following the Neighbor-Joining method with the WAG protein substitution model as shown in **figure 14**. The reliability of the generated phylogenetic tree was assessed by bootstrap analysis. The presented unrooted phylogenetic tree shows the nodes that are reliable over 95%. Remarkably, the multiple sequence alignment and the resulting phylogenetic tree clearly grouped together all sequences annotated as *ldcI* on the one side, and all sequences annotated as *ldcC* on the other side. Thus, we conclude that all modifications in gene annotations that we introduced for the sake of consistency with the genetic environment are perfectly corroborated by the multiple sequence alignment and the phylogenetic analysis. Since the regulation of the lysine decarboxylase gene (i.e. inducible or constitutive) cannot be assessed by this analysis, we call the resulting groups "*ldcI*-like" and "*ldcC*-like" as referred to the *E. coli* enzymes, and make a parallel between the membership in a given group and the ability of the protein to form a cage complex with RavA.

Organization of the *ldcA* gene from the website Pseudomonas.com was then compared to the ones of the canonical *ldcI* and *ldcC* from the analysis in enterobacteria.

N°	Organism	*ldcI (cadA)*	*ldcC*	Annotation corrected to be consistent with genetic environment	Comment
1	*Buttiauxella agrestis*	NO	YES		
2	*Cedecea davisae*	NO	YES		
3	*Citrobacter freundii*	NO	YES	YES	*WP_003829162.1 is wrongly annotated as cadA in strain 4_7_47CFAA*
4	*Cronobacter sakazakii*	NO	YES	YES	*WP_004385449.1 is wrongly annotated as cadA in strain NM1240*
5	*Edwardsiella tarda*	YES	YES	YES	*WP_034165623.1 is is wrongly annotated as ldcC in strain 80813*
6	*Enterobacter cloacae subsp. cloacae*	NO	YES	YES	*WP_014882641.1 is wrongly annotated as cadA in strain ENHKU01*
7	*Erwinia amylovora*	NO	YES	YES	*WP_004159429.1 is wrongly annotated as cadA in strain ATCC 49946*
8	*Escherichia coli*	YES	YES		
9	*Ewingella americana*	NO	YES		
10	*Hafnia alvei*	YES	NO	YES	*WP_025798687.1 is wrongly annotated as ldcC in strain FB1*
11	*Klebsiella pneumoniae subsp. pneumoniae*	YES	YES		
12	*Kluyvera ascorbata*	YES	YES	YES	*WP_035891521.1 is wrongly annotated as ldcC in strain ATCC 33433*
13	*Leclercia adecarboxylata*	NO	YES		
14	*Pantoea agglomerans*	NO	YES		*WP_010254154.1 is wrongly annotated as cadA in strain ATCC 23216*
15	*Plesiomonas shigelloides*	YES	YES		Both genes are found in operon with one *cadB* copy
16	*Rahnella aquatilis*		YES	YES	*WP_015689430.1 is wrongly annotated as cadA in strain 302-73*
17	*Raoultella planticola*		YES	YES	*WP_032697299.1 is wrongly annotated as cadA in strain ATCC 33071*
18	*Salmonella enterica subsp. enterica*	YES	YES	YES	WP_038394318.1 and WP_001021050.1 are inversely annotated in strain OLF-SE9-10012
19	*Serratia marcescens subsp. marcescens*	YES	YES	YES	*WP_025304020.1 is wrongly annotated as ldcC in strain ATCC 9150*
20	*Shigella dysenteriae*	NO	YES	YES	*WP_001021034.1 is wrongly annotated cadA in strain 155-74*
21	*Trabulsiella guamensis*	YES	YES	YES	WP_038157866.1 and WP_038155510.1 are inversely annotated in strain ATCC 49490
22	*Yersinia enterocolitica subsp. enterocolitica*	NO	YES	YES	*WP_005173170.1 is wrongly annotated as cadA*
23	*Yokenella regensburgei*	YES	YES		
	Summary	10 *ldcI*	*22 ldcC*	14 CORRECTED ANNOTATIONS	

Table 14. List of Enterobacteria presenting problems in annotations with *ldcI* and *ldcC* genes.

8.3. LdcA Phylogeny

8.3.1. Dataset assembly

Functionally characterized sequences of Ldc, Adc and Odc belonging to the AAT-fold family were retrieved from Genpep: LdcI (NP_418555.1), LdcC (NP_414728.1), Adc (NP_418541.1), OdcC (NP_417440.1), and OdcI (NP_415220.1) from *E. coli str. K-12 substr. MG1655* and LdcA (NP_250509.1) from *P. aeruginosa* PAO1). These sequences were used as seeds to query a local database containing 4,466 complete proteomes of prokaryotes downloaded from the NCBI (ftp://ftp.ncbi.nlm.nih.gov) with the BLASTP 2.2.6 software[310]. Subject sequences associated to e-values lower than visually estimated threshold were retrieved and aligned using MAFFT v7[311]. The resulting multiple alignment was used to build a HMM profile with the HMMbuild program from the HMMER v3.1b1 package[312]. This profile was then used to query the local database of complete proteomes with the HMMsearch program. Sequences with e-values lower than 9.5e-18 were retrieved. Finally, the search for potential unannotated sequences was performed using TBLASTN 2.2.6 on genomic sequences corresponding to the 4,466 complete proteomes. This work was performed in collaboration with Pierre Garcia from Céline Brochier laboratory in the University of Lyon.

8.3.2. Phylogenetic inference

Multiple alignments were built with MAFFT using the L-INS-i option that allows the construction of accurate multiple alignments and trimmed with BMGE v1.1 with matrix substitution BLOSUM30[313]. Maximum likelihood trees were inferred with PhyML 3.1[314]. The best suited evolutionary models were selected using the model test tool implemented in Iqtree v1.4.1 according to the BIC criteria[315]. The robustness of the inferred trees was assessed using the non-parametric procedure implemented in PhyML (100 replicates of the original datasets). Bayesian trees were inferred using MrBayes v3.2.6[316]. 2 runs were launched with 4 chains for each run. Starting trees are randomly generated and first 25% trees were deleted to eliminate burnin fraction. Sampling of trees depends on dataset size. Convergences of chains were observed checking Likelyhood distribution and graph of Likelyhood. Figures of trees have been generated using EvolView[317]. Reference phylogenies of species containing Ldc, Adc or Odc homologues were inferred using ribosomal proteins as suggested elsewhere[318]. Ribosomal protein sequences from the complete proteomes of these species were identified using the RiboDB database engine[319] and aligned with MAFFT

using the L-INS-i option. The resulting multiple alignments were trimmed as described above and combined to build a large supermatrix that was used to build maximum likelihood phylogenetic trees. This work was performed in collaboration with Pierre Garcia from Céline Brochier laboratory in the University of Lyon.

Part III: Results

Chapter I: The identification of the lysine decarboxylase from the AAT-fold family in P. aeruginosa

At the beginning of my thesis, at least two different genes were identified as encoding Lysine decarboxylases in *P. aeruginosa* PAO1. The first gene identified in 2010 was *PA4115,* a gene encoding a putative lysine decarboxylase, was shown to reduce the amount of persister cells after treatment with carboxypenicillins. The mutant of this gene *PA4115* in PAO1 exhibited also 25% reduction in lysine decarboxylase activity measured in whole cell extracts[239].

In 2011, a second gene, *PA1818* was identified as coding for a lysine decarboxylase. This gene was initially identified during a study characterizing the ArgR regulon and L-arginine metabolism. Due to this regulatory feature and the 41% identity of its product with *E. coli* arginine Decarboxylase AdiA, *PA1818* was thought to code for an ADC of the AAT-fold family. However, biochemical characterization of recombinant and purified PA1818 led the authors to concludethat the enzyme was only capable of decarboxylating L-Lysine and not L-Arginine. Moreover, they showed that PA1818 was necessary for the growth of *P. aeruginosa* in minimal medium with L-Lysine[235,236]. The characterization of PA1818 had opened the door to a new chapter on research of Lysine decarboxylases in bacteria outside the group of enterobacteria and makes us wonder whether this protein is playing a role in protecting *P. aeruginosa* against environmental stress in a way similar to that observed with LdcI and LdcC in *E. coli*.

1. Bioinformatics analyses of LdcI and LdcC homologues in *P. aeruginosa*

The starting point of our research began with the identification of Lysine decarboxylases that are homologues of the entebacterial LdcI and LdcC from the AAT-fold family. In order to find them, I performed BLASTP search using the FASTA sequence of amino acid sequences of LdcI and LdcC to search homologues in the genome of P. aeruginosa PAO1. By using this method, we found that indeed PA1818 (750 aa) is predicted to be a putative LAODC (Lysine/Arginine/Ornithine Decarboxylase) with an AAT-Fold and is 41.7% identical to LdcI (715 aa). The CDD (Conserved Domain Database) program from NCBI identified the three characteristics domains found in LAODCs (Wing domain: OKR_DC_1_C, PLP-binding domain:

OKR_DC_1, and C-terminal domain: OKR_DC_1_N). We also performed the same BLASTP search in the other sense, by using PA1818 to find homologues in *E. coli* and closest homologues found are indeed LdcI (41.7%), LdcC (42.23%) and also the arginine decarboxylase AdiA (40.9%).

	PA1818	**PA4115**	**PA1346**	**LdcI (*E. coli*)**	**LdcC (*E. coli*)**	**AdiA (*E. coli*)**
PA1818	100	15.96	18.96	41.67	42.23	40.90
PA4115	15.96	100	18.96	15.21	16.13	14.48
PA1346	26.94	18.96	100	26.54	27.89	26.4
LdcI (*E. coli*)	41.67	15.21	26.54	100	69.14	34.94
LdcC (*E. coli*)	42.23	16.13	27.89	69.14	100	35.22
AdiA (*E. coli*)	40.90	14.48	26.40	34.94	35.22	100

Table 15. Percent identity matrix obtained after multiple sequence alignments using the amino acid sequences of PA1818, PA4115, PA1346, LdcI, LdcC, AdiA with the program MUSCLE. Closest homologues with the highest percentage are highlighted in green and farthest one are highlighted in red.

Another unexpected gene was also found by this method: *PA1346* encodes a protein (515 aa) that appears to be 26.5% identical to LdcI. CDD showed that the predicted protein possesses also three LAODC domains: (Wing domain: OKR_DC_1_C, PLP-binding domain: OKR_DC_1, and C-terminal domain: OKR_DC_1_N). However, the PLP-binding domain is only partly present since it is 200 amino acids shorter than its homologue PA1818 and LdcI.

Although PA4115 was proposed to be a lysine decarboxylase, we could not find it by BLAST search of LdcI homologues. By using CDD, as shown in **figure 28**, we found that indeed, the protein does not have the same predicted domains and is actually 60% identical to the YgdH protein from E. coli. YghH and PA4115 belong to a family of proteins called the "Lonely Guy" proteins that possess a conserved Rossman-fold involved in the binding of nucleotides and cytokinins.

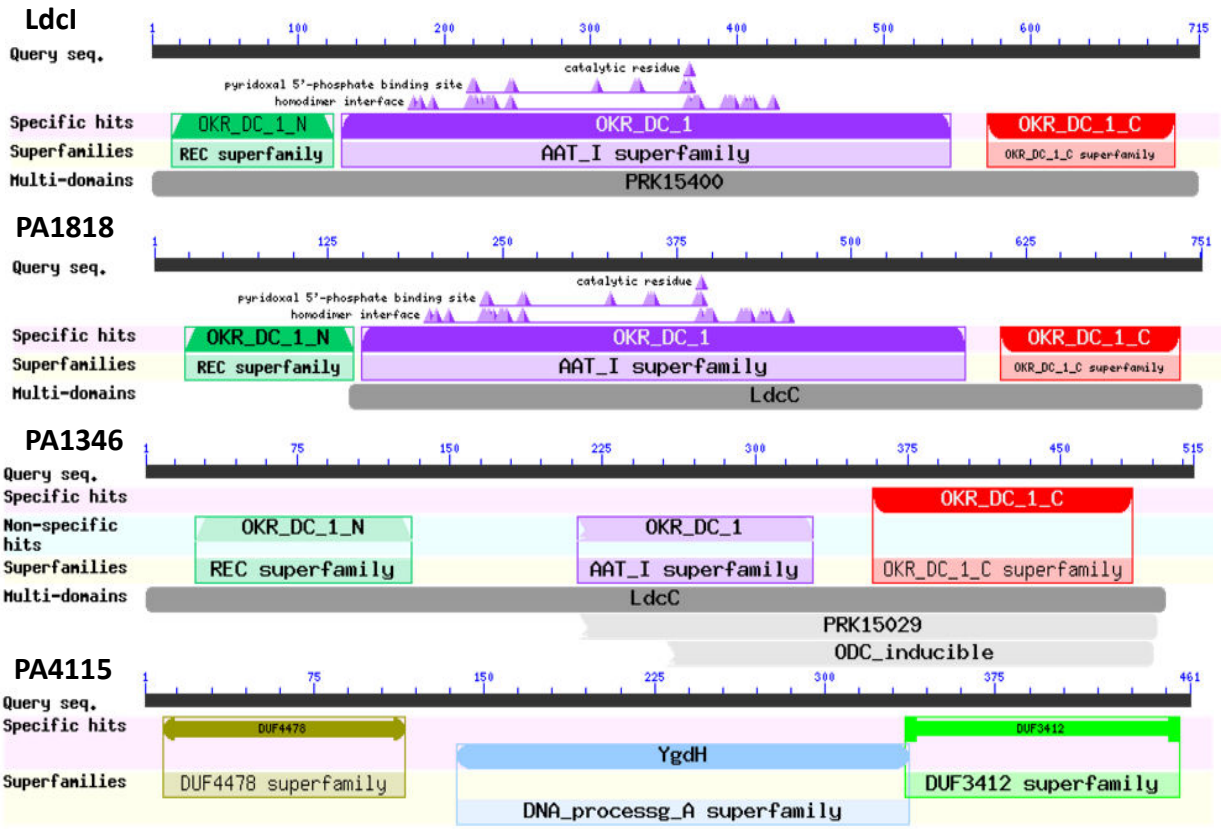


Figure 28. Conserved domain analysis of different predicted Lysine decarboxylases from *P. aeruginosa* and *E. coli* using the program CDD (Conserved domain database from NCBI). The first image corresponds to the predicted domains of LdcI whose structure is already known. Then are presented the predicted domains for PA1818, PA1346 and PA4115. LdcI from AAT-fold family, PA1818 and PA1346 possess three domains: Wing domain: OKR_DC_1_C, PLP-binding domain: OKR_DC_1, and C-terminal domain: OKR_DC_1_N). PA4115 contains also 3 domains of which a N-ter and C-ter domains of unknown function and a central YgdH domain with a conserved Rossman-fold.

With all these identified genes, we performed a deeper analysis by doing multiple sequence alignments using the amino acid sequences of PA1818, PA4115, PA1346, LdcI, LdcC, AdiA with the program MUSCLE (EMBL-EBI with standard options) **Table 15**. From this analysis, we obtained a percent identity matrix that strongly suggests that PA1818 is the closest homologue of *E. coli* LDCs. Moreover, AdiA seems to be closer to LdcA from *P. aeruginosa* than to LdcI and LdcC from *E. coli*.

Figure 29. Multiple sequence alignment of the LDC candidates. LdcA, PA1346 from *P. aeruginosa* with AdiA, LdcI and LdcC from *E. coli* focusing in the region containing the conserved Lysine needed for PLP-biding**.** LdcA possesses a conserved Lysine for PLP-binding while it is absent in PA1346.

Further analysis of the multiple sequence alignment of the putative LdcI homologues in *P. aeruginosa* PA1818 and PA1346 with LdcI, LdcC and AdiA was performed. As shown in **figure 29**, this analysis revealed that the conserved lysine 367 needed for PLP-binding in LdcI is also present in LdcA but that the alignment was not able to identify the conserved pocket with the lysine residue in PA1346. Overall the analysis indicates us that LdcA is the closest homologue of the AAT-fold LDCs from *E. coli*.

2. The genetic environment of *PA1818*

During my PhD work in Irina's lab, I could also participate in the project concerning the lysine decarboxylases from *E. coli*. As I presented in the introduction in chapter II about the Lysine decarboxylases, it was discovered that, unlike LdcI, LdcC is not capable of binding the AAA+ ATPase RavA. We wondered whether we could find a structural difference that could explain the reason why LdcC was incapable of binding RavA. Since CTD was responsible for the binding, we looked for a conserved amino acid signature in this domain. To do this, we extracted the amino acid sequences from LdcI and LdcC from a non-exhaustive database with 50 *Enterobacteraceae* that we choose randomly from the NCBI. While searching for these sequences in the NCBI database, we found that there were three groups of enterobacteria: the ones containing only LdcI, the ones containing only LdcC and the ones containing both. However, we noticed a problem with the sequence annotations. For example, in *Salmonella* sp. which contains both LdcI and LdcC, the annotations were inversed in the strain OLF-SE9-10012. The same problem was found for *Kluyvera ascorbata, Serratia marcescens, Trabulsiella guamensis*. Another problem was encountered in other species where there was

only one locus for the LDC, as it was annotated LdcI for one strain and LdcC in another one. This was the case for *Cronobacter freundii, Enterobacter cloacae, Erwinia amylovora, Hafnia alvei, Rahnella aquatilis, Raoultella planticola, Shigella dysenteriae, Yersinia enterocolitica*[297].

To overcome this problem of annotation, I analysed the genetic environment of *ldcI* and *ldcC* in all these enterobacteria and found that it was conserved for both genes, as shown in **figure 30**. In the case of *ldcI*, the gene is always associated with *cadB*, encoding the lysine-cadaverine antiporter. Upstream the *cadBA* operon is found *cadC,* coding for the low pH sensor and transcription factor and downstream are found genes encidng a dipeptide tripeptide permease and lysine t-RNA synthetase. Concerning *ldcC*, the gene is not in operon: upstream are genes like *rnhB* (ribonuclease H), *dnaE (*alpha subunit of the DNA polymerase III), and *accA* (acetyl-CoA carboxylase), and downstream we found *yaeR (*putative lyase*)* and *tilS* (ile-l tRNA synthetase).

By classifying the genes in function to their genetic environment, we could properly identify *ldcC* and *ldcI* homologues and then proceed with the phylogenetic analysis and sequence alignement that is presented in the introduction in **figure 20.** By using genetic environement classification, CTD sequence alignments, phylogenetic trees and structural analysis we could find a conserved amino acid sequence that corresponds to LdcI-RavA binding site, and that there is evolutionary preassure to keep this residues that are crucial for the formation of this complex.

With the knowledge gathered from the enterobacterial LDCs, we analysed both the sequence of *P. aeruginosa* LdcA and the genetic environment of the gene. At first, when we aligned the CTD sequence of LdcA with the ones from enterobacterial LDCs, we couldn't find any pattern to associate LdcA with neither LdcI nor LdcC. Then, the genetic environment of *ldcA* (**Figure 30**) shares characteristics with both *ldcI* and *ldcC* ones. As it is the case for *ldcI,* we found *ldcA* in operon with the lysine-cadaverine antiporter *PA1819* (that we renamed *ldcB*), but the order of genes is inversed compared to *ldcI*. However, *ldcA* is not associated with homologues of *cadC*, *dtpC* or *lysU*. Instead we can find *nhaB* (coding for sodium/proton antiporter) and a gene PA1817 with unknown function.

In another hand, we found that *ldcA* has an environment analogous to *ldcC* as it is surrounded by genes coding for DNA polymerase subunits and ribonuclease H, suggesting a putative association of *ldcC* and *ldcA* with DNA replication.

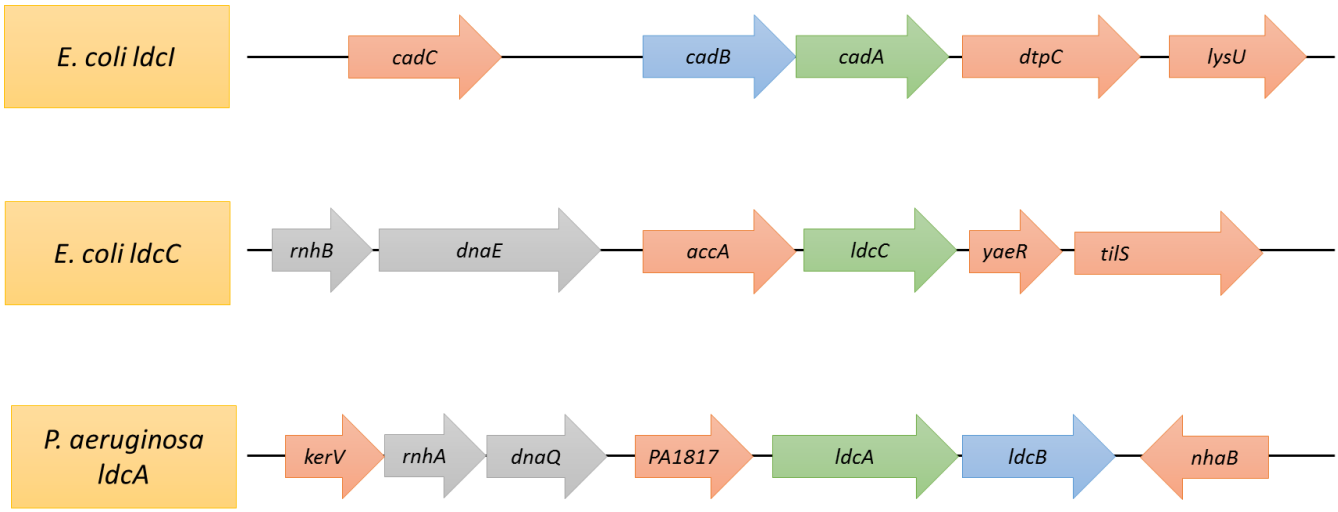


Figure 30. Genetic environment of *ldcI and ldcC* from *E. coli* and *ldcA* from *P. aeruginosa.* The green arrows represent the genes coding for lysine decarboxylases. Blue arrows depict genes coding for the lysine-cadaverine antiporter homologues. Gray arrows correspond to genes associated with DNA replication. Red arrows represent genes that don't have any homologues in the genetic environments that we compared.

3. Discussion

Our bioinformatic analysis evidenced that two proteins in *P. aeruginosa* PAO1 genome are homologues of the Lysine decarboxylases from *E. coli*. This analysis clearly shows that PA1818 is the closest homologues and is also closely related to the Arginine decarboxylase AdiA from *E. coli*. The second candidate is PA1346 that possess also the 3 domains that are characteristic for LDCs, ADCs and ODCs from AAT-fold family. However, bioinformatic prediction of domains by CDD showed a truncated PLP-binding domain in PA1346 and our multiple sequence alignments indicated that the conserved residues (STHKXL) needed for PLP-binding are absent in the amino acid sequence of PA1346. This result indicates us that current bioinformatics analyses are not suficient to tell if PA1346 is indeed a PLP-dependent decarboxylase and biochemical assays are needed to test this hypothesis.

The analysis also showed that PA4115 is not a lysine decarboxylase of AAT-fold family, and that our predictions suggest that is quite close to YgdH of *E. coli* (60%) identity. The protein contains three domains and the central domain carries a conserved PGGXGTXXE sequence that Is known to bound nucleotides such as ATP. One homologue of PA4115, the protein Cg2612 from *Corynebacterium glutamicum* has been structuraly characterized by X-ray crystallography, shows that the conserved domain with the sequence PGGXGTXXE participates in phosphorobohydrolase reactions needed for the biosynthesis of cytokinins. This information suggests us that PA4115 could be a phosphorobohydrolase involved in the biosynthesis of cytokinins or that it belongs to a completely new enzymatic architecture to perform the lysine decarboxylase reaction. Anyway, a biochemical characterization of purified proteins needs to be performed[320].

The genetic environment analysis of *ldcA* (PA1818), revealed that the gene shares characteristics with both *ldcI* and *ldcC* from *Enterobacteria*. Indeed, we found it associated in operon with a lysine-cadaverine antiporter as it is the case for *ldcI.* However, we couldn't find any homologue of *cadC* in *P. aeruginosa* genome, suggesting that *ldcA* is probably not associated to acid stress response as *ldcI*. We found that *ldcA* shares proximity with genes involved in DNA metabolism as it is the case for *ldcC*, suggesting that *ldcA* is associated and could be necessary for the proper functioning of the replication machinery. This seems coherent with previous finding that report LdcC playing a role in resistance against fluoroquinolones which are antibiotics that block DNA replication. From these informations, we hypothesize that LDCs could play an important role in DNA replication and metabolism and that it is pertinent to see whether LdcA also plays a role in protecting bacteria against fluoroquinolones.

Chapter II: The expression of the Lysine decarboxylase in P. aeruginosa

Regulation of the Lysine decarboxylase enzymes has been thoroughly studied in enterobacteria like *E. coli* and *Salmonella sp.* where two isoforms are found encoded in the chromosome (*ldcI* and *ldcC*)[257,284].

The expression of the *ldcI* gene is induced when bacteria encounter growth media with moderate acidity and when Lysine is found at millimolar concentrations under both aerobic and anaerobic conditions. As a consequence of this induction, the reaction catalyzed by LdcI consumes a significant number of protons that end up buffering the bacterial cytoplasm and the synthetized cadaverine is able to block outer membrane porins that are involved in the entrance of excessive protons in the periplasmic space[249,256,284,294]. This effective mechanism allows to counter acid stress while promoting growth. Besides the effect on porins during acid stress, the cadaverine produced by LdcI has also been shown to be part in other stress response mecanisms. For instance, it has also been shown that cadaverine is linked to the oxidative stress response by scavenging reactive oxygen species. This is the case in vibriobacteria like *V. vulnificus*, where it enhances the survival of bacteria facing oxidative stress from methylviologen[241]. In *E. coli,* cadaverine has also been shown to provide protection against reactive nitrogen species in strains causing urinary infections[266]. It has also been hypothesized that the accumulation of this polyamine during anaerobic growth would allow to protect not only the bacteria from acid stress due to fermentative metabolism but also from oxidative stress when they shift between anaerobic to anaerobic conditions[264]. Today we know that the expression of *ldcC* is dependent on the sigma factor RpoS[260,321]. *ldcC* is transcribed when the bacteria start entering stationary phase. Its expression is also known to be induced when bacteria are treated by fluoroquinolones: this induction has been hypothesized to be a response mechanism that uses cadaverine to block the outer membrane porins, thus limiting the amount of antibiotic capable of entering into the cells[260,262].

In *P. aeruginosa* genome, we have found only one gene coding for a Lysine Decarboxylase (*ldcA*) that allows the bacteria to grown in minimal medium with L-Lysine as a

carbon source. In this organism, it has been shown that *ldcA* expression is induced when L-Arginine is present in excess in minimal medium[235,322]. The induction of *ldcA* promoter is controlled by the transcription factor ArgR which also modulates the expression of numerous genes needed for the assimilation of L-Arginine and establish a link between L-Lysine and L-Arginine metabolism.

Since the previous studies focalized on the role of *ldcA* in the assimilation of L-lysine as a source of energy and the control of its expression by L-arginine, we wanted to know if this gene also participated in the stress response as it has been described for its enterobacterial orthologues (*ldcI* and *ldcC*).

In order to understand the expression and the regulation of the *ldcA* promoter, we decided to combine three different approaches: i) bioinformatic analysis of the promoter region of *ldcA* (500bp upstream of ATG) using stablished consensus sequences of binding sites of important transcription factors from *P. aeruginosa*; ii) analyzing transcription of *ldcA* under different growth and stress conditions by using promoter-reporter gene fusions; iii) correlating the transcription to the translation by performing western blot analysis with polyclonal antibodies obtained from the recombinant protein I overexpressed and purified.

1. Bioinformatics analysis of the *ldcA* promoter region

To better understand how *ldcA* expression is regulated, we performed bioinformatics analysis using the programs RSAT (Regulatory Sequence Analysis Tools; http://embnet.ccg.unam.mx/rsa-tools/) and Virtual Footprint 3.0 from the PRODORIC (Prokaryotic Database of Gene Regulation; http://prodoric.tu-bs.de/vfp/) websiteto predict putative binding sites for transcription factors in the promoter region of *ldcA*. The region that we decided to use for expression and bioinformatics analysis started at -489 bp and finished at + 12bp of the ATG of *ldcA* open reading frame.

Our analysis with RSAT consisted in searching consensus binding sequences of the main sigma factors and transcription factors that have been described in *P. aeruginosa*. The advantage of the analysis with this program is that it allows us to find consensus sequences that are strongly conserved. However, it is not well suited for the search of degenerate binding sequences because it does not take into account the probability of finding more than two alternative nucleotides in the consensus sequence.

The second program, Virtual Footprint 3.0, searches patterns by using a Position Weight Matrix of each binding sequence of the transcription factor that we wanted to search for: the results of this analysis is meant to complement the one from RSAT because Virtual Footprint is more sensible to degenerate consensus sequences.

In **table 16,** we present the results obtained after using the two programs cited above. We found the presence of a conserved site for RpoN/σ^{54} (-24/-12). One well conserved ANR site was found near the ATG codon of *ldcA*. Three ArgR sites have been predicted, with one site overlapping the -35 element of RpoD, suggesting a putative interaction between ArgR and RpoD in a complex similar to class II CAP-dependent promoters[323]. Binding sites for the QS regulators LasR and RhlR have also been found. The first highly conserved LasR DNA-binding site is found in the negative strand overlapping with ArgR binding site found in the sense strand in the same region. The second less conserved LasR site (LasR2) has also been designed to be compatible with RhlR (RhlR1) binding. Finally, a second poorly conserved RhlR (RhlR2) site is predicted to bind the sense strand of the analysed promoter.

Nevertheless, bioinformatics predictions are far from being perfect: for instance, we know from footprint studies that the transcription factor ArgR binds to the promoter region that is found at 120 bp to 81 bp upstream of the ATG start codon of the *ldcA* gene which coincides with the ArgR1 binding site predicted by our program . However, the DNAse assay didn't find other binding sites in the promoter region. This suggests that there's the possibility that the consensus ArgR binding sequence is not stringent enough or that the DNA footprint assays couldn't display ArgR binding because other factor was needed (DNA structuring, protein partners). ANR site is found very close to the ATG in the 5'-UTR region which makes it unsuitable for interacting with the RNA polymerase. In the case of RpoN, the consensus sequence contains a GGN{10}GC consensus found in *ldcA* promoter but that is not very stringent for an organism that has a genome which contains 65% GC.

Regulatory Elements	Start Position	End Position	Strand	Found Sequence	Query Sequence
RpoN	-75	-62	+	**GG**TTACGCGCGT**GC**	**GG**N{10}**GC**
ANR	-15	-2	+	C**TG**CTCGGG**ATCA**A	**TTGA**N{5}**ATCA**
ArgR1	-116	-101	-	G**GTCGC**GGCAACAC**AA**	**TGTCGC**N{8}**AA**
ArgR2	-189	-174	+	**TGTCGC**TCCGTAGC**AG**	**TGTCGC**N{8}**AA**
ArgR3	-427	-412	+	**TGTCGC**CGCCGTCG**A**T	**TGTCGC**N{8}**AA**
LasR1	-184	-169	-	**CTG**C**C**CTGC**T**ACGG**AG**	**CTNCC**N{5}**T**N{3}**AG**
LasR2	-218	-203	+	**CTG**A**C**GATCCGATA**AG**	**CTNCC**N{5}**T**N{3}**AG**
RhlR1	-218	-203	+	**CTG**ACGATCCGATA**AG**	**CTN**{5}**G**N{7}**AG**
RhlR2	-393	-378	-	**CTG**GGAG**G**GCTTCAGG	**CTN**{5}**G**N{7}**AG**

Table 16. Predicted Regulatory Elements found by bioinformatics analysis: performed by RSAT and Virtual Footprint 3.0. ArgR site highlighted in blue has been experimentally described before. +: sense, -: antisense. Start and ending positions are shown as a position from ATG of the *ldcA* gene.

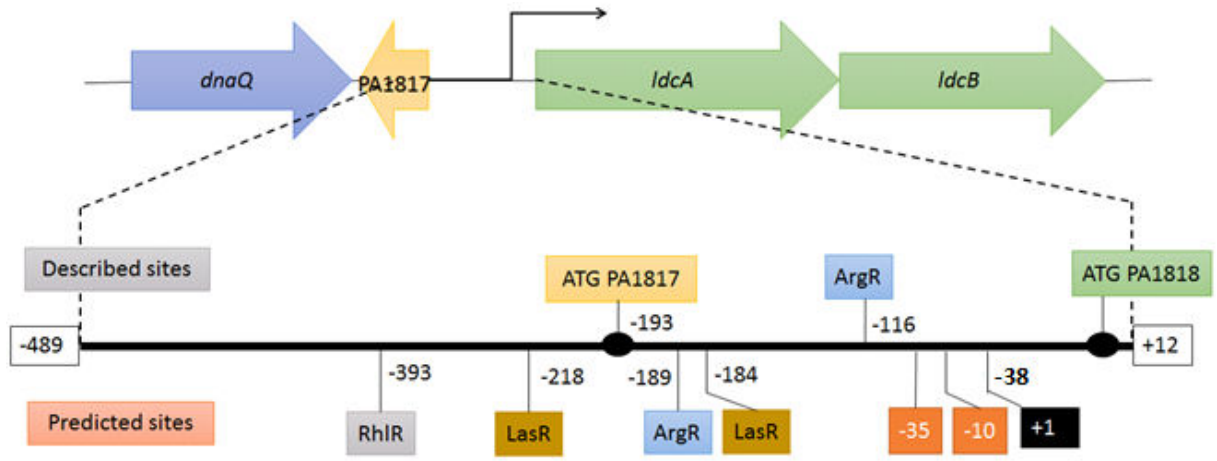


Figure 31. **Bioinformatics analysis of the promoter region of *ldcA* gene** that was used for expression studies using promoter-reporter gene fusions (-489 bp to + 12 bp from the ATG site). Features that have been previously described are found in the upper part of the line representing the promoter region. Features that have been predicted by our analysis are shown below. The gene *PA1819*, has been annotated as *ldcB*. The intergenic space between *PA1818* and *PA1817* corresponds to a distance of 192 nucleotides. The +1 of transcription was extrapolated from PA14 transcriptomic data[324].

These prediction results suggest us a possible involvement of other regulatory factors governing the expression of LdcA besides ArgR. The finding of putative LasR and RhlR binding sites suggest us that expression of *ldcA* could be regulated by quorum sensing. Nevertheless, transcriptomic data of quorum sensing regulated genes do not show changes in the expression of *ldcA*[74,75]. This contradictory result tells us to be cautious with this bioinformatic predictions and that further investigation of a role of QS in *ldcA* expression is only interesting if *ldcA* shows a population-dependent or growth phase dependent expression pattern.

Overall this information tells us that there could be an implication of quorum sensing in the expression of *ldcA* and in a lesser degree by ANR.

2. Constructions of different *ldcA* promoter fusions

After the identification of putative regulatory sites in the sequence of the *ldcA* promoter through bioinformatics analysis as shown in **figure 31**, we decided to study its expression by using promoter-reporter gene fusions. The fusions were made by amplifying the promoter region (-489 to + 12 bp from the ATG) by PCR and cloning it in an integrative plasmid containing a promoter-less reporter gene. The resulting miniCTX P_{ldcA}-reporter gene vector, bearing an *attP* site and integrase-encoding gene, was capable of integrating at the *attB* site found in the chromosome of the PAO1 strain. This system has two advantages: i) only one copy of the promoter fusion is present in the bacterium that avoids any titration effect of the transcription factors regulating the *ldcA* promoter, ii) by using FLP recombinase, we are able to get rid of the antibiotic marker of the miniCTX plasmid that could modify the physiology of our organism and the expression of our promoter.

Since we wanted to conduct experiments in similar conditions to the ones that have been performed for *E. coli*, we wanted to test which reporter gene would be the most suited for testing *ldcA* expression in LB medium but also in minimal medium.

For our study, we had three different options for reporter gene: genes encoding i) the GFP, ii) the β-galactosidase and iii) the luciferase.

First, we decided to test the *gfp* and *lux* fusions, that have the advantage of being suited for kinetic experiment that allow you to follow expression levels throughout cell grow without heavy experimental procedures. Moreover, the *gfp* and *lux* reporters are convenient for high throughput experiments that give great amounts of data for statistical analysis.

For the experiments using the GFP reporter, we used the strains: PAO1 (wild type) and PAO1::*gfp* (integrative vector) as controls without GFP production, and PAO1:: P_{ldcA}-*gfp* to test the promoter activity. In parallel, we checked the expression of *ldcA* by using the luciferase as a reporter system in the same growth conditions. The miniCTX lux bears the *luxCDABE* genes and codes for the luciferase system. The system is composed by the luciferase protein which is a heterodimer formed by the luxA and luxB gene products. The *luxC, luxD*, and *luxE* gene products encode for a FMN reductase, transferase, and synthase respectively, that work together in a single complex to generate an aldehyde substrate for the bioluminescent reaction. In *P. aeruginosa,* an endogenous Flavin reductase is in charge of the regeneration of the required $FMNH_2$ substrate. Together with molecular oxygen, these components are all that are required to produce a bioluminescent signal and no exogenous substance are needed for the functioning of the process.

We used the strain PAO1::P_{ldcA}-*lux* to assay promoter activity and the PAO1::*lux* as a negative control of light production. These two experiments were done by growing bacteria for 6 hours at 37°C at 100 RPM in a Fluoroskan Ascent (ThermoFischer Scientific) that is capable of measuring fluorescence and light production in 96-well plates.

The third method that we used for measuring the expression of *ldcA* was the *lacZ* system that uses the β-galactosidase. For this assay we studied the strain PAO1::*lacZ* as a negative control and the PAO1::P_{ldcA}-*lacZ* to assess the activity of the promoter. The strains were grown in LB and the expression was measured 6 hours after the start of the culture.

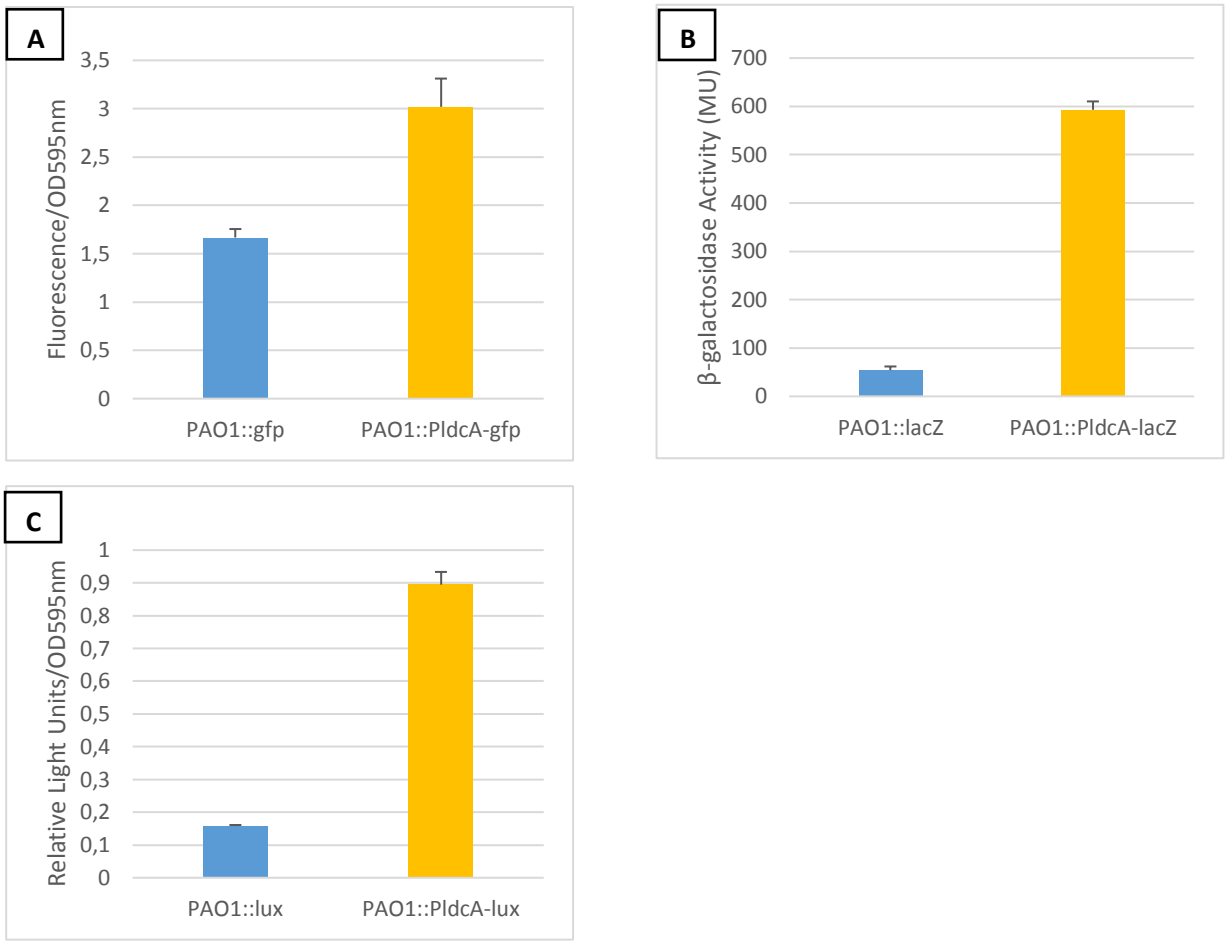


Figure 32. **Assay of promoter-reporter gene fusions between *ldcA* and *gfp, lacZ* and *lux* operon. A)** Measurement of fluorescence of GFP normalized to OD_{595nm} (wavelength: 520-560nm) produced by the different strains of PAO1::*gfp* (control without promoter) and PAO1::P_{ldcA}-*gfp*. when growing in LB medium. **B)** Measurement of the light produced by the luciferase normalized to OD_{595nm} in the strains PAO1::*lux* (control without promoter) and PAO1::P_{ldcA}-*lux*. **C)** Measurement of the β-galactosidase activity of the strains PAO1::*lacZ* (control without promoter) and PAO1::P_{ldcA} -*lacZ*. The measurements were done after 6 hours of growth in LB medium, when cells are at the transition phase between exponential and stationary phase and the signal was higher for all assays. Error bars correspond to the standard deviation obtained from 3 independent experiments.

The results are shown in **figure 32,** where the *ldcA* expression measured with the three reporters is compared. The reporter activities are comparable because each reporter activity (fluorescence, luminescence and β-galactosidase activity) is normalized to the number of bacteria (OD595nm for *gfp* and *lux* and OD600 for β-galactosidase activity).

The results showed that while all the reporter gene fusions were capable of detecting a transcriptional activity for the *ldcA* promoter, the signal-to-noise ratio was different for each reporter system. For instance the measurement of GFP fluorescence exhibited the worst ratio, due to a low expression of *ldcA* promoter that is masked by LB medium and *P.*

aeruginosa pigments autofluorescence . For this reason we decided not to continue to use the GFP reporter because it is not suitable to detect the weak *ldcA* expression when bacteria grow in LB medium. The two other reporters, luciferase and β-galactosidase, presented a better "signal-to-noise" ratio compared to GFP, making them more sensitive and adapted to study the activity of the promoter when bacteria grow in LB medium. Even though the luciferase reporter presents the advantage of being suitable for kinetics and high throughput experiments, it depends on energy metabolism (ATP, NADPH, $FMNH_2$) that are affected when bacteria are struck by acid and oxidative stress[325]. For this reason, the β-galactosidase reporter was chosen to conduct promoter analysis under stress. Nevertheless, the luciferase reporter was used to confirm the expression in non-stressed growth conditions. Finally, another argument for the use the β-galactosidase as a reporter of *ldcA* expression is that this methodology has already been used with success to study the expression of *ldcA* in *P. aeruginosa* and of *ldcI* and *ldcC* in enterobacteria[235,321,326].

3. Characterization of anti-LdcA antibodies for Western Blot analysis.

We decided to obtain anti-LdcA antibodies to visualize whether protein synthesis followed the pattern of activation of the *ldcA* promoter. The pET system was used to create a plasmid with recombinant Histagged LdcA **(figure 33 A).** The protein was overexpressed in *E. coli* and was further purified by affinity chromatography, as shown in **figure 33 B.** Afterwards a final step of purification including a size exclusion chromatography was added and the purest fractions, in which only LdcA was visible, were observed by negative staining electron microscopy. **In figure 33 C**, we could observe the presence of decamers resembling the LdcI from *E. coli.* **In figure 33 D**, the results from using anti-LdcA antibodies against recombinant Histagged LdcA and another cytoplasmic protein PcrV, showed that they can detect up to 10ng of LdcA in a transfer membrane.

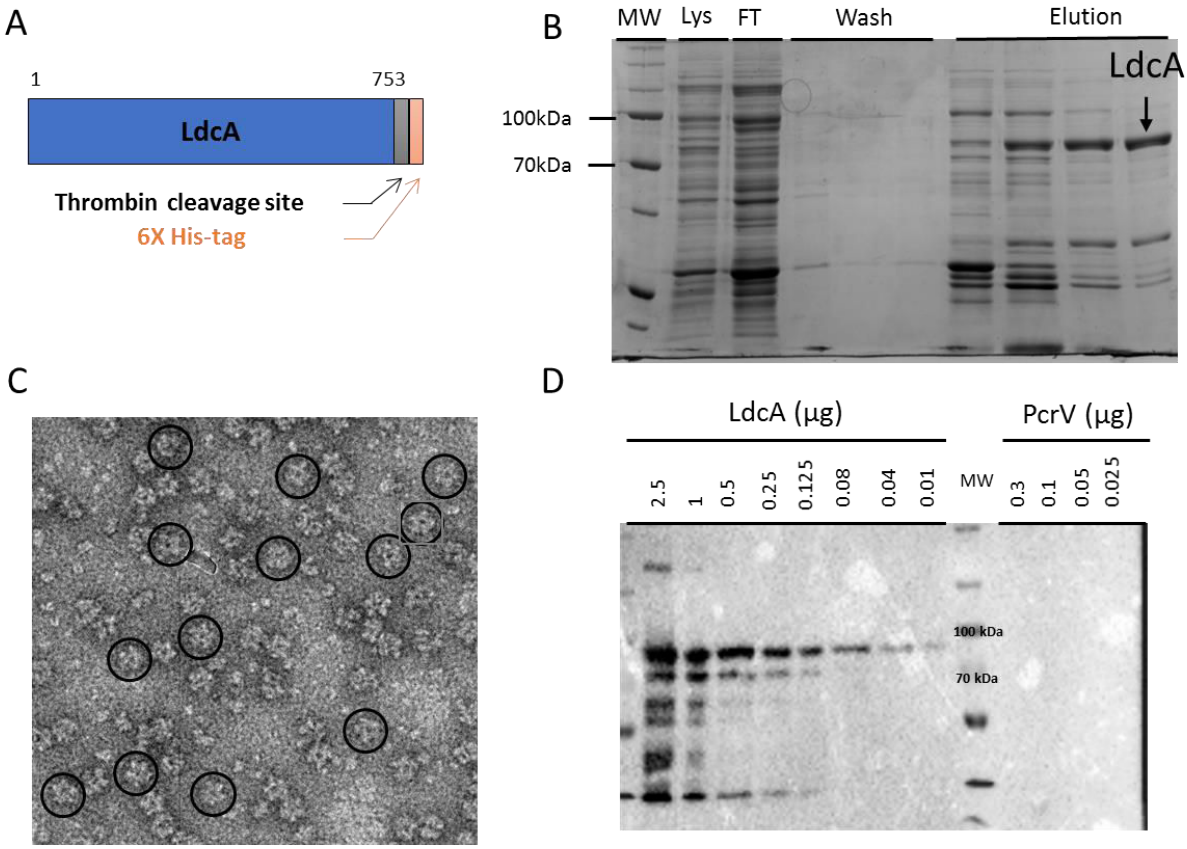


Figure 33. Design and Purification and western blotting of recombinant LdcA. A) Schematicof the recombinant His-tagged LdcA; **B)** SDS-PAGE gel showing the nickel purification of recombinant LdcA, aliquots corresponding to **Lys:** total cell lysate, **FT:** flow-throught: **Wash:** Buffer with 50mM Imidazole, **Elution**. Purest fractions were pooledd concentrated and injected for further purification by Gel filtration; **C)** Negative stain of LdcA containing fractions obtained after Gel filtration, five-order symmetric structures resembling LDC decamers are surrounded by black circles; **D)** Polyclonal antibodies obtained from immunized rabbits were tested against purified LdcA and purified PcrV as control by western blotting. Primary anti-LdcA antibodies from rabbit were used at 1/1000 dilution and commercial secondary anti-rabbit HRP-coupled antibodies from mice were used at 1/50000 dilution.

4. Expression of *ldcA* promoter in minimal medium

We decided to test the *ldcA* promoter under the same medium used in other studies (MMP medium). The characterization of the *ldcA* promoter activity was performed by using both the β-galactosidase and the luciferase reporter fusions.

In **figure 34 A**, we show experiments that were performed in MMP complemented with Glutamate (20mM). Under these conditions we observed effect of the addition of Lysine or Arginine (20mM) during the exponential growth phase. Arginine was the only

amino acid to induce the expression of *ldcA* for our strain PAO1::P_{ldcA}-*lacZ*, which is in accordance with already published results.

In **figure 34 B**, we observed the expression of *ldcA* using the lux reporter in a 96-well set up. We tested our PAO1::P_{ldcA}-*lux* strain in MMP Glutamate but also when we added either 1mM or 20mM Arginine. The expression of *ldcA* progressively increased with cell density. We observed also that arginine induces *ldcA* expression in a dose-dependent manner, the highest expression value being obtained in presence of 20mM Arginine. **Figure 34 B** also shows that the induction peak of *ldcA* expression is reached earlier when higher concentrations are present. Then the expression decreased during the stationary phase.

The results obtained with the strain harbouring the P_{ldcA}-*lacZ* fusion are presented in **figure 34 C**: in this experiment, the bacteria were grown in erlenmeyers containing MMP Glutamate + Arginine (20mM). The results are similar to those obtained with *lux*, as we observed the same tendancy, a progressive induction throught out growth.

The differences in behaviour in **figure 34 B and C**, could be accounted for the different set ups of growth. Lux experiments in 96-well plates do not have neither the same oxygenation nor growth rate as those experiments done in erlenmeyers. This suggests that factors affecting growth have great impact in the acquisition of arginine and the activation of ArgR. Another possibility is that other regulators besides ArgR regulate the expression of *ldcA*, such as QS systems or sigma factors like RpoS.

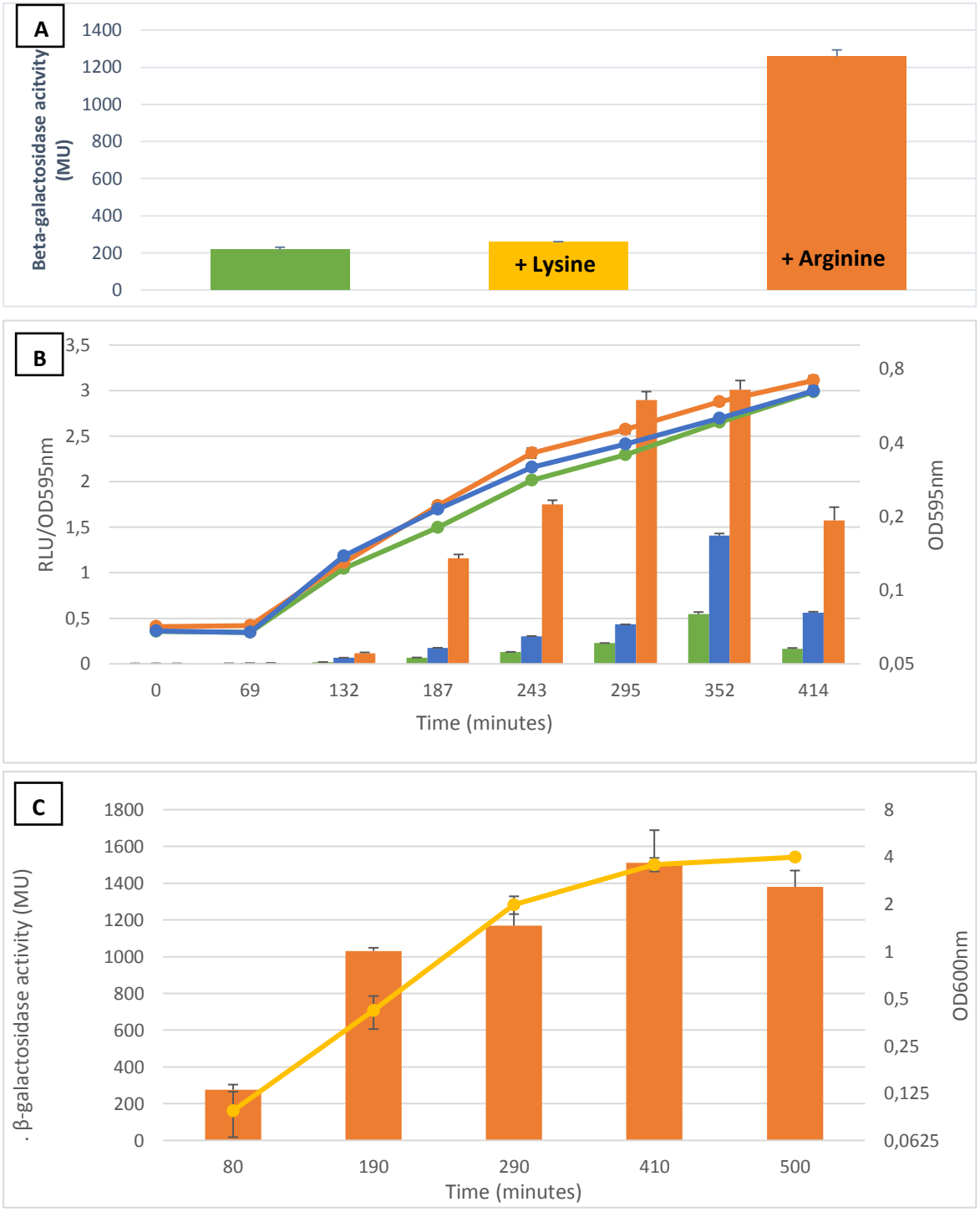


Figure 34. Activity of *ldcA* promoter in MMP minimal medium. A) β-galactosidase reporter activity of of PAO1 P_{ldcA}*::lacZ* in minimal medium P (MMP): containing 20mM L-Glutamate (green), plus 20mM Lysine (blue) or 20mM Arginine (orange) when indicated. The activity was assayed with bacteria at exponential growth phase (OD600nm: 0.6) at 37°C, 300 RPM. **B)** Growth curves and Luciferase reporter activity of the strain PAO1 P_{ldcA}*::lux*: Growth 20mM L-Glutamate (green), 20mM L-Glutamate + 1mM L-Arginine (blue) , 20mM L-Glutamate + 20mM Arginine (orange) @ 37°C, 100RPM in 96-well plates. **C)** Growth curve and β-galactosidase reporter activity of *ldcA* expression of the strain PAO1 P_{ldcA}*::lacZ*: Growth on MMP 20mM Glutamate 20mM Arginine at 37°C, 300 RPM

5. Expression of *ldcA* promoter in rich LB medium

The expression profile of the *ldcA* promoter in rich medium was assessed as for MMP medium, by using both β-galactosidase and luciferase reporters, as shown in **figure 35.**

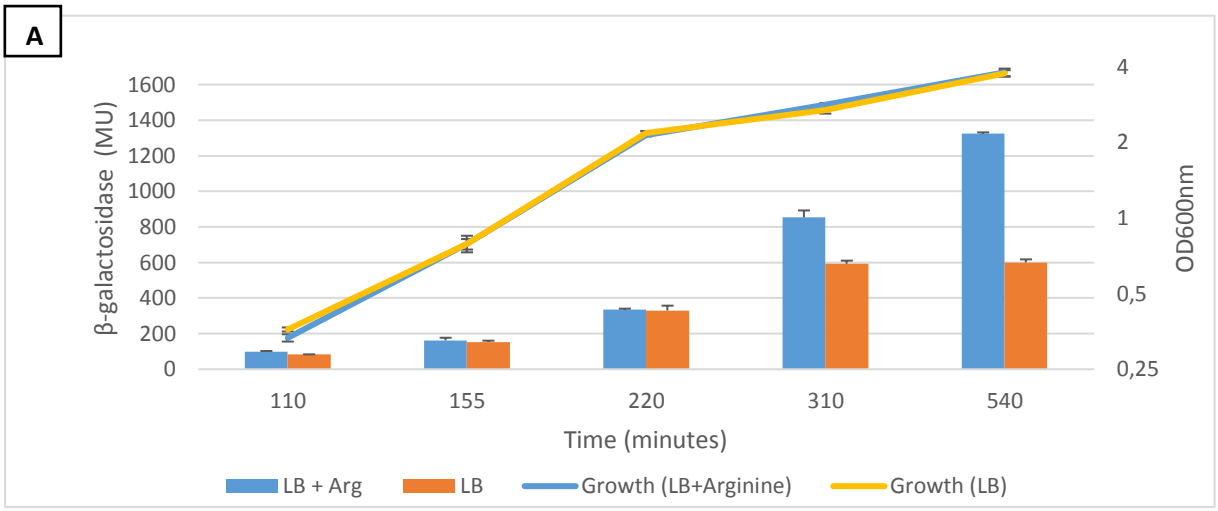
The β-galactosidase reporter showed that promoter activity was minimal during exponential phase and then progressively increased when the bacteria entered the transition phase between exponential growth and stationary phase, reaching a plateau of activity, as shown in **figure 35 A**. The maximum activity value obtained from overnigh cultures was comparable with the plateau observed in early stationary phases, suggesting that the expression was kept probably constant throughout the stationary phase during the night.

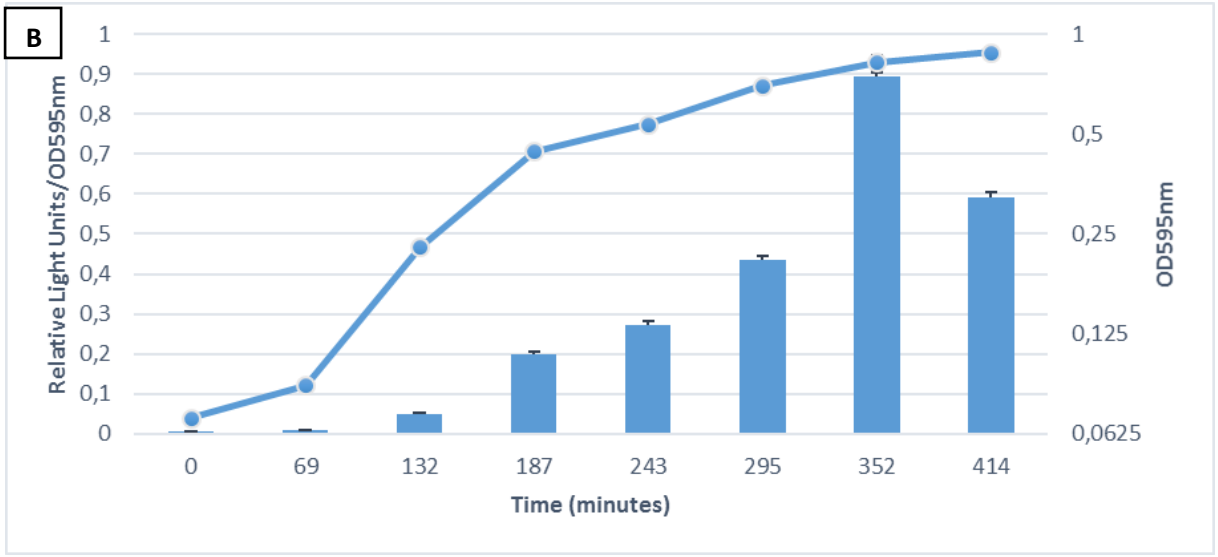
The same pattern was observed with luciferase activity **in figure 35 B**, although the activity started to decay after reaching a peak in the early stationary phase. Western blotting of cytosolic extracts was performed in parallel **(figure 35 C)** and showed that protein synthesis correlated with the results obtained from the β-galactosidase: LdcA is at undetectable amounts during the exponential phase and its synthesis progressively increases and reaches its maximum at high cell densities.

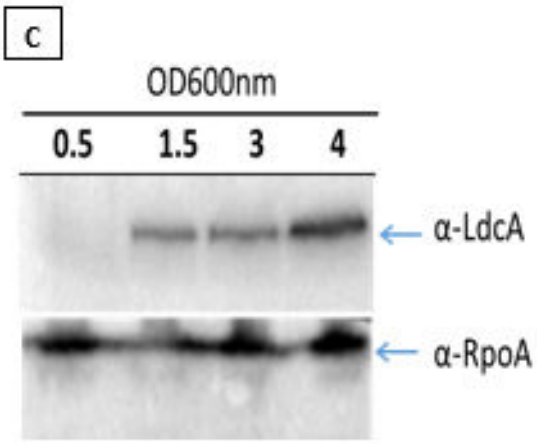
The experiments in LB showed that *ldcA* expression is maximal at high cell densities. But we wanted to see how the presence of L-Arginine would influenced the expression profile in rich medium **(Fig 29 A).** The experiments showed us that the addition of L-Arginine didn't change growth but affected the expression of *ldcA* only during the transition to the stationary phase. The β-galactosidase activity level found in LB + 20mM Arginine (1500-1300 MU) is in accordance with the maximum level observed during the growth in minimal medium with 20mM Arginine (1250 MU), suggesting that the concentration of arginine is the main factor determining the level of induction.

The influence of growth phase of *ldcA* expression indicated that another factor than ArgR could control *ldcA* expression, even though **figure 35 D** clearly shows that ArgR is indeed the main regulatory factor of *ldcA* expression. Moreover our bioinformatics analysis suggested that quorum sensing (QS) regulators (LasR and RhlR) could be involved in *ldcA* regulation. We also found in literature that *ldcC,* a homolog in *E. coli*, has a growth phase dependant expression which is controlled by the sigma factor RpoS. For all these reasons, we

decided to check out the influence of QS and RpoS in the expression profile of *ldcA* in rich medium.







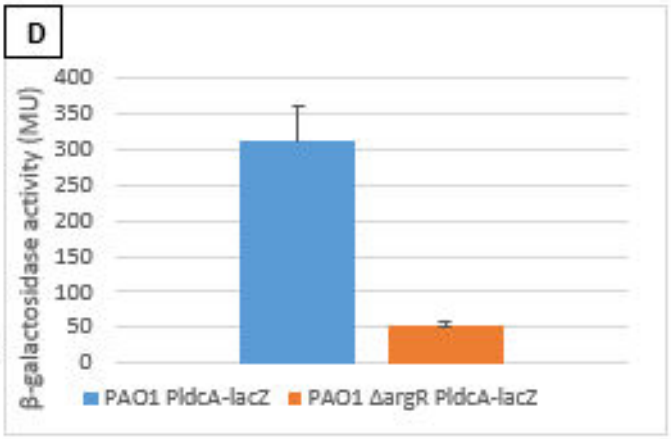


Figure 35: **Expression profil of *ΔldcA* and *ΔargR* in rich medium LB. A)** β-galactosidase activity of PAO1::*PldcA-lacZ* grown in LB: + or - 20mM Arginine in well aerated cultures. Growth of bacteria in each condition is represented on the right Y-axis as the absorbance at OD_{600nm}, *ldcA* promoter expression is shown as Miller Units on the left Y-axis. **B)** Luciferase activity assay of PAO1::*PldcA-lux* grown in LB medium in 96 well-plates. Growth of the bacteria is representent on the right Y-axis measured as absorbance at OD_{595nm}. *ldcA* promoter expression is represented in Relative light units/OD595nm on the left Y-axis. **C)** Western Blot analysis of cytoplasmic fractions of PAO1 at different growth phases in LB medium OD_{600nm} of 0.5 (mid-exponential); 1.5 (late-exponential); 3 (early stationary); 4 (stationary phase). **D)** β-galactosidase activity of PAO1::*PldcA-lacZ* and PAO1 *ΔargR*::*PldcA-lacZ* grown in LB medium. The assays where done at the early stationary phase for a $OD600_{nm}$ of 3.5 for the WT and 2.6 for the *ΔargR*.

6. Seek for regulators of *ldcA* expression

In Ina Attrée's lab, we have a collection of mutants in different *P. aeruginosa* backgrounds. One of these strains is called CHA (mucoid) and was isolated from the lung of a patient suffereing of cystic fibrosis (CF). We decided to test the expression of *ldcA* by introducing our promoter-reporter fusions in the following CHA mutants: CHA *ΔrpoS,* CHA *ΔlasI-rhlI*, CHA *ΔpqsA.* The first mutant CHA *ΔrpoS,* has a deletion that affect the expression of the *rpoS* regulon that includes genes in metabolism but also in stress response as already exposed in the introduction in chapter I. The mutant CHA *ΔlasI-rhlI* has deletions in the genes responsible for the synthesis of the autoinducers 3-oxo-C12-AH and C4-AHL that are needed for the functioning of the *las* and *rhl* QS systems. The mutant CHA *ΔpqsA* has a deletion in the gene necessary for the synthesis of anthranilate-CoA needed to produce 2-heptyl-3,4-dihydroxyquinoline, which is the autoinducer for the PQS system.

In **figure 36 A,** we present the growth curves and β-galactosidase reporter activities of the wild type and mutants CHA strains containing chromosomally-encoded *pldcA-lacZ* fusion when growing in LB medium. The experiments showed us that the growth fitness of the mutants was roughly the same compared to the wild-type strain and that the expression profile of *ldcA* in CHA wild-type strain is similar to the one observed for PAO1.However, the maximal expression of *pldcA-lacZ* in CHA is slightly lower and exhibits in average 25% less β-galactosidase activity compared to PAO1.

The expression profile of *ldcA* promoter in the *rpoS* and *lasI-rhlI* backgrounds appeared to be comparable to the profile exhibited by the wild-type strain, as shown in **figure 36 A**. These measurements suggested us that neither *rpoS* nor the *las/rhl* systems

were involved in the regulation of *ldcA* promoter in these conditions. Only the *pqsA* mutant showed a difference in its expression profile compared to the wild type: indeed we noticed that even if the expression also increases during growth, it displayed a lower level of activity compared to the control, as shown in the **figure 36 B**. We further compared the activity of *ldcA* promoter in CHA grown either in LB or in minimal medium containing 20mM L-Glutamate and 20mM L-Arginine (MMPGR), and observed an increased of around 1,5 fold. In the CHAΔ*pqsA* mutant, roughly the same β-galactosidase activity was measured in the two different media. As we see in **figure 36 C,** not only expression of *ldcA* is lowered in a *pqsA* deleted background, but the strain has also lost the abitilty to induce *ldcA* expression in response to Arginine.

Our results suggest that PQS plays a role in the regulation of the *ldcA* promoter, the complementation of *pqsA* mutant needs to be done in order to confirm the resultat. In parallel, the role PQS needs to be also assessed in PAO1 to verify that the effect is not particular to the CHA strain.

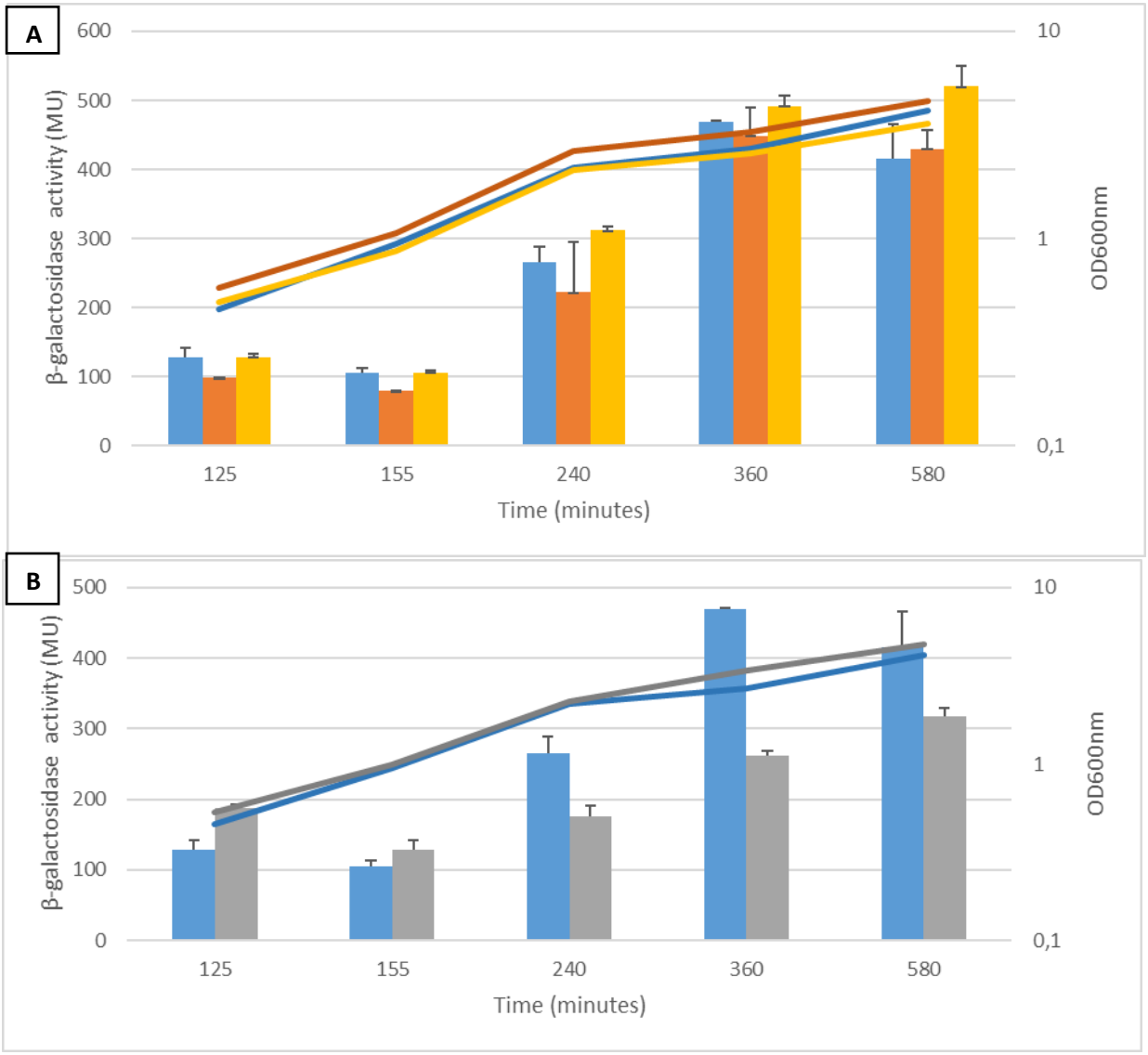


Figure 36. Expression profile of *pldcA-lacZ* fusions in CHA strains in different media. A) β-galactosidase activity of CHA:: *pldcA-lacZ (blue),* CHA *ΔrpoS ::pldcA-lacZ (yellow)* and CHA *ΔlasIΔrhlI ::pldcA-lacZ (orange)*, when growing in LB medium (well-aerated cultures). **B)** β-galactosidase activity of CHA::*pldcA-lacZ (blue) and* CHA *ΔpqsA::pldcA-lacZ (gray),* when growing in LB medium (well-aerated cultures) and **C)** in stationary phase (overnight cultures) in either LB or Minimal medium with 20mM Glutamate + 20mM Arginine. For A) and B), growth curves are represented in the same graph in the secondary OD600nm axis.

7. Characterization of *ldcA* expression in biofilm-like and anaerobic conditions.

Since polyamines have been shown to be important for the creation of bacterial biofilms, we wondered if *ldcA* was expressed when PAO1 grew under biofilm-like conditions. In **figure 37 A,** we show the average activity of the *ldcA* promoter in cells that grew as a bacterial lawn on LB for 24 or 48 hours. The bacterial lawn was resuspended and lysed in order to performn β-galactosidase activity assays. The β-galactosidase activity was at slightly lower level than the one displayed by planktonic cultures in stationary phase. Since the physiological state of bacterial colonies has been linked to the planktonic stationary phase[327], it will be important to verify the expression on air-liquid biofilm assays to see the expression of *ldcA* in a well defined biofilm growth setting.

Polyamines have also been shown to be important to protect bacteria agains the toxic effects of oxygen after experiencing anaerobic growth as it is the case for *E. coli*. Moreover, LdcI, one the homologues of LdcA in enterobacteria is up-regulated in anaerobic conditions[243]. We wondered whether *ldcA* could also be overexpressed when *P aeruginosa* is present in anoxic environments. PAO1 is capable of growing under anaerobic conditions when nitrate is present, we decided to use a homemade set up in which the bacteria grew in 15ml falcons filled with LB + KNO_3 up to the hermetically sealed plug. The set up allowed us to do comparable one time activity-measurements because each tube shared the same population and solution composition from the beginning. Once the falcons are sealed, bacteria start consuming the dissolved O_2 in the medium, causing its depletion and forcing bacteria to use NO_3^- present to gradually start anaerobic respiration. We obtained a growth rate of 0.74 h^{-1} (1.35 h of doubling time) that was measured between 3h and 6h of growth. This value is very close to the reported 0.7 h^{-1} (1.4 h of doubling time) growth rate value found in nitric oxide anaerobic respiration of *P. aeruginosa* and suggests us that our bacteria are indeed under anaerobic metabolism.

As shown in **figure 37 B**, *ldcA* expression in anaerobic conditions is slightly decreased during exponential phase and then recovered its maximum expression level after 24H. While the maximum level of expression is the same than the one found in aerobic liquid cultures, we do not see the initial strong decrease in β-galactosidase activity as found in the exponential phase in aerobic LB cultures. The expression of *ldcA* under this condition showed us that ANR is not affecting the promoter activity as suggested by the fact that the ANR binding site was in a position that would unlikely modify the expression of *ldcA* (Part II, chapter II).

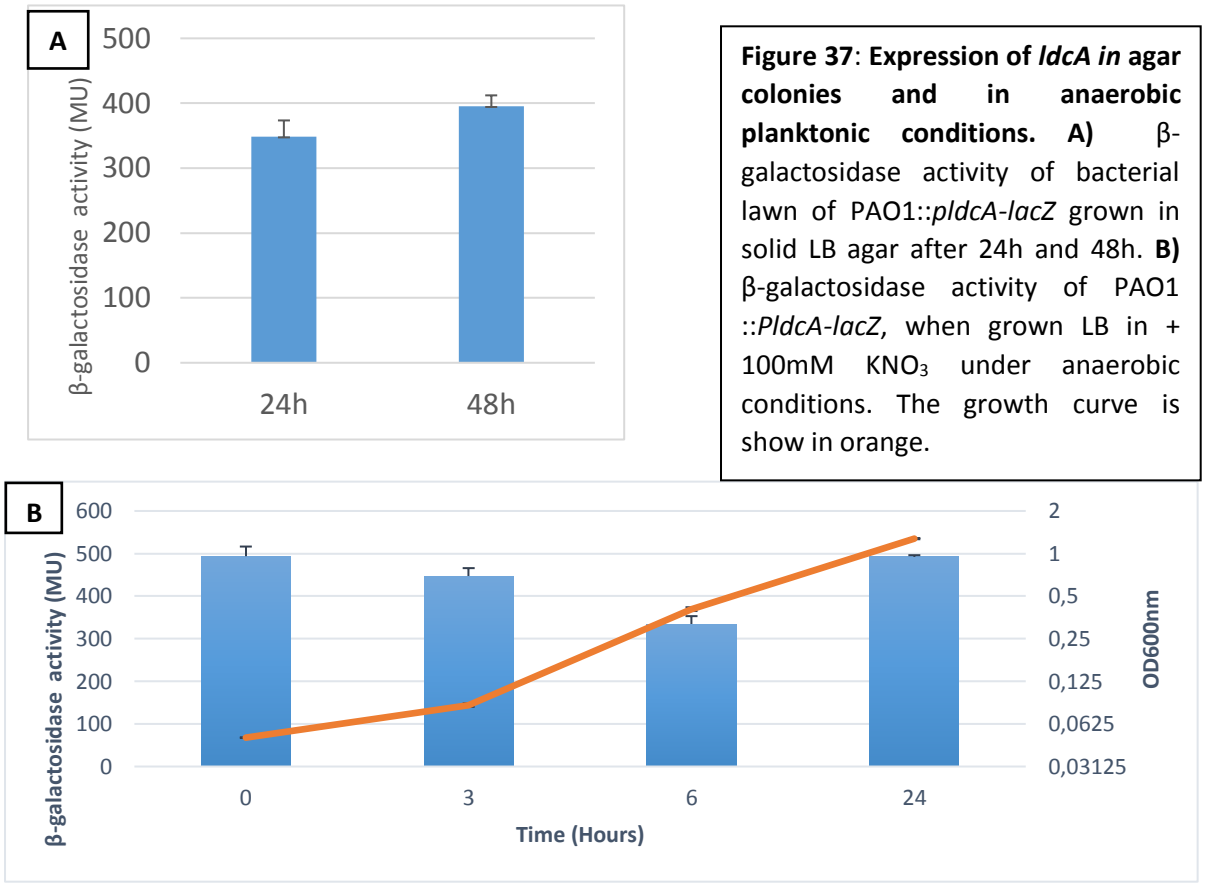


Figure 37: **Expression of *ldcA in* agar colonies and in anaerobic planktonic conditions. A)** β-galactosidase activity of bacterial lawn of PAO1::*pldcA-lacZ* grown in solid LB agar after 24h and 48h. **B)** β-galactosidase activity of PAO1 ::*PldcA-lacZ*, when grown LB in + 100mM KNO_3 under anaerobic conditions. The growth curve is show in orange.

8. Effect of acid and oxidative stress on the *ldcA* expression

In *Enterobacteria,* the expression of *ldcI (ldcA* homologue*)* is governed by CadC. Growth condititions involving low pH and excess lysine activate CadC, which activates the full expression of *cadBA* operon under aerobic conditions. Even though, there is no CadC homologue in *P. aeruginosa,* we wanted to know if acid stress could activate the expression of *ldcA* in a CadC-independent manner[78–80].

In **figure 38 A and B** we show experiments where we induced acid stress by decreasing the pH of the medium to a value of 5, during the exponential phase of growth.

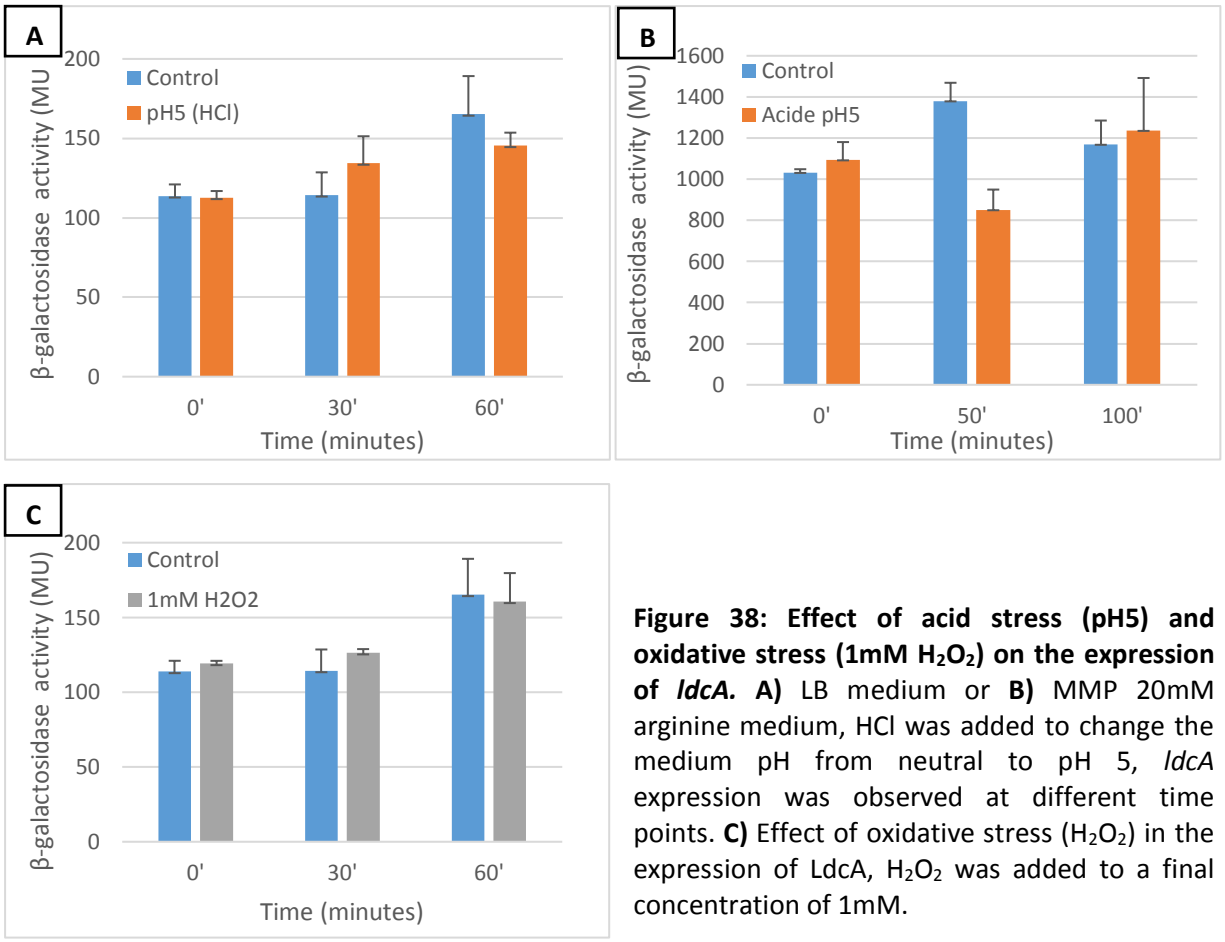


Figure 38: Effect of acid stress (pH5) and oxidative stress (1mM H_2O_2) on the expression of *ldcA*. A) LB medium or **B)** MMP 20mM arginine medium, HCl was added to change the medium pH from neutral to pH 5, *ldcA* expression was observed at different time points. **C)** Effect of oxidative stress (H_2O_2) in the expression of LdcA, H_2O_2 was added to a final concentration of 1mM.

In **figure 38 A**, we found no significant difference in *ldcA* expression between the HCl treated (pH5) and the neutral pH control medium after 60 minutes. The pH of the media was also monitored during growth and showed that *P. aeruginosa* was capable of buffering its medium in less than two hours. The same experiment was performed in in minimal medium. In **figure 38 B**, we observed that 50 minutes after acid shock, there is no induction of *ldcA* activity and what we actually observe is a temporary reduction of the β-galactosidase activity. the activity is then reestablished for the next time point where pH has risen up to 7.

These experiments allowed us to confirm that low pH do not activate *ldcA* expression and that *P. aeruginosa* is not recruiting LdcA for acid stress response.

Literature about lysine decarboxylase from *Vibrio vulnificus* showed that SoxR is responsible for the induction of *ldcI* during H_2O_2 stress. We decided to check if *ldcA* expression is induced by H_2O_2 at a final concentration of 1mM in growth medium, a condition that is known to induce oxidative stress response for *P aeruginosa* by the activation of $O2^{-}$ and H_2O_2 sensors such as OxyR and SoxR. Our experiments in **figure 38 C,** show that the β-galactosidase activity ofthe control and H_2O_2 treated bacteria are roughly the same and demonstrate that *P. aeruginosa* does not overexpress *ldcA* to respond against oxidative stress conditions.

9. Discussion

Analysis of the promoter-reporter gene fusions indicated that *ldcA* expression in *P. aeruginosa* in minimal medium was indeed activated by exogenous L-Arginine but not by L-Lysine , in accordance with published results [322]. By monitoring the expression during the growth curve, we noticed that *ldcA* expression increased progressively and reached a maximum during the stationary phase in minimal medium in presence of L-Arginine.

We also performed for the first time, an analysis of *ldcA* expression during growth in the rich medium LB. A progressive increase in *ldcA* expression through out growth in LB, was observed for both PAO1 (non-mucoid) and CHA (mucoid) strains. By comparing experiments done with lux reporter we observe that the maximum value reached in LB was lower to the ones obtained for the growth in MMP containing Glutamate 20mM + 1mM Arginine, suggesting that in LB medium, arginine was present but in lower concentrations.

Even though ArgR is the main factor of *ldcA* expression, we think that other factors could be involved in *ldcA* expression. Because when L-arginine was added to the rich medium LB we observed that the inductive effect of the amino acid began only when bacteria reach the early stationary phase because . From this observation the following hypothesis can be suggested:

i) L-Arginine acquisition is growth-phase dependant and could be regulated by quorum sensing or the stationary phase sigma factor RpoS ;

ii) L-Arginine acquisition is inhibited in exponential phase (catabolite repression) and blocks indirectly ArgR activation;

iii) ArgR needs a cofactor (protein) to activate *ldcA* transcription and this cofactor is produced in the transition to stationary phase;

iv) A repressor inhibits the transcription of *ldcA* (direct inhibition) or *argR* (indirect inhibition) in exponential phase.

In our lab (PBRC, BCI), we had the tools to test whether the RpoS the sigma factor (regulator of *ldcC* in *E. coli*), and the QS systems *las/rhl* and the PQS were involved in the expression of *ldcA* in the strain CHA.

Our results showed that *lasI* and *rhlI* mutants did not have any effect on *ldcA* expression despite of the fact that bioinformatics analyses suggested a putative role due to several predicted DNA-binding sites. Furthermore CHA∆*rpoS* mutant did not show any difference compared to CHA wild-type. Only the CHA∆*pqsA* mutant exhibited a a slightly lower level of *ldcA* expression compared to that of the wild-type one when entering the transition to stationary phase. The induction in minimal medium by adding L-Arginine, was also impaired in CHA ∆*pqsA* strain. Moreover, CHA ∆*pqsA* exhibitied the same level *ldcA* expression when the bacteria grew in LB or in minimal medium with L-Arginine.

Even though the role of PQS needs to be confirmed by complementing the *pqsA* mutant and by reproducing the same experiments in PAO1 strain to exclude any strain-specific effect, the putative effect of PQS in *ldcA* regulation seems consistent with its growth phase dependent profile of expression observed in both PAO1 and CHA strains.

Even though our bioinformatics analyses did not find any PqsR DNA-binding site in the promoter region, experimental confirmation needs to be done in order to check if PqsR plays a direct role in the regulation of *ldcA* expression.

Nevertheless, an indirect role of PQS in *ldcA* regulation can be hypothesized based on data in literature, as the QS system seems to affect L-Arginine acquisition. Indeed transcriptomic analysis of PQS regulated genes in PAO shows that the *arcD* gene, which encodes an L-arginine-ornithine antiporter, is upregulated by addition of PQS at the transition phase during LB growth, with a timing similar to *ldcA* expression (5h after the start of growth)[237,328]. It has been described that ArcD activity is essential for *P. aeruginosa* during L-arginine anaerobic growth and is important for L-arginine uptake. Since ArgR role is to sense

intracellular L-arginine concentrations, a default in L-arginine uptake should downregulate ArgR depended genes as a consequence.

Even though PQS is not synthetized under anaerobic conditions because oxygen is necessary[329], L-Arginine acquisition is regulated by ANR in order to support anaerobic L-arginine fermentation, this would explain why *ldcA* expression is kept more or less constant under anaerobic conditions[236,237].

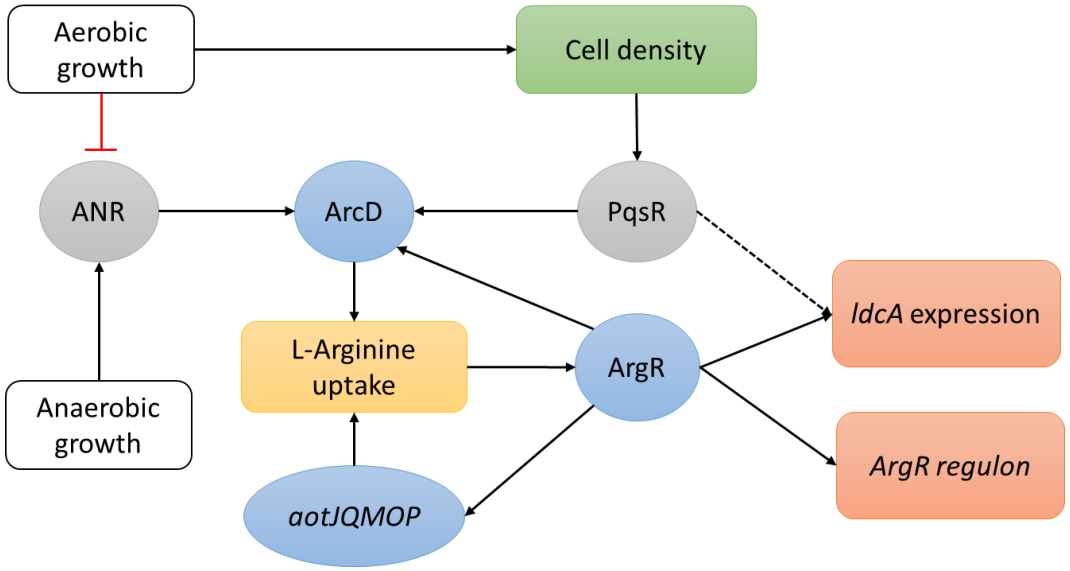


Figure 39: Interaction network of *ldcA* expression and regulation. ArgR is the principal regulator of *ldcA* expression, ArgR activation is dependent on intracellular L-Arginine concentrations. The L-Arginine pool would rely on *arcD* expression that is regulated by ArgR but also by PqsR. Under anaerobic conditions, ANR induces *arcD* expression to favor arginine mode of growth. This model suggests a novel role for PQS system that induces *arcD* when intracellular concentrations of L-arginine are too low to activate the *aotJQMOP* operon.

One way to prove this hypothesis would be to follow the expression of *arcD* and isotope labeled L-arginine uptake to see if it correlates with the *ldcA* expression, or to express *arcD* constitutively and to see how this affects L-arginine induction of *ldcA* expression when *P. aeruginosa* grows in LB. Another possibility would be to see the effect of AHQs in the expression of *arcD* and *aotJQMOP*.

During our study, we also checked the effect of acid stress and oxidative stress conditions on the expression of *ldcA* promoter. Despite of the fact that bioinformatics analysis found that

no CadC homologue was encoded in *P. aeruginosa* genome and that we did not found any SoxR binding site predicted in *ldcA* promoter, we wanted to see if *ldcA* could be activated by acid stress or oxidative conditions.

Since *ldcA* expression is at its minimum when bacteria are in exponential phase, we tested if an acid shock or hydrogen peroxide shock was capable of activating *ldcA* promoter in the growing phase as it has been described for *ldcI* in *E. coli* and *V. vulnificus.* By adjusting pH to 5 in LB and minimal media, we observed the absence of induction by acid stress This result confirms that LdcA is not part of stress response mechanisms during acid shock.

Oxidative stress was assessed by adding concentrated H_2O_2 at a final concentration of 1mM in the growth medium. We observed no effect in the expression pattern under these conditions where redox sensors such as OxyR and SoxR activate the transcription of genes involved in oxidative stress response.

Overall, our results showed that *ldcA* regulation differs from the regulation of its previously described homologues *ldcI* and *ldcC*. It is mainly regulated by ArgR but could involve other factors such as PQS system. The expression pattern of *ldcA* expression shows a parallel with the one described for *ldcC* in *E. coli*[321,326]*,* and suggests that under normal growth conditions both *E. coli* and *P. aeruginosa* accumulate cadaverine during the stationary phase.

Chapitre III: The Role of the lysine decarboxylase in P. aeruginosa

A lysine decarboxylase (LdcA) belonging to the amino-aspartate-transferase fold (AAT-fold) family of amino acid decarboxylases has been found in *P. aeruginosa* and was partly characterized by Chou et *al. 2010*[330]*.* LdcA is the key enzyme for the first step of L-Lysine assimilation pathway and is needed for the decarboxylation reaction of L-lysine into cadaverine which is further degraded in a pathway that generates energy by producing acetyl-CoA. However, literature suggested that LdcA could play another role us that its role could go beyond L-Lysine assimilation. For instance, LdcA homologues in enterobacteria are known to be important for acid and oxidative stress responses. In *E. coli,* this effect relies in detoxifying effect of cytoplasmic buffering by proton consumption and the ROS scavenging properties of cadaverine. Moreover, the sigma factor RpoS, which is a main regulator for coordinating cellular mechanisms to face stress response is induced by the presence of polyamines[331]. It is thus possible that *P. aeruginosa* could be using the lysine decarboxylase to promote its fitness when it encounters both stressful conditions as it is the case in the lungs of CF patients.

Another context in which LdcA could play a role is in antibiotic resistance. One homologue of LdcA, LdcC, is known to be overexpressed in *E. coli* during antibiotic treatment with fluoroquinoles leading to enhanced resistance[260,262]. The protective effect is thought to be explained by the capacity of cadaverine to block outer membrane porins such as OmpF and OmpC[294]. However, the addition of exogenous polyamines such as cadaverine, putrescine and spermidine in the growth medium of *P. aeruginosa* have been shown to sensitize bacteria to antibiotics such as β-lactams but increasing the resistance to quinolones, cationic peptides and aminoglycosides. This paradoxical effect is explained by the fact that polyamines affect the expression of outer membrane proteins such as OprD and OprH[332,333]. LdcA activity could modify antibiotic resistance byaffecting the expression of this family of genes.

A study about bacterial persistence against the antibiotics carboxypenicillins has shown that a putative lysine decarboxylase PA4115, played a role in modulating the amount of persister cells, this effect was attributed to cadaverine. Since the deletion of PA4115 diminished

cadaverine production by 25%, we expect that the remaining 75% can be attributed to other lysine decarboxylase like LdcA. Based on this information it seems reasonable that LdcA should play a similar role than PA4115 in the persistence phenomenon.

Literature of polyamines in *P. aeruginosa* show also that putrescine and spermidine are needed for growth and affect mechanisms like protein synthesis[334,335]. However, the role of cadaverine is barely explored in this organism and since cadaverine has similar chemical properties than putrescine and spermidine we believe that LdcA could play a role in promoting growth fitness.

In this chapter, we present an extensive study in which we assessed the role of LdcA in growth fitness, stress response, antibiotic resistance and persistence, and virulence: the goal of this work was to better understand the importance of lysine decarboxylases in bacterial physiology and the properties they have acquired during evolution.

1. **Assessment of the role of LdcA in bacterial stress response by high throughput method.**

To study the role of the LdcA, I first constructed a suicide plasmid containing a truncated allele of *ldcA* gene by the technique SOE-PCR. This plasmid was transferred to our PAO1 strain by triparental mating and by homologous recombination, the truncated allele replaced the wild type allele without the need of inserting cassettes that could create a polar effect because of the operon organization of *ldcA* and *ldcB* genes. To complement our mutant strain, we introduced one copy of *ldcA* with its own promoter in the *attB* site of PAO1 chromosome. This complementation avoided any titration effect of activating transcription factors while producing the LdcA at endogenous levels.

After successfully obtaining *ldcA* mutants (PAO1 *ΔldcA)* and their complemented strains (PAO1*ΔldcA ldcA*), we decided to use a high throughput method (Biolog) to systematically test a large number of stress conditions in a systematical and reproducible manner. To do this we started a collaboration with Dr. Rémi Peyraud at INRA Toulouse where I went for a week to perform the experiments. The Biolog system allowed to understand whether LdcA played a role in detoxifying and protecting *P. aeruginosa* during acid, alkaline and oxidative stress, antibiotic treatment and toxic molecules causing DNA damage, nitrosative stress, membrane destabilization.

The Biolog method measures the absorbance at 590 nm from cultures in the different conditions found in 96-well plates. The measured parameter at OD_{590nm} is the concentration of reduced tetrazolium. Tetrazolium is a dye that exists in two oxido-reduction states; when the molecule is reduced after reacting with NADH from cellular metabolism, it becomes violet. Therefore, tetrazolium is proportional to metabolic activity but also to the number of cells in culture.

The preparation protocol is quite simple and consisted in diluting overnight grown bacteria at initial OD_{600nm} of 0.001 in minimal medium IF-0a GN/GP containing the tetrazolium dye (as explained in part II); the mixture is then deposited in each well and the plates are put inside the incubator for the duration of the experiment. The obtained growth curves can then be analyzed by different methods but the most useful method is to compare the Area Under the Curve (AUC) between the control strain and the mutant.

The conditions selected to test bacterial fitness were held in three different plates:

1. **Plate PM10**: designed for testing the **growth fitness** in different pHs (3 to 10); the presence of **amino acid decarboxylases and other enzymes** with acid buffering capabilities; the presence of **amino acid deiminases and other enzymes** with alkaline buffering capabilities as shown in **table 10.**
2. **Plate PM11C: 24 different antibiotics** that target the outer membrane, protein synthesis, cell wall synthesis, DNA topoisomerase and that cause DNA oxidation, **table 11.**
3. **Plate PM15B: toxic molecules** that target cell membrane, create DNA damage, abolish respiration and cause oxidative stress, **table 12.**

1.1. Effect of pH on the growth fitness of *P. aeruginosa*

AUC analysis of the growth fitness of our strains (wild type, mutant and complemented mutant) showed that *P. aeruginosa* could grow optimally between a pH range from 5 to 10 without any effect on the metabolism and biomass growth **(figure 40).** We also observed that, between pH 4 to 5, the bacterial growth started to be strongly inhibited and the strains were unable to grow below pH 4, which explains why acetic acid (pH 3)[336,337] is an effective disinfectant of *P. aeruginosa* wound infections that are resistant to antibiotics. This set of

experiments didn't show any significant difference between our mutant strain and the wild-type or the complemented mutant and confirmed what we observed in LB medium.

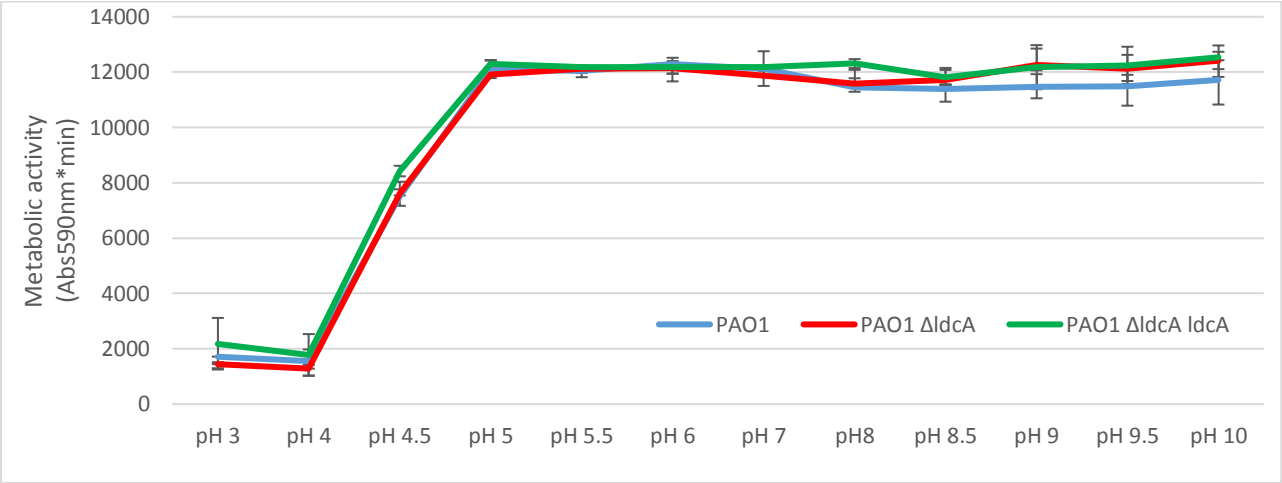


Figure 40. **Growth fitness experiments of *P. aeruginosa* in minimal medium at different pHs**. Each point corresponds to the area under the curve **(AUC)** obtained from the growth of the bacteria after 24H. In blue: PAO1 (WT), in red: PAO1*ΔldcA*, in green: PAO1*Δldc ldcA*. Experiments were performed twice for each strain.

1.2. Effect of decarboxylases in promoting fitness at pH 4.5

Amino acid decarboxylases systems, such as glutamate, arginine, ornithine and lysine decarboxylases, have been shown to significantly promote acid stress survival and fitness in bacterial families such as *vibrio, enterobacteria and lactobacilli*[248,253,338,339]. The PM10 plate was designed to test the presence of these well-known amino acid decarboxylases but also to test if the organism uses other enzymes capable of decarboxylating amino acids and other compounds to buffer the pH 4.5 of the growth medium. With our strains, we can obtain two different informations: i) we can compare the growth fitness of the wild type PAO1 at pH 4.5 with different amino acids; ii) we can see whether the mutant PAO1 *ΔldcA* has less fitness compared to the wild type, suggesting a role of LdcA in promoting growth fitness. Besides LdcA, *Pseudomonas aeruginosa* possesses other genes encoding for enzymes with amino acid decarboxylating activities such as *speA* (arginine), *speC* (ornithine), *panD* (aspartate), *gcvP1/2* (glycine), *gs* (glutamate).

The decarboxylase experiments in **figure 41,** showed us that the LdcA didn't affect the acid stress response of *P. aeruginosa* as the wild type and mutant had no differente in growth

fitness. These results were consistent with the fact that acid stress didn't induce any change in the expression of the *ldcA* promoter (as shown in **figure 38**). Moreover, enzymatic activity assays from *Chou* et al. showed that LdcA activity is maximal at pH 8.5, with its activity decreasing rapidly towards neutral pH. Overall, the experiments demonstrated that LdcA from *P. aeruginosa* is not being used to promote fitness during acid stress response despite of the fact of being a close homologue of LdcI from enterobacteria. The lack of involvement in acid stress suggests that LdcA would rather participate in polyamine metabolism and homeostasis.

The rest of the experiments revealed that the only amino acid that had a positive effect on fitness under acid conditions was L-Norvaline, a non-proteinogenic amino acid that is a byproduct of L-Valine biosynthesis in *E. coli* when there is an overflow of pyruvate in metabolism[340]. Up-to-date no norvaline decarboxylase has ever been described but *P. aeruginosa* encodes a putative amino acid decarboxylase with unknown function (*PA1346*). Nonetheless, further experimental proof would be necessary to show whether this amino acid is produced under acid conditions.

Another explanation for the advantageous effect of L-norvaline comes from the fact that the amino acid has been described to be a competitive inhibitor of the ornithine carbamoyl transferase, a pathway that produces protons in the cytoplasm and that is needed for the biosynthesis of citrulline[341,342]. The inhibition of this pathway would also increase the amount of available ornithine in the cytoplasm inducing the activity of the ornithine decarboxylase (ODC) which is known to consume protons. Nevertheless, the experiments in which ornithine was added to the medium displayed no change in the acid stress response and suggests that this pathway is probably not responsible for the fitness increase.

We also discovered that several amino acids and other molecules decreased the viability of the bacteria when dealing with acid stress. Indeed, these compounds include L-isoleucine, L-leucine, L-norleucine, L-tryptophan, anthranilate, L-5-hydroxy-tryptophan and p-amino-benzoate.

By checking the metabolism database (Metacyc, Kegg), we found that the degradation of these compounds didn't produce extra protons in cytoplasm. Nevertheless, they are quite hydrophobic molecules and could be responsible for permeating protons from one side of the membrane to the other, sabotaging buffering mechanisms. Another possibility is that

they inhibit the function or expression of proteins involved in adapting *P. aeruginosa* metabolism to acid stress conditions.

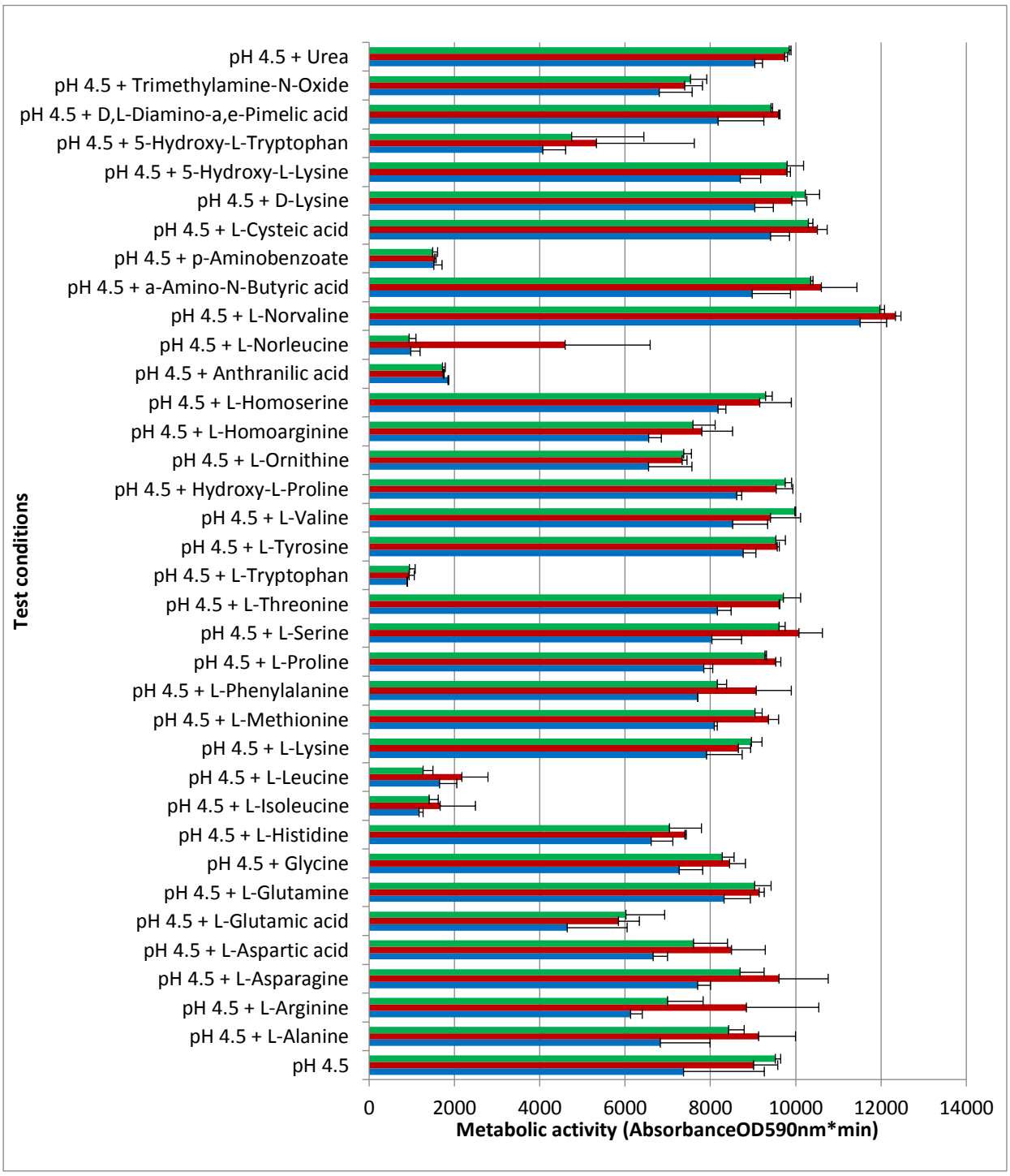


Figure 41. Effect of decarboxylases in promoting fitness at pH 4.5. Bars represent Area Under the Curve obtained from growth curves after 24h for our strains. In blue: (PAO1 WT), in red: PAO1 *ΔldcA*, in green: PAO1 *ΔldcA ldcA*. Error bars (standard deviation) were obtained from duplicate experiments from each strain. Compounds had a concentration of 2mM.

1.3. Effect of deiminases in promoting fitness at pH 9.5

The plate PM10 presented also screening conditions test the effect of amino acid deiminases involved in alkaline resistance at pH 9.5 as shown in **table 10**. Even though the lysine decarboxylase was not expected to have a protective effect under these conditions, we took advantage of this test to see whether LdcA played a role when bacteria faced alkaline conditions. Moreover, literature has shown that the pH optimum of LdcA was quite high (8.5) and it would relevant to see if cadaverine had an impact in alkaline stress physiology.

Since *P. aeruginosa* metabolism and biomass growth didn't change much between pH5 and 10, the growth conditions at pH 9.5 didn't allow us to see much of the positive effects of amino acid deiminases. Nonetheless by comparing the wild types in different growth conditions that are displayed in **figure 42**, we could observe that L-Tyrosine had a small positive effect on growth that would need to be confirmed, while L-Tyramine and b-Phenylethylamine exerted a strong negative effect on fitness and we also observed a slight negative effect of L-Tryptophane.

We checked on metabolic databases (Kegg & Biocyc) and we found that the degradation of L-Tyrosine generates two H^+ per molecule of L-Tyrosine that could explain the small positive effect on fitness that we observed. In the case of L-Tyramine we know that *P. aeruginosa* is properly equipped to degrade this compound but paradoxically its degradation creates H^+ and should have a positive effect or neutral effect on fitness. Finally, we found in literature that no enzymes degrading b-Phenylethylamine have been described in *P. aeruginosa* and the molecule is having a negative effect by an unknown mechanism.

Concerning the role of LdcA, we observed that the mutant behaved in the same way than the wild type in all conditions suggesting that LdcA does not play a role in promoting fitness during alkaline conditions.

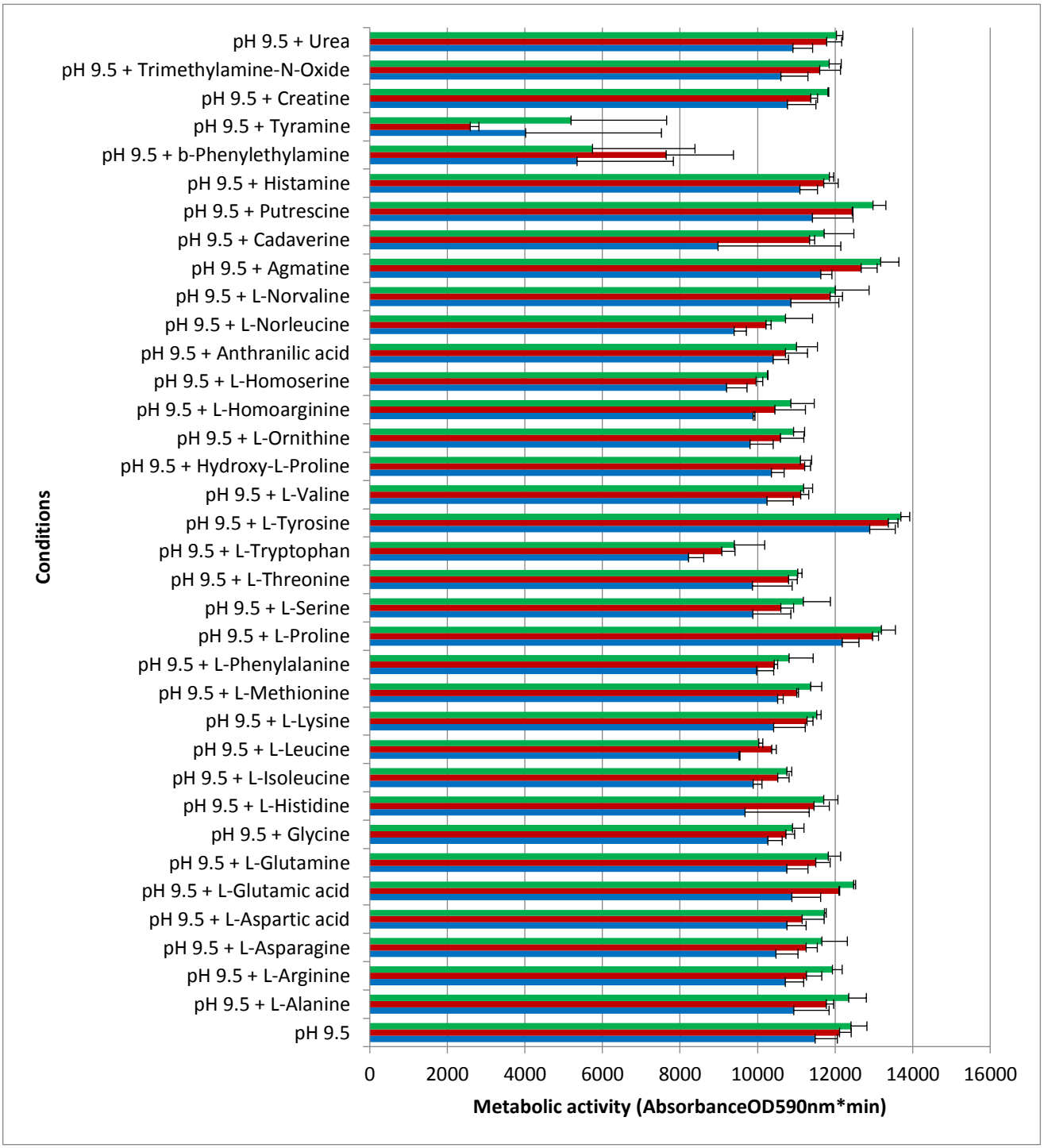


Figure 42. Effect of deiminases in promoting fitness at pH 4.5. Bars represent Area Under the Curve obtained from growth curves after 24h for our strains. In blue: (PAO1 WT), in red: PAO1 *ΔldcA*, in green: PAO1 *ΔldcA ldcA*. Error bars (standard deviation) were obtained from duplicate experiments from each strain. Compounds had a concentration of 2mM.

1.4. Effect of LdcA in antibiotic resistance, oxidative stress response, and detoxification of other harmful molecules

To assess a putative effect of LdcA in antibiotic resistance, we induced the expression of *ldcA* in the minimal medium IF-0a GN/GP by adding 1mM of L-Arginine. We observed that the PAO1 strain was resistant to 21 out of 24 antibiotics at the given concentrations: we only observed sensibility for colistin, nafcillin and minocycline. Nevertheless, no significant difference was observed between the wild-type, mutant and complemented strains under these conditions. Finally, in **figures 43 to 46** we show the results from the assays with the wells containing toxic molecules. The experiments revealed that only 8 of them had an effect in reducing *P. aeruginosa* viability. Nevertheless, we didn't found any difference between our tested strains which meant that LdcA does not play a significant role in resisting to nitrosative stress (nitroimidazole), protecting membrane from destabilizing agents like EDTA, domiphen bromide, procaine, dimethoxybenzyl alcohol or the chaotropic agent guanidine chloride and the antifolate inhibitor hydroxyurea.

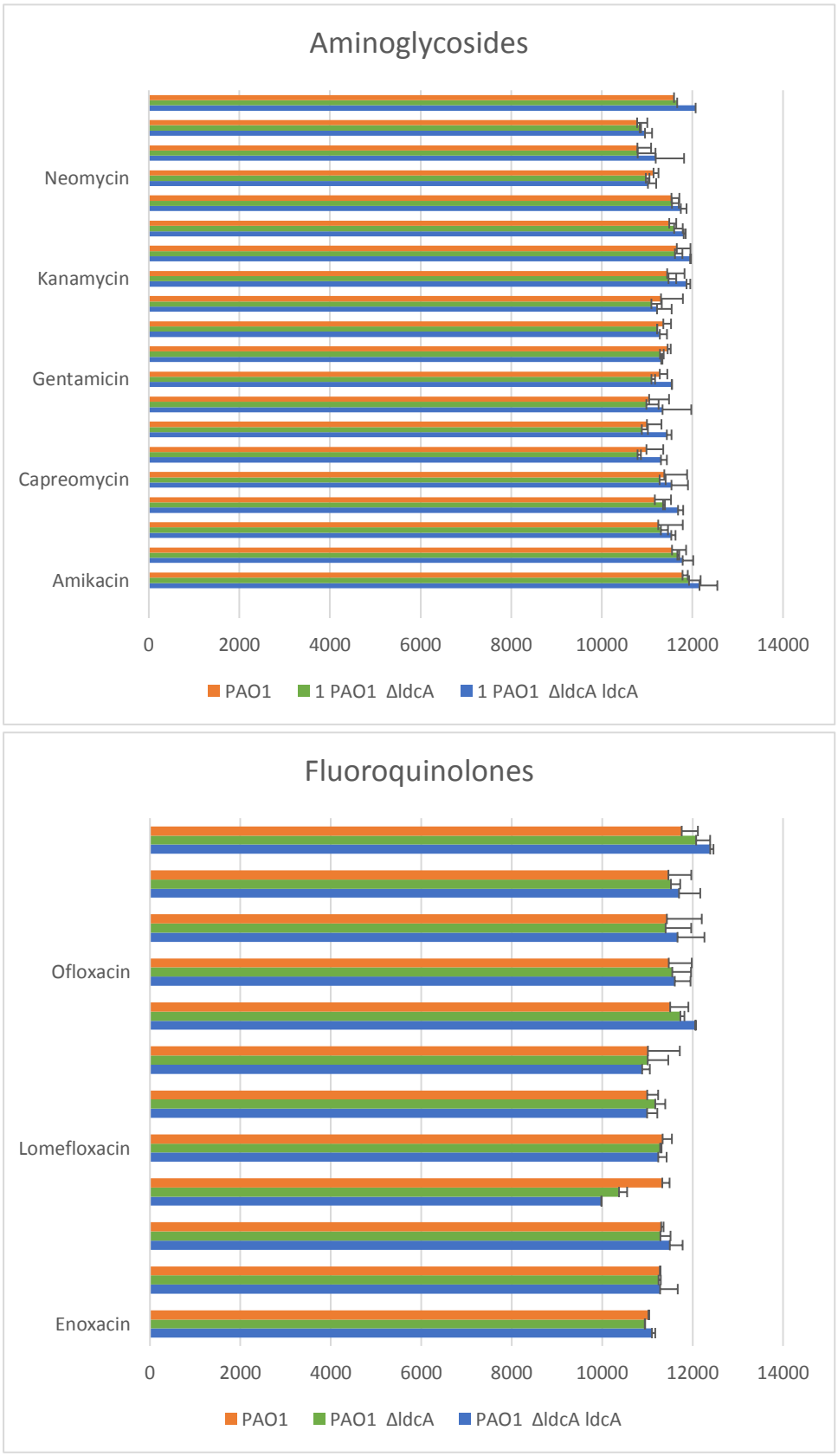
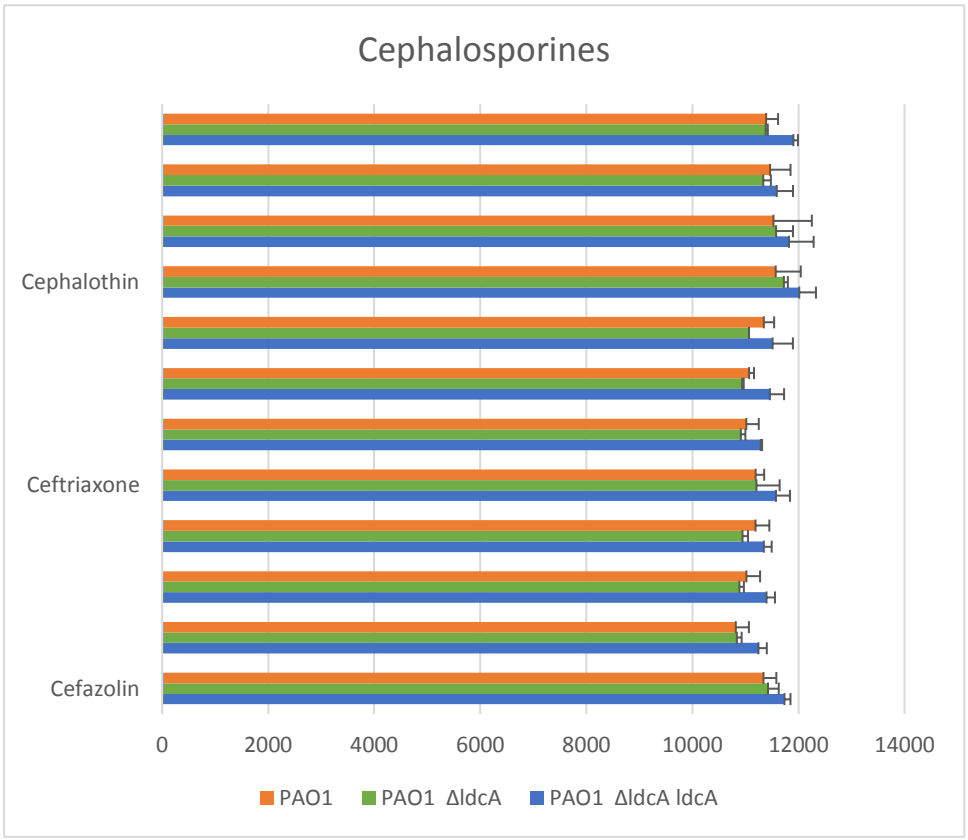


Figure 43. Effect of Aminoglycosides and Fluoroquinolones in growth fitness of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA.*



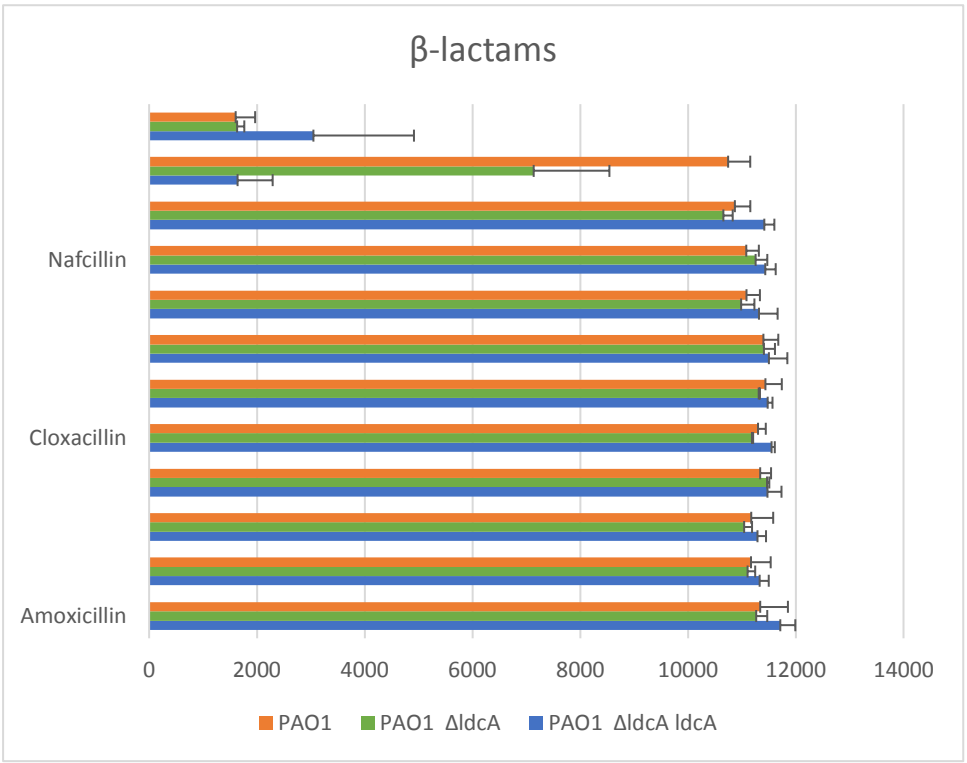


Figure 44. Effect of Cepholosporines and β-lactams in growth fitness of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA*.

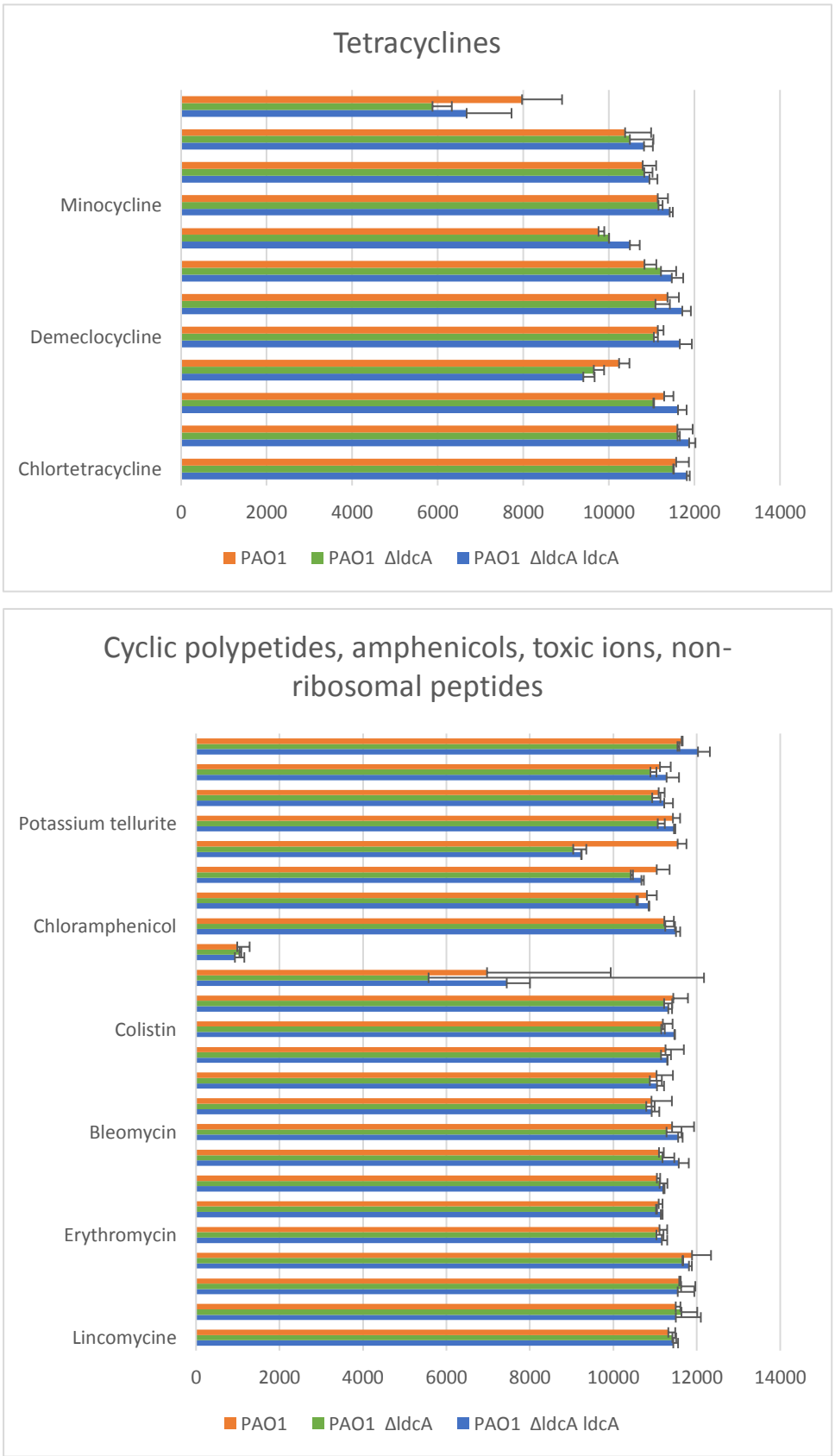


Figure 45. Effect of Tetracyclines and cyclic polypeptides, amphenicols, toxic ions, non-ribosomal peptides in growth fitness of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA*.

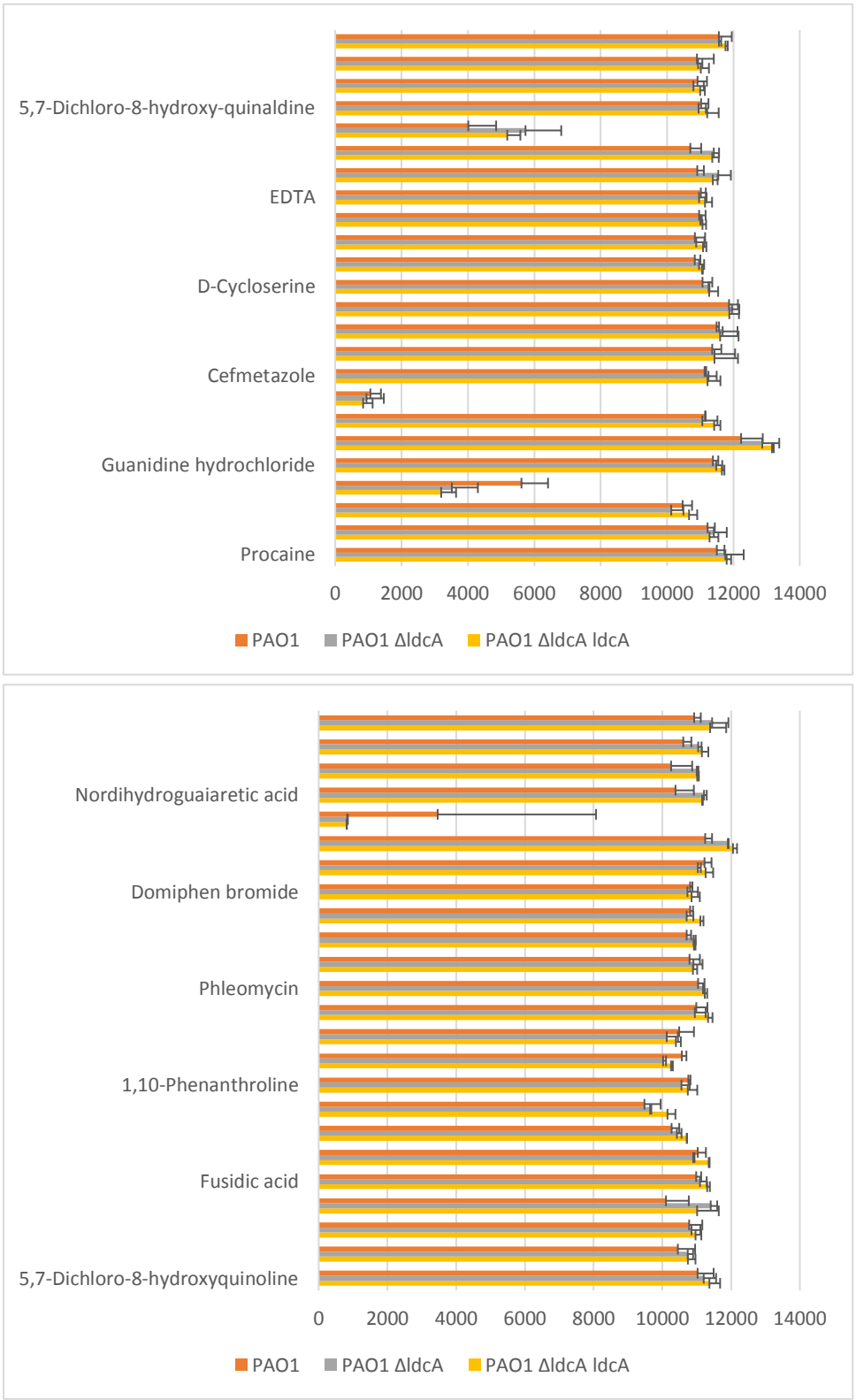


Figure 46: Effect of toxic molecules in the growth fitness of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA*, part 1.

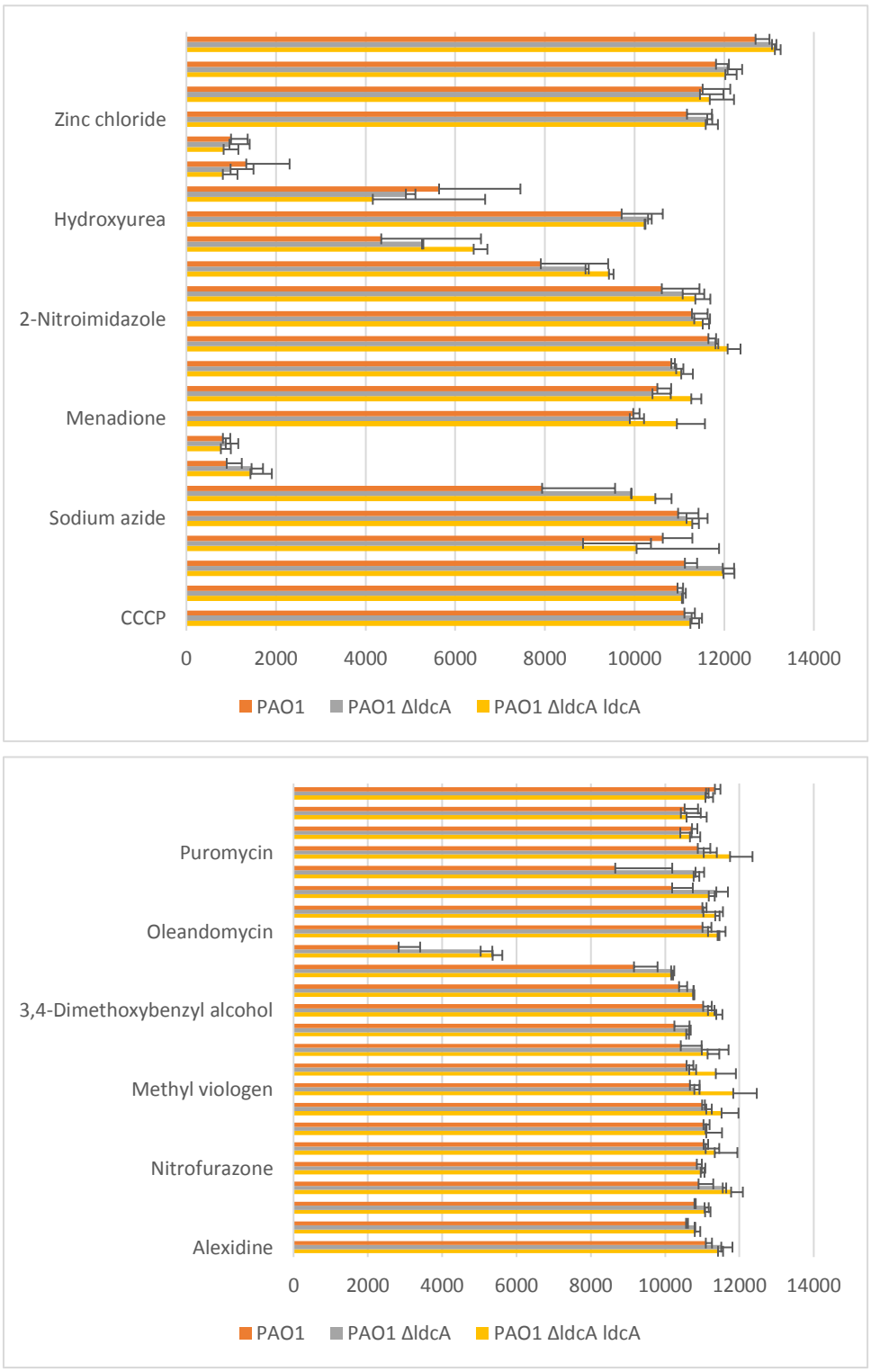


Figure 46: Effect of toxic molecules in the growth fitness of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA,* part 2.

2. The role Lysine decarboxylase and polyamines during growth

2.1. Importance of Lysine decarboxylase in carbon metabolism

While we were characterizing the expression of *ldcA* during the growth of *P. aeruginosa* by western blot, we observed that the mutant had problems when growing in minimal medium (MMP) with L-Glutamate as a carbon source. More precisely, the mutant showed a biphasic growth curve behaviour where bacteria had a long lag phase before entering exponentional growth compared to the WT and the complemented strain.

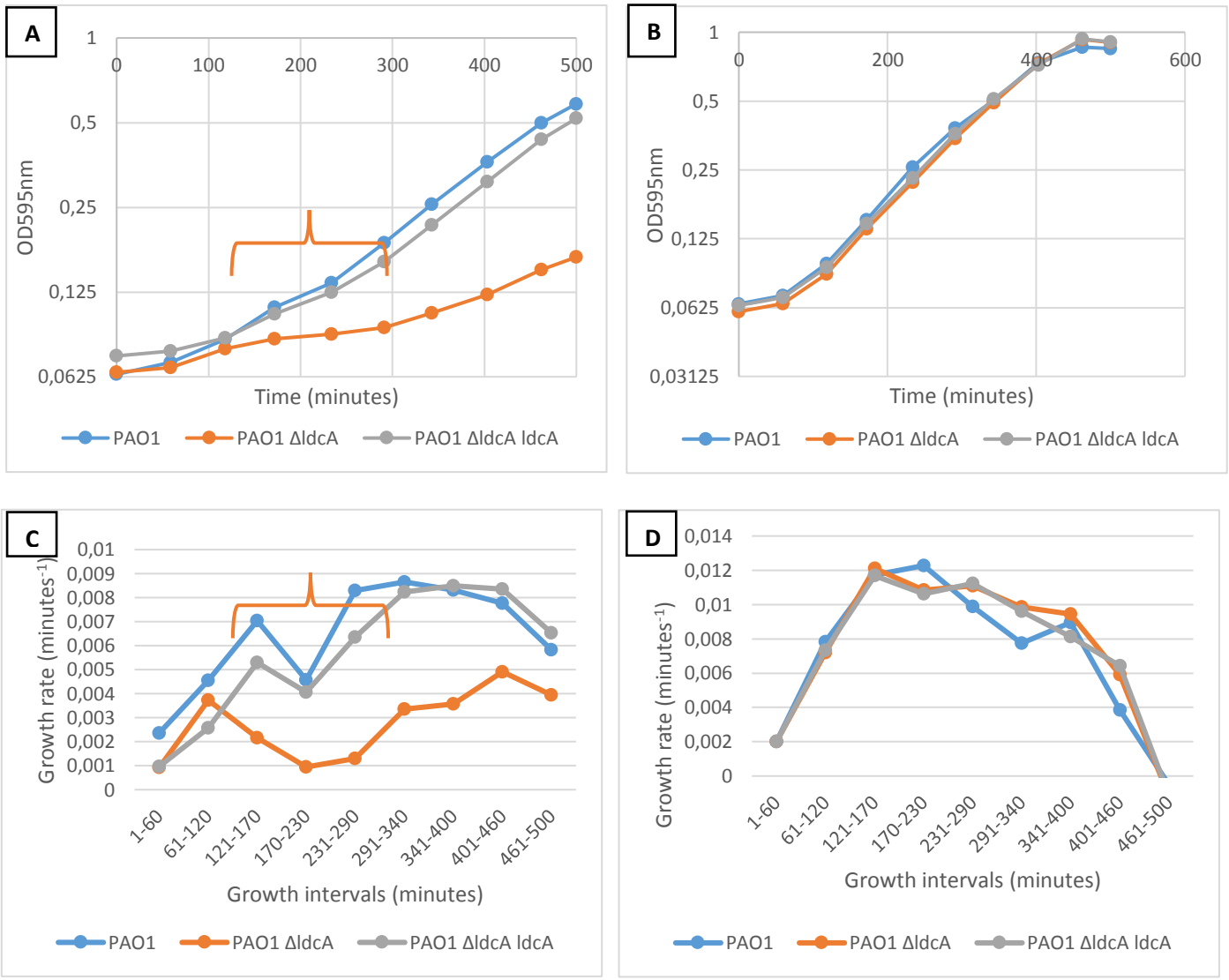


Figure 47. Growth of PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA in* minimal medium with Glucose or Glutamate. A) Growth curve in MMP Glutamate 20mM. **B)** Growth curve in MMP Glucose 20mM of the same strains. **C)** and **D)** Curves representing the growth rate of each growth interval of curves A and B respectively. Curves C and D allow to see the variation of the growth rate throughout cell growth. Experiments were performed at least 3 times with two independent mutants and their complement strains.

We confirmed the growth defect of the mutant by growing the bacteria in MMP Glutamate and MMP Glucose in 96-well plates at 37°C at 250RPM. We estimated the growth rate (R) for each condition by using the formula that was described by J. Monod:

$$dR = \frac{\log_2 dN}{dt}$$

In **figure 47 A**, we monitored the growth of our strains in MMP glutamate and we detected indeed that the mutant presented an odd growth curve in which bacteria started growing during the 60-120 minute interval and then suddenly slowed down the growth during the 120-290 minute interval, afterwards we observed the resuming growth and the entrance into what it seemed to be the exponential phase. For the other two strains, we see a similar behaviour where the lag time is not as long as the mutant. We decided to plot the growth rate for each time interval of the curve to see more clearly the changes in growth behaviour, in a similar way, as shown in **figure 47 C**. The growth-rate curve allows to see more clearly that the mutant had a period between 120-290 minutes in which growth rate falls and remains constant before starting to accelerate and reaching a peak around 400 minutes of the experiment. The behaviour of the wild type and the complemented strains is quite similar, we observe a strong short acceleration during the 60-120 minutes' interval and a growth deceleration in the 170-230 minute interval before reacceleration and entering the exponential phase, from this curve we took the highest growth rate for each growth curve.

The estimated growth rate for PAO1 and PAO1 *ΔldcA ldcA* was 0.00865 and 0.0849 minutes^{-1} respectively while for the PAO1 *ΔldcA* was 0.049 minutes^{-1} when growing on MMP Glutamate. Interestingly, experiments on MMP Glucose exhibited that the three strains (wild type, complemented and the mutant) behave similarly. The growth-rate curves show bell-shaped curves and with growth rates (peak values) around 0.12 minutes^{-1}.

From these experiments, we observed three different things:

i) the effect of LdcA depended on the carbon source and coul be linked to production of secondary metabolites important for growth fitness from cadaverine[12].

ii) we observe an effect of LdcA despite the fact that it is barely expressed under MMP Glutamate and Glucose; which can be explained by the fact that precultures could have accumulated intracellular cadaverine during the stationary phase.

iii) The effect appears between the lag phase and the exponential phase, suggesting a stimulating role for cadaverine in the beginning of cell growth

By using the information available in the KEGG and Metacyc databases, we designed diagrams of the carbon metabolism of *P. aeruginosa* when growing on Glutamate and Glucose to better understand the difference in the metabolic pathways used for the assimilation of each substrate. The objective was to select other substrates that would show the same growth defect as seen in Glutamate and to identify if the growth defect was present to one or more metabolic pathways.

Our diagrams **(annexe I and II)** show that one fundamental difference between both metabolisms is that when growing in Glutamate, *P. aeruginosa* pursues neoglucogenesis to supply sugars for the synthesis of nucleotides and polysaccharides, while growth on Glucose activates glycolysis through the Entner Doudoroff Pathway. Another fundamental difference when growing in glucose is that *P. aeruginosa*, assimilates ammonium (NH3) in three different nodes of the metabolic network (Glutamate Dehydrogenase: GDH, Glutamine Synthase: GS, Carbamoyl Phosphate Synthetase: CPS). While when growing in Glutamate, it only assimilates ammonium at two points (GS, CPS) but releases ammonium in one point (NAD-GDH).

The consequence of pursuing these two opposed pathways is that cellular metabolism requires a reorganization of the enzymes needed to generate energy and biomass which will impact the flow of key metabolites such as malate, pyruvate and phosphoenol pyruvate, glucose and glutamate but also the management of ammonium by GDH, GS and CPS. We also added the effect of the presence of Arginine (the inducer of *ldcA* expression) in our diagram of glutamate metabolism (**annexe II** blue arrows). L-Arginine shares the same pathways as L-glutamate but increases the synthesis of polyamines (putrescine, spermidine, cadaverine) and replenishes the amount of succinate and glutamate available, additionally it also increases the amount of excreted ammonium.

From this analysis, we decided to test three different types of carbon sources classified per the pathways they use:

i) Krebs cycle substrates: because they share the same properties of ammonium assimilation (GDH, GS, CPS) of the glucose pathway but use neoglucogenesis as the glutamate pathway;

ii) aminoacids sharing the degradation pathway of glutamate: because they can tell if the growth defect depends on the transport of glutamate; they assimilate ammonium at one point (GS);

iii) amino acids independent of the degradation pathway of glutamate: they end up in the same pathways as the krebs cycle substrates but assimilate less ammonium (GS, CPS).

By taking into account this information, we chose Krebs cycle substrates such as **citrate** which comes from the condensation of acetyl CoA and oxaloacetate, **α-ketoglutarate** which comes from the degradation of L-glutamate and **succinate** which is *Pseudomonas sp.* preferred carbon source[343].

In figure 48, we present the degradation pathway of amino acids amino acids that were directly linked to glutamate metabolism, they included **L-proline**, **L-glutamine**, **L-histidine** and **L-arginine.** The first three compounds possessed an intermediate metabolism between them and glutamate but the main difference is that they use other transporters than L-glutamate.

L-arginine is degraded by four different pathways. The AST pathway is the most important leading to the production of L-glutamate, succinate and ATP. The ADI pathway allows to ferment L-arginine producing L-ornithine and ATP.Finally, the ADC pathway generates additional agmatine and putrescine while the ADH pathway generates supplementary succinate.

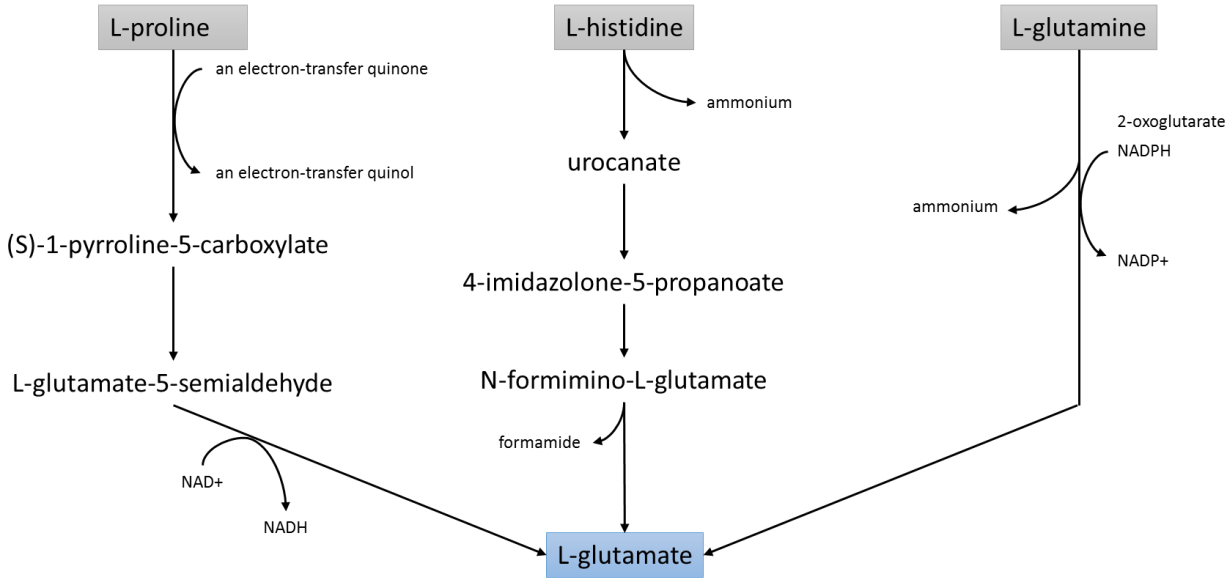


Figure 48. Catabolic pathways of L-proline, L-histidine and L-glutamine. L-proline is assimilated by two steps of oxidations leading to L-glutamate (L-glu). L-glutamine can be converted into L-glu by the action of the GOGAT or by the glutaminase. L-histidine is converted into L-glu after 4 reactions in which ammonium and formamide are relased. Adapted from Kegg and Metacyc.

The amino acids chosen for not sharing the same degradation pathway of glutamate (NAD-GDH) were **L-alanine** and **L-aspartate** and **L-phenylalanine.** They two first substrates are linked to L-glutamate metabolism by transamination reactions (Alanine Aminotransferase: ALAT, Aspartate Aminotransferase: ASAT)**.** **L-aspartate** also provides information about amino acid transport because it shares the same negative charge as L-glutamate.

L-phenylalanine as an amino acid which is not directly linked to L-glutamate metabolism and its degradation pathway ends up in the formation of acetyl-CoA and succinyl-CoA.

We also checked the effect of nitrogen surplus by adding (40mM) ammonium to the strains growing in glucose. Finally, we verified if the L-glutamate growth phenotype was still present in minimal medium with different compositions (Part II**: Materials and methods)**

			Doubling time (minutes)		
Rank	Medium	Carbon source (20mM)	PAO1	PAO1 ΔldcA	PAO1 ΔldcA ldcA
1	MMP	α-ketoglutarate	57	58	59
2	MMP	Succinate	58	59	52
3	MMP	Glutamine	68	65	62
4	MMP	Glucose	70	64	71
5	MMP	Citrate	74	76	81
6	MMP	Glucose (40mM NH4)	74	73	74
7	MMP	Arginine	82	84	81
8	MMP	Histidine	82	80	77
9	MMP	Glutamate	104	189	103
10	MMP	Proline	115	104	108
11	M63	Glutamate	134	183	134
12	MMP	Glutamate (no NH4)	143	205	158
13	M9	Glutamate	154	185	133
14	MMP	Alanine	186	182	232
15	MMP	Aspartate	264	392	243
16	MMP	Phenylalanine	365	340	322

Table 17. Growth fitness of the strains PAO1, PAO1 *ΔldcA*, PAO1 *Δldc ldcA* in different media. The effect of ammonium concentration is also assayed and the minimal medium composition (MMP, M9, M63). Results were obtained from duplicates (2 independent *ldcA* mutants and their complemented strains) in 96-well plates at 37°C with agitation (250 RPM). Doubling times are calculated by diving 1/R (growth rate).

For practical reasons, we decided to show the doubling time of the strains in minutes instead of the growth rate which is expressed as $minutes^{-1}$ or $hours^{-1}$. **In table 17 and figure 49,** we observed that substrates that conferred the fastest generation time corresponded to metabolites from the Krebs cycle. The fastest growth rate was obtained for α-ketoglutarate that exhibited a doubling time of 57', 58' and 59' for the the wild type, the mutant and the complemented strain respectively. None of the substrates of Krebs cycle showed any difference in growth for the three strains.

Amino acids conferred a slower growth rate compare to Krebs cycle metabolites and glucose. However, L-glutamine which showed a division rate comparable to the one exhibited by cultures growing on glucose. We observed a growth defect of *ΔldcA* mutant in minmal medium with **L-glutamate,** where the mutant had a doubling time of 189' and the

wild type and the complemented strain had 104’ and 103’ respectively. A similar phenotype was observed in **L-aspartate** where the mutant had a very slow growth of 392’ and the wild type and complemented had 264’ and 243’, respectively.

The L-glutamate phenotype appeared in all three-minimal media (MMP, M9 and M63) but also when L-glutamate was utilized as a carbon and nitrogen source. The division rate of the mutant is roughly the same in the three media, but the wild type and the complemented strain lost fitness in M63 and M9 media explaining a more pronounced difference in growth fitness in MMP.

Even though the three media (MMP, M9 and M63) were slight different in their mineral composition, one major difference is the amount of iron available. M9 had no iron in its composition while M63 had the half of the iron concentration of MMP.

The unexpected similar behaviour of the mutant in these three media suggest that other factors such as iron availability mediates the benefits of cadaverine in growth fitness.

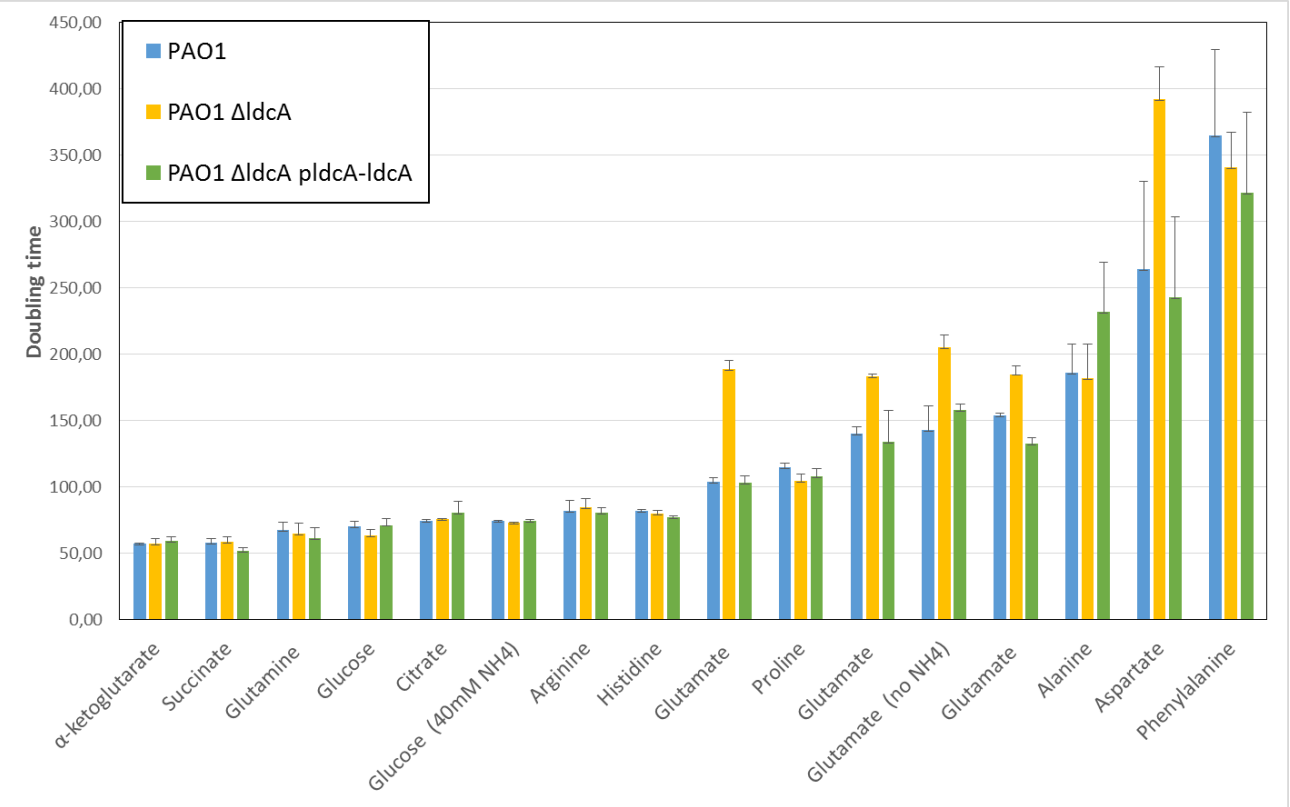


Figure 49. Doubling time (minutes) of the strains PAO1, PAO1 *ΔldcA*, PAO1 *Δldc ldcA* in growth medium with different carbon sources. The effect of ammonium concentration is also assayed and the minimal medium composition (MMP, M9, M63). Glutamate was also used as a carbon and nitrogen source (no NH4 condition). Results were obtained from duplicates (2 independent *ldcA* mutants and their complemented strains) in 96-well plates at 37°C with agitation (250 RPM).

2.2. The importance of polyamines when growing on L-glutamate

After we realized that our growth defect was only seen when growing in two amino acids (Glu and Asp), we wondered whether we could restore a normal growth phenotype in the mutant by adding polyamines in the solution.

In figures 50 A,C,E, we show three growth curves from cultures in MMP Glutamate where we included 1mM Cadaverine, 1mM Putrescine and 1mM Spermidine from the beginning of the culture. We observed that the three polyamines could reduce the growth defect of the *ldcA* mutant. Putrescine had the most potent effect on restoring growth, followed by Spermidine and then by Cadaverine.

We also plotted the growth rate for each growth interval for every experiment in the same way we did it for MMP Glutamate and MMP Glucose **in figure 45.** The addition of 1mM Cadaverine didn't completely abolish the growth delay at the transition between lag and exponential phase in the intervals (120-340), **figure 50 A**, However, we observe that the curves shows a biphasicexponential phase. The first region is observed in the 120-290 minutes interval and the second part is observed afterwards. The growth rate for each interval is 0.043 $minutes^{-1}$ for the first part of the curve and 0.09 $minutes^{-1}$ for the second part of the curve.

The addition of 1mM Putrescine (**figure 50 C**) was sufficient to abolish the growth difference between the mutant and the other strains, it was almost the case for1mM Spermidine **(figure 50 E).** Interestingly as for the case of cadaverine we see the appearance of an exponential phase with two parts exhibiting different growth rates. The addition of these polyamines created a strong acceleration in the intervals between 60-170 minutes, which diminishes afterwards leaving the place for a second part of the exponential phase with a slower but more constant growth rate.

The results obtained from the experiments suggested us that polyamines are limiting factors when *P. aeruginosa* grow on L-glutamate.

Figure 50. Effect of polyamines when growing minimal medium with glutamate for strains PAO1, PAO1 *ΔldcA,* PAO1 *ΔldcA ldcA.* A) MMP Glutamate 20mM + 1mM Cadaverine; C) MMP Glutamate 20mM + 1mM Putrescine; E) MMP Glutamate 20mM + 1mM Spermidine. B) D) F) Plots of growth rate at each growth intervals from the growth curves A, C, E. respectively. Experiments were done in duplicates (2 independent mutant strains and their complemented strains), only one representative

experiment per strain is shown for clarity. Experiments were done in 96-well plates at 37°C,250 RPM.

To continue with our study about the importance of polyamines for *P. aeruginosa* when growing on Glutamate, we decided to test whether the addition of: i) L-arginine or; ii) synthetic polyamines or; iii) 5-aminovalerate could complement the mutant phenotyp and enhance growth fitness as the three polyamines cadaverine, putrescine, and spermidine.

The interest in arginine relied in the fact that it activates the ArgR regulon that involves several enzymes implicated in the synthesis of polyamines such as the arginine decarboxylase (*speA),* the agmatinase (*speB*) and the lysine decarboxylase (*ldcA*). We wondered whether L-arginine had the same effect as the addition of exogenous polyamines (cadaverine, putrescine, spermidine) in boosting the growth fitness by inducing the endogenous synthesis of polyamines (putrescine, spermidine, cadaverine and agmatine)[236,344].

We combined the arginine and exogenous polyamines to attain the maximum growth fitness for our strains. We decided to also try the addition of 2 synthetic polyamines (diaminohexane and diaminoheptane) to see if they could induce an effect on growth fitness. We wondered whether different polyamines have comparable effects on …….

Finally, we tested the effect of 5-aminovalerate, one of the metabolites downstream in the cadaverine pathway, on growth fitness . It is known that 5-aminovalerate not only serves as an energy source but also regulates several transport operons such as the *agtABCD* and the *agtSR* operons[345].

Our results in **table 18** showed that the addition of 5mM arginine was capable of boosting the growth rate of all our strains similarly to the effect seen with exogenous polyamines in **figure 50.** However, the growth curve when bacteria were cultured on arginine was not bi- but monophasic.

The combination of 1mM Putrescine with 5mM Arginine allowed the highest growth rate and shortest doubling time for the *ldcA* mutant (60'). Also, comparable growth rates for the wild type (61') and the complemented strain (60') were obtained under these conditions. The combination of 1mM Cadaverine with 5mM Arginine had also a high boost in growth fitness but not as important as the one observed for putrescine.

		Doubling time			% of doubling time from control		
Additive 1	Additive 2	PAO1	PAO1 ΔldcA	PAO1 ΔldcA ldcA	PAO1	PAO1 ΔldcA	PAO1 ΔldcA ldcA
Control Glutamate 20mM only		104	189	103	100%	100%	100%
Spermidine 1mM	-	53	73	53	51%	39%	51%
Putrescine 1mM	-	61	69	58	59%	37%	56%
Cadaverine 1mM	-	82	111	84	79%	59%	82%
Arginine 1mM	Cadaverine 5mM	65	69	64	63%	37%	62%
Arginine 1mM	Putrescine 5mM	62	65	65	60%	34%	63%
Arginine 1mM	Spermidine 5mM	65	71	67	63%	38%	65%
Arginine 1mM	5-Aminovalerate 5mM	71	68	68	68%	36%	66%
Arginine 1mM	Diaminohexane 5mM	72	75	76	69%	40%	74%
Arginine 1mM	Diaminoheptane 5mM	77	72	81	74%	38%	79%
Arginine 1mM	Lysine 5mM	80	103	83	77%	54%	81%
Arginine 5mM	-	69	76	67	66%	40%	65%
Arginine 5mM	Cadaverine 1mM	74	69	74	71%	37%	72%
Arginine 5mM	Putrescine 1mM	61	60	60	59%	32%	58%
Arginine 5mM	Lysine 1mM	73	69	69	70%	37%	67%

Table 18. Growth of the strains PAO1, PAO1 *ΔldcA*, PAO1 *Δldc ldcA* in different growth media. The growth fitness is evaluated by the doubling time of the strains in each growth medium. The effect of different additives to MMP Glutamate was tested in duplicates (2 independent *ldcA* mutants and their complemented strains). The boosting effect of the additives is shown as a % of the doubling time of the strains in MMP Glutamate only. Experiments were performed in 96-well plates at 37°C at 250 RPM. Since the curves for MMP Glutamate and MMP Glutamate + SPD/PUT/CAD exhibit two different growth rates, the fastest one has been taken into account for comparison.

By doing the reciprocal combination of Arginine and polyamines we obtained roughly the same values of generation time. Indeed, the supplementation of 1mM Arginine with 5mM Lysine, boosted the doubling time of the wild type (82') and the complemented strain (84') to similar values to the ones obtaines for 1mM Cadaverine. The mutant was unable to use Lysine for cadaverine synthesis but could use 1mM Arginine, and exhibited a shorter doubling time of 103' instead of 189' in glutamate only. The reciprocal combination of arginine and lysine showed similar growth rate values to the ones obtained for 5mM Arginine in all the strains.

The addition of synthetic polyamines (Diaminohexane and Diaminoheptane) or 5-aminovalerate to 1mM Arginine boosted the growth rate of all the strains in a similar way than the observed for the addition of the 3 polyamines (PUT, CAD, SPM).

The results of the experiments in **table 18** suggested that addition of both exogenous polyamines and arginine has the same effect in boosting the growth rate of all the strains growing in glutamate: this indicates that polyamines are needed for optimal growth and they are in limited amounts when growing in glutamate.

The beneficial effect of diaminohexane and diaminoheptane could be explained by two hypotheses:

1. The growth fitness in glutamate depends on the expression of proteins and enzymes that are regulated by polyamines (polyamine modulon).
2. Their catabolism of polyamines enhances the metabolism of glutamate by the production of dicarboxylic acids that can enter the Krebs cycle or by boosting its acquisition by mechanisms such as gamma-glutamylation.

As the supplementation with 5-aminovalerate (5-AMV) helped to boost the growth of all the strains reinforces the hypothesis that polyamines are important because they replenish the Krebs cycle with dicarboxylic acids. However, the observation that the mutant growing in glutamine, proline and histidine doesn't exhibit any growth defects favours the hypothesis of the role of polyamines in the acquisition of glutamate.

3. The role of LdcA in the persistence to carbenicillin.

A transposon mutant in the *PA4115* gene of *P. aeruginosa* PAO1was reported to exhibit 20-fold and 48-fold more persister cells than wild type after 10 hours of ticarcillin and carbenicillin antibiotic treatment, respectively[239]. Since bioinformatics database of the NCBI predicted that the protein encoded by *PA4115* had a "lysine decarboxylase-like fold", they tested the lysine decarboxylase activity in whole cell extracts of both wild type and mutant. Their experiments showed a 25% decrease in the activity of *PA4115* mutant. They also added 0.5mM cadaverine in combination with the antibiotic treatment and they observed that the

mutant was complemented when treated with ticarcillin and only partially when treated with carbenicillin[239].

Since the persistence phenotype was attributed to the cadaverine synthesized by PA4115, we wondered whether *ldcA* mutant could also exhibit a higher number of persister cells. Moreover, their experiments suggested that PA4115 is responsible for 25% of the total cadaverine synthesis, suggesting that LdcA could synthesize the remaining 75% considering that there are only two LDC in *P. aeruginosa* genome.

Before starting to address the effect of *ldcA* in the persistence phenotype, we checked whether *ldcA* deletion modified the sensitivity to carbenicillin. We proceeded by determining the minimal inhibitory concentration (MIC) by using CLSI microdilution method[239].

As shown in **figure 51**, the determined MIC for all the strains was 64 µg/ml, 51µg/ml and 48µg/ml for the wild type, the mutant and the complemented strain respectively. Even though, the average values show that the mutant and the complemented strain show a tendency to be more sensitive to carbenicillin, the difference is not significant and suggests that the absence of *ldcA* does not impact the MIC of carbenicillin. Furthermore, it shows that the manipulation of PAO1 and mutagenesis protocols for obtaining the *ldcA* mutation did not change the sensitivity to carbenicillin.

As shown in literature, we assessed if the exogenous addition of cadaverine could sensitize our strains to to carbenicillin treatment.[332] To do so, we added 20 mM Cadaverine into the test medium and calculated the MIC of the different strains. Indeed, the exogenous cadaverine increased the sensitivity for all the strains, showing similar MIC for the wild type (12 µg/ml), the mutant (16 µg/ml) and the complemented strain (16 µg/ml).

The experiments in **figure 51,** showed that 20mM of cadaverine had an effect on antibiotic sensitivity and confirmed what was already described in literature[332]. However, the lack of significant difference between the wild type strain and the *ldcA* mutant suggested that cadaverine production by LdcA is not sufficient to induce a change in antibiotic sensitivity of PAO1 or, for other reasons the effect happens when cadaverine is exogenous.

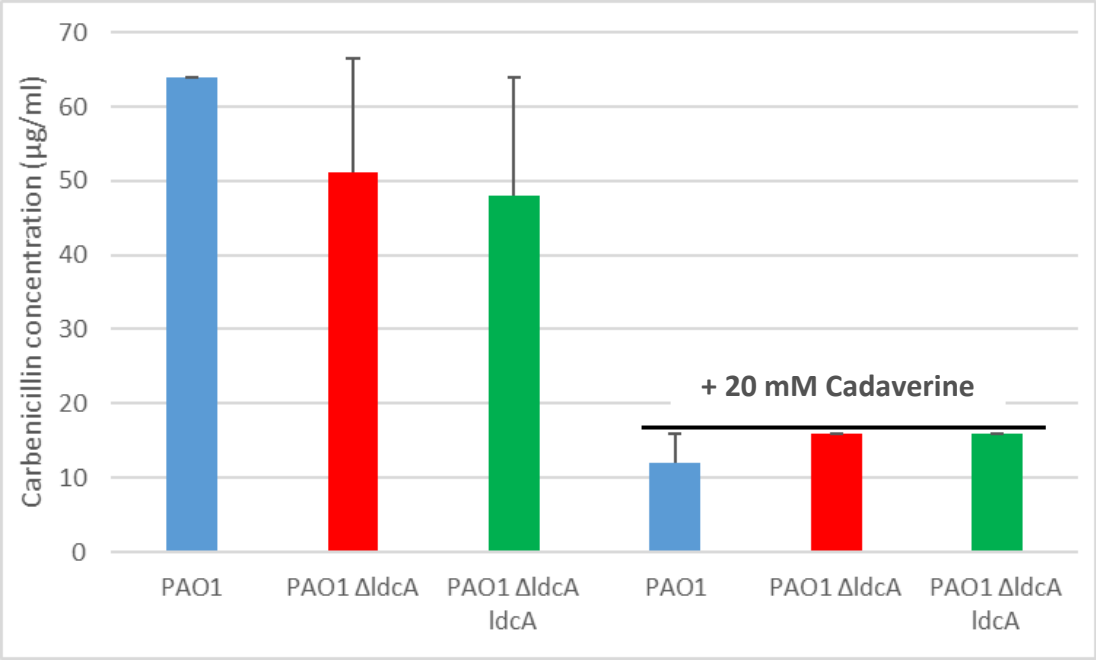


Figure 51. Minimal inhibitory concentrations (MIC) of carbenicillin against *P. aeruginosa* in Mueller Hinton Broth. In blue: PAO1 WT, in red: PAO1 *ΔldcA.* The strains have been treated with 20mM Cadaverine (CAD) when indicated**.**

After checking that our strains have a similar MIC for carbenicillin, we decided to use the same experimental settings that were described on *PA4115* research paper[239] to assess the amount of persisters for our strains when treated with carbenicillin (as shown in Part II). Two different growth media were used for the test: the cation adjusted Mueller Hinton Broth (MHB, Rich Medium), which was used to find the *PA4115* mutant phenotype[239], and the minimal medium MMP containing 20mM Glutamate, 1mM Arginine and 5mM Lysine as carbon sources, which showed a growth defect for the mutant strain (**Table 18**). Since literature about bacterial persistence establishes that slower metabolism and growth fitness favor bacterial persistence[89], we were wondering if PAO1*ΔldcA* would exhibit a stronger persistence phenotype when its growth fitness was lower than the wild type.

Our persistence experiments were performed as the following: briefly, we used overnight grown bacteria that were used to start cultures at OD600nm of 0.05 in Erlenmeyer flasks under aerobic conditions; once the cultures reached exponential phase (OD600nm 0.4-0.6), we added carbenicillin at a final concentration of 500 µg/ml which corresponds to 8X the MIC for the antibiotic. Finally, we assessed the amount of persisters by doing CFU after either 6h or 24h of treatment.

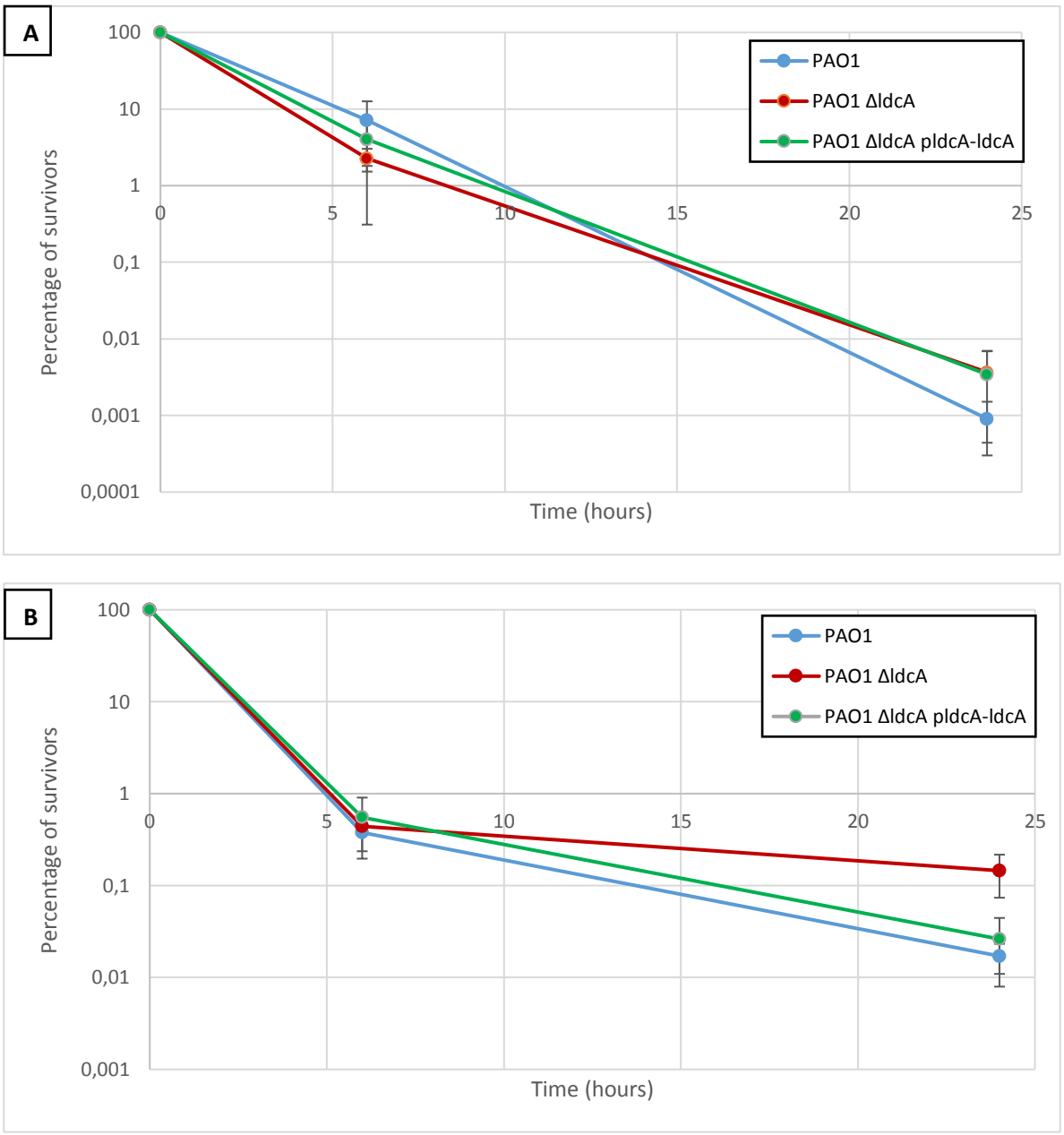


Figure 52. Persistence assays in two different growth media (rich medium MHB and minimal medium MMP) treated with 500µg/ml of carbenicillin A) Percentage of survivors in Minimal medium (MMP Glutamate 20mM Arginine 1mM Lysine 5mM) after 6h and 24h of carbenicillin treatment. **B)** Percentage of survivors in rich medium (cation adjusted Mueller Hinton Broth) after 6h and 24h of carbenicillin treatment. Growth was performed in quadriplicates in erlenmeyer flasks at 37°C with agitation (300 RPM). Perfectage of survivors was calculated from CFU counting before and after 6h and 24h of antibiotic treatment.

Figure 52 A), presents the assays performed in minimal medium. The first samples were taken 6 hours after carbenicillin addition and showed that 93% to 98% of bacteria were killed: no significant difference was observed between the 3 strains. The next samples were taken after 24h showed that bacteria continued to be killed, but the number of survivors

was not significantly different between the strains. Other experiments were done in minimal medium butno significant difference had ever been observed up to now.

		% of survival		
Medium	Strain	Control	6h	24h
MMP	PAO1	100	7.2 ± 5.4	0.00091 ± 0.00061
	PAO1Δ*ldcA*	100	2.3± 0.7	0.0036 ± 0.0032
	PAO1Δ*ldcA ldcA*	100	4.0±3.7	0.0034 ± 0.0035
MHB	PAO1	100	0.37 ± 0.14	0.017±0.006
	PAO1Δ*ldcA*	100	0.19 ± 0.17	0.145±0.071*
	PAO1Δ*ldcA ldcA*	100	0.55 ± 0.35	0.026±0.018

Table 19. Survivors from antibiotic persistence experiments in **figure 54**. Statistical analysis using Krustal Wallis test showed that the mutant had a significantly higher amount of persisters compared to the wild type and the complemented strain when the experiments where done in MHB medium. (* = $p<0.05$).

When the assay was performed in rich MHB medium **Figure 54 B),** no significant difference between the strains was again observed after 6 hours of treatment. However, after 24 hours of treatment, strain PAO1Δ*ldcA* exhibited in average 8.5 times more persisters than the wild type and 5.5 times more than the complemented strain (see Table 10). A Kruskal-Wallis test, conducted on the quadruplicates, concluded that the difference in peristers was statistically significant. To conclude, the mutation in *ldcA* impacts significantly persistence mechanism as the mutant exhibits a higher number of persiter cells. This is in agreement with the proposal from Manuel et *al.,2010*[239] that establishes that cadaverine affects the amount of persisters. However, in our hand the effect caused by cadaverine was not as strong as theirs. By using two different growth media, we also could see that the effect of the cadaverine depends on the composition of the growth medium.

4. Assessment of the importance of LdcA in virulence of *P. aeruginosa*

It has been shown that the lysine decarboxylase can exert a negative effect on the pathogenicity of enterobacteria such as *Shigella sp.* and Shiga-Toxin producing *E. coli* (STEC). One of the common features of this bacteria is that they lost the expression of *ldcI* operon [346,243]. When the expression of *ldcI* is restored in *Shigella flexneri* the Shiga-Toxins are inhibited and the bacteria are unable to lyse the phagolysosome of the host macrophages

and bacteria were unable to induce PMN transepithelial migration[243,271]. In pathogenic *E. coli*, it has been described that *ldcI* has been lost in shigatoxin-encoding strains and that cadaverine inhibits the expression of intimin, an adhesin required for full pathogenicity[243]. The pernicious effect of cadaverin and LDC in pathogenicity suggests that the loss of LdcI was a pathoadaptive evolution event required for displaying full pathogenicity of STEC and *Shigella* [243].

Another relevant information in literature pointed out that exogenous spermidine induced the expression of the T3SS in *P. aeruginosa*. Even though exogenous cadaverine was not capable of inducing T3SS, it is nevertheless possible that endoneous cadaverine affect the expression of T3SS[347].

For these reasons, we wondered whether *ldcA* affected T3SS dependent virulence in *P. aeruginosa*.

DelaVega et *al., 1995*[348] showed that the blocking of porins by cadaverine affected the swarming motility of *E. coli*. Since the motility test are very simple to do, we tested *ldcA* affected the mechanisms of motility in the same way as in *E. coli*.

In **figure 53**, we present experiments of cytotoxicity of *P. aeruginosa* PAO1 against murine macrophages. After 3 experiments, observed no significant difference between our strains. Even though we didn't measure the expression of *ldcA* in the wild type, the culture medium for macrophages DMEM (Dulbecco's Modified Eagle's Medium) contains 0.5 mM of Arginine and 1mM of Lysine implying that *ldcA* expression is induced and has enough Lysine to produce cadaverine. Nevertheless, the expression of *ldcA* needs to be verified to conclude that *ldcA* does not affect T3SS dependent virulence under these conditions.

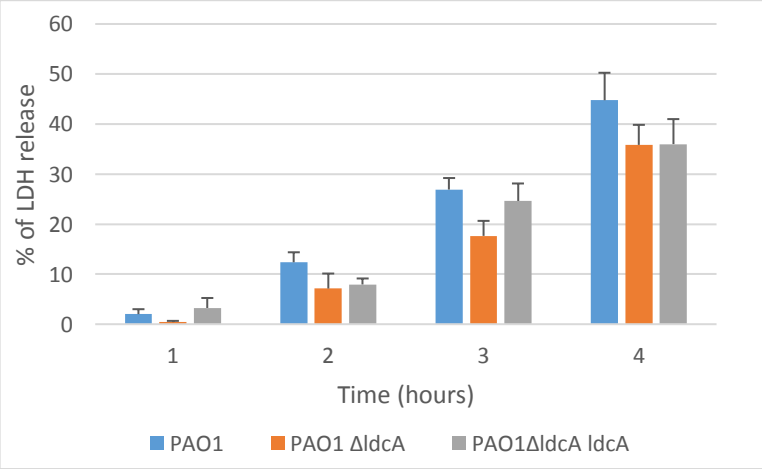


Figure 53. LDH release by murine macrophages RAW 264.7 infected with PAO1, PAO1 *ΔldcA*, PAO1 *ΔldcA ldcA*. Cells were infected for 4 h at an MOI of approximately 5. LDH enzyme is released by macraphoges upon cell death. Error bars represent standard errors of the means for experiments performed in triplicate.

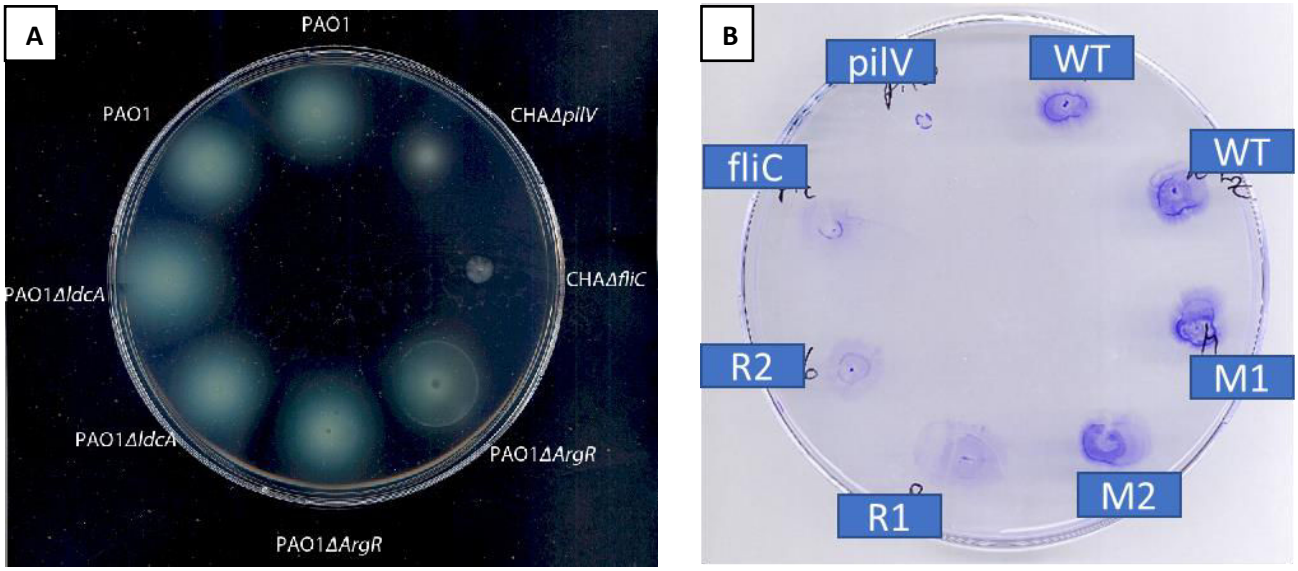


Figure 54: Macroscopic motiliy assays of *P. aeruginosa*. A) Flagellum-mediated motility assay: swimming zones were measured 24h after inoculating 0.3% agar plates with Tryptone 10g/L, 85mM NaCl; zone diameters (means ± standard deviations; n= 2) of PAO1 (3.2 ± 0.2 cm), PAO1 *ΔldcA* (3.4 ± 0.2 cm), PAO1 *ΔargR* (3.3 ± 0.2 cm), CHA *ΔpilV* (1.05 cm), CHA *ΔfliC* (1.5 cm). B) Twitching motility assay. Cells were stabbed into LB agar plate (1.5% agar) and incubated at 37°C for 48h, afterwards twitching zone was revealed by addition of coomassie blue; zone diameters of of PAO1 (WT) (1.4 ± 0.1 cm), PAO1 *ΔldcA* (M1 & M2) (1.45 ± 0.1 cm), PAO1 *ΔargR* (R1 & R2) (1.4 ± 0.2 cm), CHA *ΔpilV* (pilV) (0.35 cm), CHA *ΔfliC* (fliC) (0.63 cm). CHA *ΔpilV* and CHA *ΔfliC* were used for negative controls for twitching and swimming experiments respectively.

We also performed experiments as shown in **figure 54,** to see whether *ldcA* had an impact in the motility of *P. aeruginosa*. We observed that the lack of *ldcA* did not have any significant difference in the size of the swimming diameter or in the twitching diameter compared to the wild type PAO1. Suggesting that LdcA activity does not affect the activity of the flagellum or the type IV pili.

Even though our virulence experiments didn't show any effect of *ldcA* in T3SS and motility, it will be necessary to performn experiments in entire organisms in which the expression of *ldcA* is governed by biologically relevant conditions and not just a customized growth medium as the ones used for macrophage growth or LB medium. We believe that LdcA and cadaverine could play a role in other virulence mechanisms in which polyamines are involved such as PMN migration, carcinogenesis, cell apoptosis, biofilm formation and attenuating innate defence mechanisms such as the inducible nitric oxide synthases[118].

5. Discussion

5.1. *P. aeruginosa* LdcA does not participate in active stress response mechanisms.

The high throughput phenotypical analysis performed with the Biolog system allowed us to test whether LdcA was being part of stress response mechanisms. The 3 different screening plates, with 144 different experimental conditions that we used, allowed us to test whether *ldcA* played a role in acid, alkaline, oxidative stress response and antibiotic resistance. The results of this analysis indicated that LdcA didn't have any significant effect in enhancing fitness during stressful conditions. This was consistent with the fact that our expression experiments showed that *ldcA* is not overexpressed during acid stress response as it is the case for *ldcI* in *E. coli*.

Even though these results seem surprising because cadaverine from LdcI has been reported to scavenge Reactive Oxygen Species (ROS) and protect *Vibrio vulnificus* and *E. coli* from oxidative stress[218,241], this difference could be explained by the fact that defense mechanisms change from one bacterial species to the other. For instance, the mechanism by which enterobacteria use LdcI to fight oxidative stress consists in blocking the porins (OmpC, OmpF) with the synthesized cadaverine[294]. The fact that OmpC or OmpF homologues do not exist in *P. aeruginosa,* suggest that cadaverine could play a different role in enterobacteria compared to *P. aeruginosa*.

However, it is still possible that cadaverine produced by LdcA and accumulated by the bacteria during stationary phase or arginine induction could still enhance the survival of *P. aeruginosa* under stress conditions.

The Biolog system was not effective in delivering much information about the effect of cadaverine in the resistance of *P. aeruginosa* against antibiotic treatment. Only 3 antibiotics, Colistin (polypeptide antibiotic: solubilizes the cytoplasmic membrane), Nafcillin (beta-lactam antibiotic: inhibits cell wall synthesis) and Minocyclin (tetracycline-type antibiotic: protein synthesis inhibitor by binding 30S ribosomal subunit) were toxic to *P. aeruginosa* and there was no significant difference of their toxicity in the wild type or *ldcA* mutant strains. We realize that the antibiotic concentrations stablished by the Biolog system were too low for our strain of *P. aeruginosa,* which is known to contain several efflux systems to expel toxic molecules and antibiotics outside the cell and low membrane permeability. Unluckily,

because Biolog products are protected by patents, we couldn't know the concentrations of the molecules we used. The company would only share this information if a publication is based on their information.

In the same way, the screening of toxic molecules showed that only 8 of them out of 24 had an effect in reducing *P. aeruginosa* viability but no difference was significant between our different strains: this suggests that LdcA does not play a significant role in resisting to nitrosative stress (nitroimidazole), protecting membrane from destabilizing agents like EDTA, domiphen bromide, procaine, dimethoxybenzyl alcohol or the chaotropic agent guanidine chloride and the antifolate inhibitor.

In overview, the results that we obtained from the high throughput experiments showed us that *P. aeruginosa* is not using LdcA as a stress response enzyme against acid stress conditions or different kinds of oxidants, because there was no difference in the growth fitness between the wild type and the *ldcA* mutant in our experiments. Furthermore, it didn't affect the sensitivity towards different classes of antibitiocs. Even though, these experiments show that the cadaverine produced by LdcA does not enhance the fitness of *P. aeruginosa* under stress conditions, this doesn't mean that cadaverine could not play a role in enhancing the survival of bacteria to stress conditions as it as been shown in E*. coli* and *V. cholera.*

Since it is well known that *P. aeruginosa* creates complex biofilms to respond to a wide variety of stress challenges, it would be pertinent to analyse whether LdcA play a protective role under these conditions.

5.2. The cadaverine is needed for optimal growth in L-glutamate and L-aspartate

Since the cadaverine degradation pathway is not directly related to the assimilation of L-glutamate, it was surprising to discover that *ldcA* mutant had lower growth fitness under this condition compared to the wild-type strain. This suggests that cadaverine and its degradation pathway do not only participate in the creation of energy for cell growth but could also influence the efficiency of amino acid metabolism.

By understanding the different assimilation pathways of L-glutamate and L-glucose (no *ldcA* phenotype), we could choose different carbon sources to test whether L-glutamate degradation pathway or transport was affected. The growth rate analysis showed that amino acids using the same degradation pathway as L-glutamate (L-proline, L-arginine, L-glutamine,

L-histidine) didn't present a growth defect as it was seen when bacteria grew in the presence of L-glutamate. This result suggested that the defect of *ldcA* mutant seems to be linked to the transport of the amino acid into the bacterial cytoplasm. Since this entrance relies on different specific ABC transporters, it seems plausible that cadaverine and polyamines are playing a role in the transport of acidic side chain amino acids such as L-glutamate and L-aspartate by an unknown mechanism. Today only one transporter (PA1340) has been described as necessary for acidic amino acids and L-glutamine, but another homolog exists (PA5076) and it would be interesting to probe if this transporter needs polyamines for functioning properly.

AMINO ACID	TRANSPORTER
L-PROLINE	PutP
L-HISTIDINE	HisJ
	HisQ
	HisM
	PA5504
	OpdC
L-ARGININE	AotJ
	AotQ
	AotM
	AotP
L-GLUTAMINE	AatM
L-GLUTAMATE	AatM
L-ASPARTATE	AatM
	PA5076

Table 20. List of amino acid transporters linked to L-glutamate metabolism.

The "transporter" hypothesis is also consistent with the observation that adding 1mM of polyamines in the L-glutamate growth medium enhanced growth rate of all the strains. This observation also suggests that polymines are one of the limiting factors when bacteria are growing in a medium containing L-glutamate. Nevertheless, our general knowledge about polyamine metabolism is still limited and even though the main pathways and regulators of polyamine biosynthesis have been described, the role of other important regulatory mechanisms such as polyamine acetylation and deacetylation are completely unknown in *P. aeruginosa*. Literature from *E. coli* shows however that acetylation and deacetylation are essential processes in the homeostasis of polyamines and help to fine tune the intracellular

concentrations and function of this compounds, they also block the toxic effect of polyamines at low temperatures by changing their molecular properties.

Quite recently two new genes, *PA0321* and *PA1409,* and their protein products have been described to be necessary for the growth of *P. aeruginosa* on substrates such as acetylcadaverine and acetylputrescine[349]. Moreover, the fact that there could be at least 4 possible polyamine acetylase counterparts (*PA1377, PA1472, PA3944, PA4114*) shows that the field of polyamine physiology is far from being completely understood. Therefore, it remains possible that cadaverine participates in L-glutamate metabolism in other mechanisms beyond its transport.

5.3. The role of cadaverine in bacterial persistence.

The experiments assessing whether LdcA modified the amount of persister cells after antibiotic treatment showed us 3 different things:

1. The dynamics of cell death and persistence depended on the growth medium, which was in accordance with the fact that metabolism had a decisive impact in regulating the amount of persisters. By comparing the shape of the curves of surviving bacteria in both rich and minimal media, we realized that there were more persisters after 6h of antibiotic treatment in minimal medium compared to the rich medium. However, this tendency reversed after 24h of treatment as there were more survivors in rich medium than in minimal medium. The other point was that *ldcA* mutant showed a significant difference in persister level with the wild type and the complemented strains only in the rich medium.

   Another factor that could affect the dynamics of killing came from the fact that ppGpp levels are higher when nutrients are in limited amounts. Since ppGpp regulates essential processes such as protein and DNA synthesis, we expected to have a higher amount of persisters in minimal medium. Nevertheless, our experiments showed that other factors in the growth medium were implicated in this phenomenon because at the end we saw more survivors in rich medium MHB after 24H of antibiotic treatment.
2. The effect of LdcA is lower compared to that of PA4115. The experiments in MHB showed that *ldcA* mutant exhibited 8.5 more persisters in average compared to the

wild type strain, while in the case of PA4115 mutant there is 48-fold difference. This discrepancy could come from the fact that we actually don't know the expression level in this medium nor the amount of cadaverine accumulated in the strains.

3. We have strong doubts whether PA4115 is really a lysine decarboxylase as proposed[239]. By doing bioinformatics analysis of PA4115, we find in the NCBI database a "lysine decarboxylase like fold" that has never been shown to be capable of this enzymatic activity. The consensus sequence found in PA4115 belongs to proteins having a Rossmann fold capable of binding nucleotides[350]. Moreover, a close homologue in *Corynebacterium glutamicum,* the protein *Cg*2612 which is also describedin the database as having a "lysine decarboxylase like fold" was purified and never exhibited LDC activity but instead showed a phosphoribohydrolase activity[320]. In fact, the crystal structure of the protein showed that the conserved Rossmann fold is capable of binding AMP. Data from literature altogether suggest that this family of LOG proteins is involved in the biosynthesis of cytokinins and not into lysine decarboxylation activities. This information opens the possibility that *PA4115* could actually be synthetizing a growth factor that could have an impact in the amount of persister cells independent or not from cadaverine.

5.4. The role of cadaverine produced by LdcA in virulence

Experiments in which cytotoxicity of the different *P. aeruginosa* strains (PAO1, PAO1*ΔldcA*, PAO1*ΔldcA::ldcA*) was tested on murine macrophages RAW showed that presence of LdcA is not required in this T3SS-dependent infection model and that T3SS is not affected by the cadaverine produced by LdcA. Furthermore, we demonstrated also that LdcA didn't play any role in modulating the swimming and twitching mobility. Finally, we wanted to test the toxic effect of polyamines in more relevant virulence models such as the chicory leaves, but preliminary results were not conclusive and the experiments must be repeated. Since our expression experiments showed that LdcA is expressed in stationary phase and air-surface biofilms, it would be very interesting to see whether LdcA is necessary for biofilm formation, this would rejoin previous observations in literature that show that polyamines are necessary for the maturation of biofilms.

Chapter IV: Phylogenetic analysis of the Lysine decarboxylase from P. aeruginosa

During my PhD thesis, we decided to complete the biochemical characterization of LdcA by purifying the recombinant LdcA in order to see its oligomeric state, enzymatic activity, the effect of ppGpp inhibition. We also decided to solve the structure of LdcA by different structural biology methods such as X-ray crystallography and cryo-Electron microscopy. This structural characterization would be important to bring new elements of comparison with enterobacterial lysine decarboxylases and would also allowed us to see the differences that arrived during the evolution of the LDC in a different taxonomic order of proteobacteria.

To complement this analysis, we decided to establish a scientific collaboration with Celine Brochier-Armanet from University of Lyon to perform phylogenic analysis of LdcA. This analysis would allow us to better understand the evolutionary history and ancestral relationship with the other well-known lysine decarboxylases of the AAT-fold family.

1. Phylogeny of Lysine decarboxylases LdcA

In collaboration with Celine Brochier-Armanet from University of Lyon, we decided to understand the relationship of LdcA with its homologues in other bacteria. An in-depth survey of 4,466 proteomes was performed and revealed 4,090 homologues of Ldc, Adc and Odc. Representatives of this family were mainly present in Firmicutes, Actinobacteria, Cyanobacteria and Proteobacteria, the family was called as the L/A/Odc family of proteins. A maximum likelihood tree inferred with a representative subsample of sequences showed that Ldc, Adc and Odc found in Proteobacteria grouped together, with Ldc and Adc being more closely related, while sequences from other bacterial phyla appeared more distantly related and corresponded likely to distantly related subfamilies. Monophyly of OdcI/C, LdcA and LdcI/C groups are supported but not for Adc (pp=0,72), as shown in **figure 55.**

Our phylogenetic analyses indicated that LdcA was ancestral in Betaproteobacteria and in Pseudomonadaceae, while LdcI/LdcC and OdcI/Odc originated likely in bifurcation between Aeromonadaceae and others Gammaproteobacteria and in bifurcation between Vibrionaceae and others Gammaproteobacteria, respectively. Finally, we highlighted also several putative horizontal gene transfer events, especially in the Adc subfamily. Recently, it has been shown that L/A/Odc from Burkholderia sp. AIU 395 has Lysine decarboxylase and Oxydase activity[301]. This sequence is found to be in the group of Adc suggesting that this group is maybe composed mainly of Ldc and that Adc activity is an exception inL/A/Odc family.

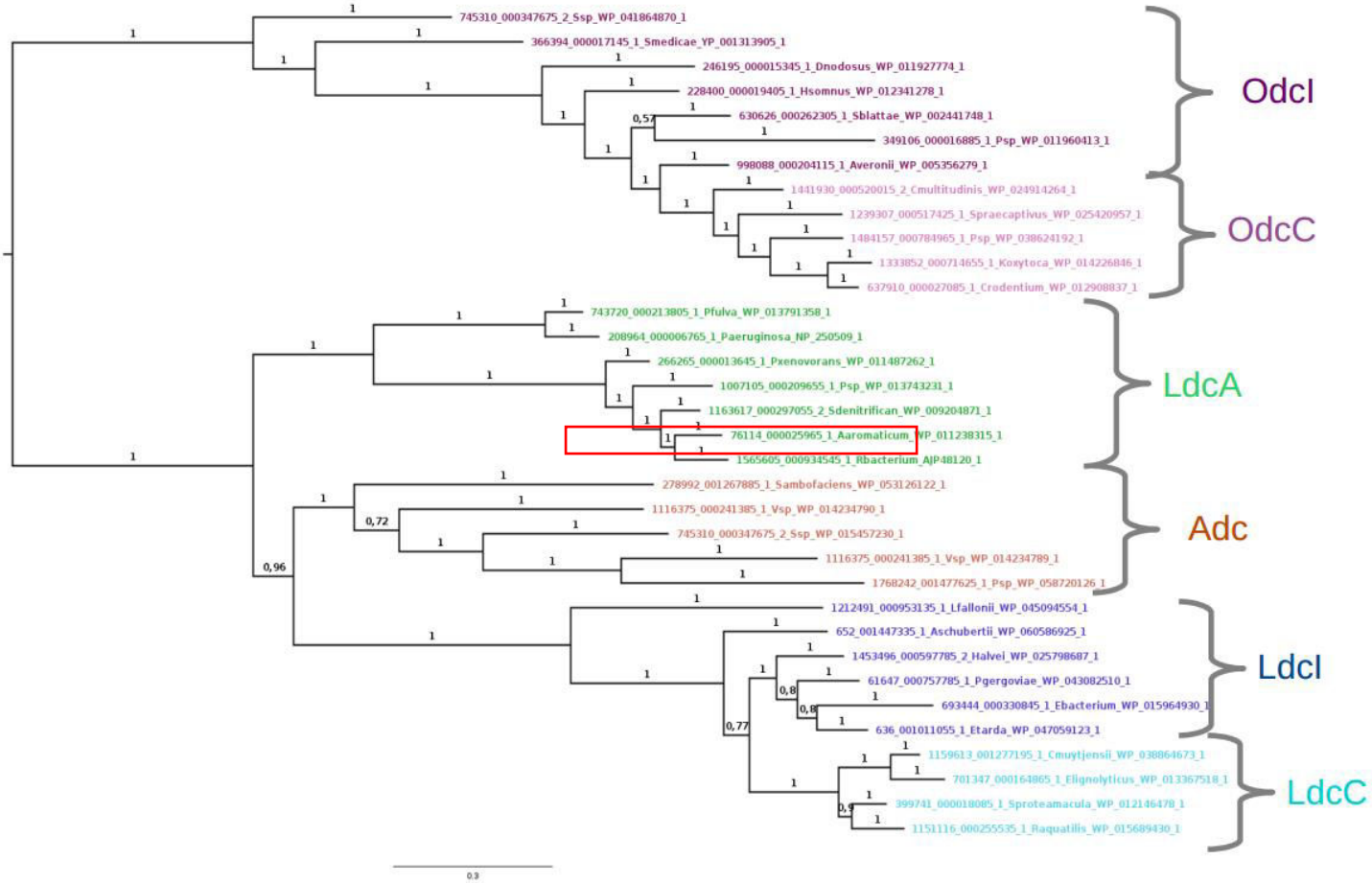


Figure 55. Phylogenetic analysis of Lysine, Arginine and Ornithinine Decarboxylases in the bacterial kingdom. LdcA from *P. aeruginosa* is highlighted in red.

2. Discussion

Phylogenetic analysis show that indeed LdcA belong to a monophyletic group different to LdcI and LdcC which is also close to ADC. However, this analysis could not tell us whether the LdcA group was ancestral to ADCs or vice versa. Since ADC group seem to be paraphyletic, it is more possible that this group appeared from a succession of horizontal gene transfers from LDCs and subsequent mutations that lead to this heterogenous group carrying LDCs and ADCs alike.

Part IV: Conclusions and perspectives

1. **The genetic environment of the *ldcA* shares characteristics with enterobacterial LDCs**

The gene PA1818 encode a lysine decarboxyse of the AAT-fold family, and it has been called *ldcA* by Chou in 2010. Our genetic environment analysis revealed that *ldcA* shares characteristics with both of its two homologues, *ldcC* and *ldcI* from *Enterobacteria*. *ldcA* is associated with *ldcB,* a lysine-cadaverine antiporter by forming the *ldcAB* operon which is similar to the *cadBA* operon of enterobacteria, but with a switched order. However, the main difference between *ldcAB and* the *cadBA* operon is that *P. aeruginosa* do not possess *CadC* regulator which is crucial for the expression of the *cadA* in Enterobacteria. There are also some interesting similarities between the genetic environment of *ldcA* and *ldcC*. They are both near two genes that encode products that are important for DNA replication: the ribonuclease H and one subunit of the DNA polymerase III. This proximity in the genetic environments between *ldcA* and *ldcC* suggest that the lysine decarboxylases could be associated or coordinated with the DNA replication process. Moreover, *ldcC* has been described as a factor needed for fluoroquinolone resistance. Since fluoroquinolones are antibiotics that block DNA synthesis, the idea that lysine decarboxylases could participate in DNA replication process becomes more plausible.

Even though there's no direct experimental proof for this argument, it has been shown that other two polyamines, putrescine and spermidine, can enhance or inhibit or change the specificity of the ribonuclease activity[288,351,352]. For instance, it would be pertinent to see whether cadaverine affects the activity of ribonuclease H.

2. **The expression of the LdcA is associated to the stationary phase.**

Our expression studies showed that ArgR is indeed the main factor regulating the expression of *ldcA* (Annexe III). However, we could observe for the first time how the gene is expressed through growth in different media. During planktonic growth, we observe that *ldcA* expression increases progressively and reaches its maximum when *P. aeruginosa* enters the stationary phase. This expression pattern, suggested that another transcription factor could participate in the expression of *ldcA*. We confirmed in the strain CHA, that RpoS doesn't have a role in *ldcA* expression, as it is the case for *ldcC* expression. Other factor such as QS signaling systems were tested, and we found that the mutant of *pqsA*, incapable of synthetizing PQS, was not able to fully express *ldcA*. However, the role of PQS needs to be

confirmed in PAO1 and in the complemented strains of CHAΔ*pqsA*. We also confirmed that acid stress or oxidative stress didn't play role in *ldcA* expression which is the case for *ldcI.* The expression of *ldcA* was also checked in colonies growing in agar (48h) and anaerobic conditions, and we found that the level of expression didn't present strong variations and seemed constitutive. Overall, these results suggest that *ldcA* does not seems to be an important factor during exponential growth of the planktonic lifestyle. Since it is mostly expressed during the stationary phase, and in anaerobic or agar colonies, our expression assays suggest that its role could be more important during the sessile lifestyle.

3. **The role of LdcA in persistance**

Even though we didn't found any effect on antibiotic resistance of the *ldcA* mutation, we observed that LdcA plays a role in the phenomenon of persistence against carboxypenicillins. We found out that the mutant had 8 times more persisters than the wild type and the complemented strain after 24h in presence of 8X the MIC of carbenicillin. This result is coherent with the findings of Manuel, showing that PA4115 (putative LDC) and exogenous cadaverine reduce the amount of persister cells. Two different hypothesis could explain the effect of cadaverine in persistence:

i) Cadaverine could modify the expression of porins that are involved in the uptake of carboxypenicillins.
ii) Cadaverine could modify the activity of ribonucleases or antitoxin RNAs from Toxin-Antitoxin modules that arrest growth and generate persistence.

4. **The role of LdcA in growth fitness**

A growth defect was detected for the *ldcA* mutant when growing in L-glutamate. The mutant exhibited a prolonged lag phase then an exponential phase with a slower growth rate. The growth defect of *ldcA* mutant could be compensated by the addition of exogenous polyamines such as cadaverine, putrescine and spermidine, suggesting that defect depended

on a general mechanism common to all polyamines. Two hypothesis could explain this phenotype:

i. Polyamines modulate the expression of genes that are involved in the assimilation of L-glutamate.
ii. Polyamines are need for the correct functioning of the central carbon/nitrogen metabolism when growing on L-Glutamate.

The growth phenotype seems contradictory with the fact that *ldcA* is barely expressed during exponential phase. However, cadaverine accumulates during the stationary phase of our precultures, and it seems that the cadaverine in the inoculum is enough for us to see a phenotype[12]. Overall, the growth phenotype in Glutamate suggests that polyamines are needed for the transition between the lag phase and the entrance into the exponential phase.

5. LdcA and virulence

We also observed no implication of LdcA in macrophages T3SS dependent killing. This result also confirms the publication that shows that spermidine but not cadaverine activates the T3SS[347]. We also checked if *ldcA* affected the motily of PAO1, but we didn't find any difference between mutant and wild type. Since the virulence assays rely in chemical defined mediums and in vitro lab conditions, it would be necessary to see whether LdcA participate in acute infections or in chronic infections. Studies in *E. corrodens* show that LDC can also be a virulence factor in biofilms by depleting the available L-lysine from the epithelium[244], generating inflammation and enhancing the damage done by the biofilm, this aspect seems particularly interesting in the context of Cystic Fibrosis where *P. aeruginosa* is known to generate chronic infection by establishing biofilms.

6. LdcA and biofilms

Since *ldcA* is weakly expressed during exponential growth and does not seem to participate in acute virulence, it seems possible that the gene could be more important during the biofilm lifestyle. Even though, during my thesis we could not verify this hypothesis, we started a collaboration with Nassos Typas group from EMBL Heidelberg, where they test the role of genes in biofilm, and stress response. Preliminary results using a *ldcA* mutant,

obtained by transposon mutagenesis, showed that their mutant was more sensitive to fluoroquinoles and had a default in growth in biofilm conditions.

These promising results could open the door for new discoveries of the importance of *ldcA* in the biofilm lifestyle and antibiotic resistance. The modulating effect of cadaverine in antibiotic persistence could also be exploited for antibiotic treatment.

7. Bioinformatic analysis of the ppGpp binding site

During the first chapter of the thesis we performed bioinformatic analysis that involved multiple sequence alignments between LdcA and its homologues in *E. coli* LdcI, LdcC and AdiA. This analysis allowed us to identify that LdcA (PA1818) was indeed the only homologue from the AAT-fold family in *P aeruginosa*. In Irina's lab, Isai Kandiah, performed a deeper analysis of the alignment between these 4 proteins focused in the residues needed for ppGpp binding, as shown in **figure 56 A and B**. They observed that the conserved residues for ppGpp binding in LdcI and LDcC: L562, R565, N568 and R585 are replaced by an alanine, proline, aspartate and glycine respectively in LdcA and AdiA. This similary with AdiA is not without consequence, because the protein does not bind ppGpp and as a consequence its enzymatic activity is not affected by ppGpp. Overall this result suggests us the possibility that LdcA activity is not affected by ppGpp.

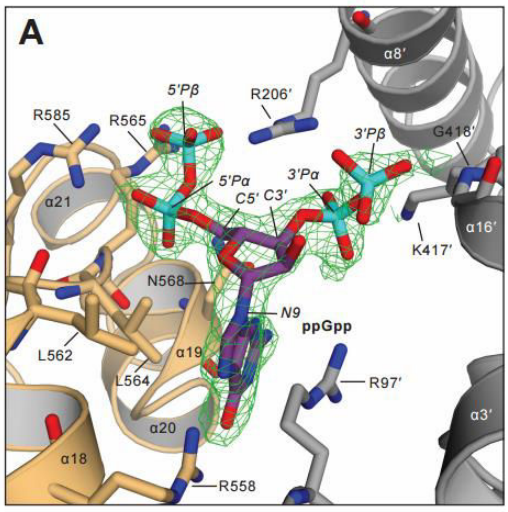


Figure 56. 3D structure of the ppGpp binding site in LdcI. A) The key residues for ppGpp binding are indicated in the figure: R97, R206,G416,K417,L562, L564, R565, N568, R585. **B) Sequence alignment of LdcA (*P. aeruginosa*) and LdcI, LdcC, AdiA (*E. coli*).** We find that the conserved L562, R565 and R585 are replaced by alanine, proline, and glycine respectively in LdcA. The analysis was performed by I. Kandiah.

B

```
AdiA, E.   TRGKWGTLVNTLCSFKRHYDANTPLAQVMPELVEQYPDTYANMGIHDLGDTMFAWLKENN  647
LdcA,P.    TKGKWSTLVTELLEFKRCYDANLPLLDVLPSVAQAGGKRYNGVGLRDLSDAMHASYRDNA  638
LdcI, E.   DKTKALSLLRALTDFKRAFDLNLRVKNMLPSLYREDPEFYENMRIQELAQNIHKLIVHHN  601
LdcC, E.   DKTKAMGLLRGLTEFKRSYDLNLRIKNMLPDLYAEDPDFYRNMRIQDLAQGIHKLIRKHD  601
           : *   *:  * .*** :* *  : :::*.:     . *  :::::*.: :.    ..
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8. Enzymatic activity of LdcA and the role ppGpp

After I. Kandiah showed that the ppGpp binding site of LdcA was similar to the one of ADC that does not bind to ppGpp, we wanted to verify whether the hypothesis that LdcA was not affected by alarmone was true. The enzymatic activity of recombinant LdcA and LdcI in presence and absence of ppGpp was tested by Jan Felix, who is also a member of Irina's Lab. The purified proteins wereoverexpressed from pET29-LdcA and pETTEV-LdcI plasmids transformed in ppGpp-null *E.coli* MG1655 strains. The purified proteins were then used for enzymatic activity assays with Isothermal Titration Calorimetry (ITC) technique. For the experiments, 2.5 nM of LdcI reacted with increasing concentrations of Lysine at pH6.5 in presence or withouth 1µM of ppGpp. Similar experiments with 2nM of LdcA were carried out at pH8.5 with or without ppGpp. The enzymatic reaction of LDCs were assumed to be described by Michaelis-Menten kinetics and biochemical parameters K_m and K_{cat} were calculated accordingly. Since the mechanism of inhibition by ppGpp is not completely understood, apparent K_m and K_{cat} values were calculated for reactions with ppGpp. The results in the **Table 21** and **figure 57,** confirmed that LdcI was inhibited by ppGpp in a significant manner. Indeed, the apparent Km for LdcI in presence of ppGpp was in average 10 times greater than LdcI without ppGpp. In the case of LdcA we obtained that the K_m was not significantly different in presence of ppGpp. This told us that ppGpp was not an inhibitor of LdcA and confirmed the suspicions from bioinformatic analysis of I. Kandiah.

	LdcI (No ppGpp)	**LdcA (No ppGpp)**
K_m (in µM)	225 +/- 20	447+/-62
K_{cat} (s^{-1})	439 +/- 37	344+/-36
	LdcI (1 µM ppGpp)	**LdcA (1 µM ppGpp)**
K_m (in µM)	1920 +/- 383	361+/- 38
Kcat (s^{-1})	541 +/- 37	277+/- 11

Table 21: Enzymatic parameters of LdcI and LdcA in presence or absence of ppGpp. The parameters were calculated assuming Michaelis-menten kinetics.

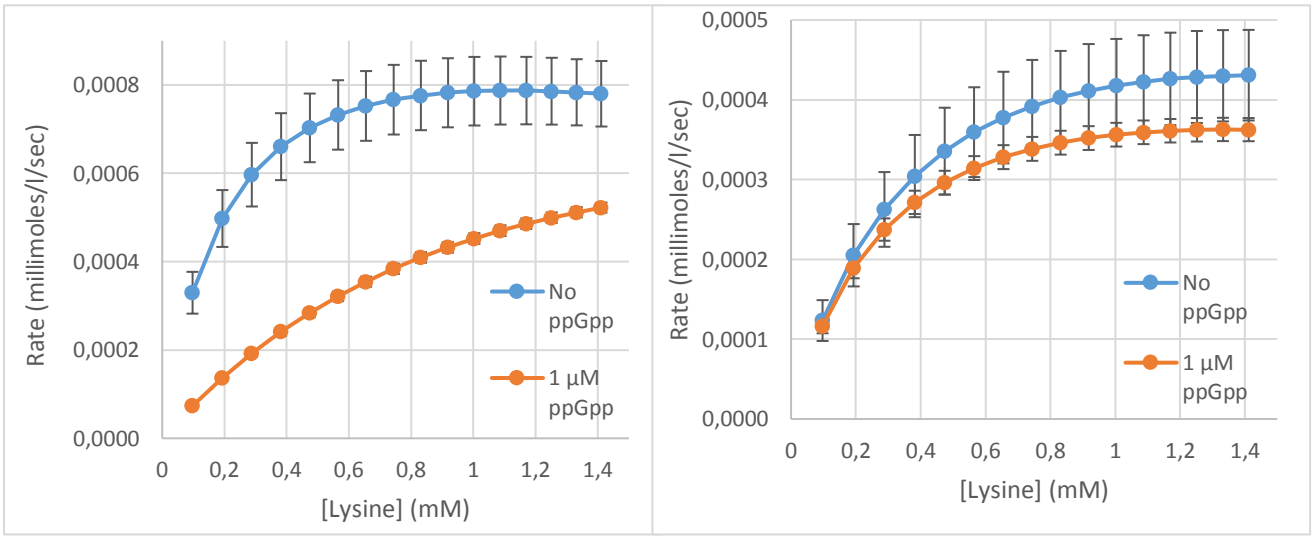

Figure 57. Enzymatic activity of LdcI and LdcA in presence and absence of ppGpp. A) Enzymatic activity assays of LdcI. **B)** Enzymatic activity assays of purified LdcA. 2.5nM of LdcI and 2nM of LdcA were used for the ITC experiments. The experiments were performed by Jan Felix.

9. Structural determination of LdcA

In Irina's lab, we also determined the structure of LdcA and compare it to LdcI and LdcC. Recombinand Histagged LdcA was overexpressed in Rosetta 2 cells transformed with pET29-LdcA (kan^R) plasmids. As shown in **figure 58,** the recombinant protein was purified and verified by negative stain electron microscopy analysis. Indeed, the peaks contain either decameric or dimer species of LdcA. Crystallization attempts of the decameric forms of LdcA were performed and gave crystals that were diffracted at the ESRF, Grenoble. LdcA crystals appeared to diffract at a resolution of 9A which was insufficient to provide sufficient resolution for structure solving. Images of LdcA using the Cryo-Electron Microscopy technique were obtained by I. Gutsche and allowed the reconstruction of electron density maps with a resolution of 4.2 A. The determination of LdcA structure is currently being solved by I. Kandiah and preliminary figures show a structure highly similar to the one obtained for LdcA homologues LdcI and LdcC.

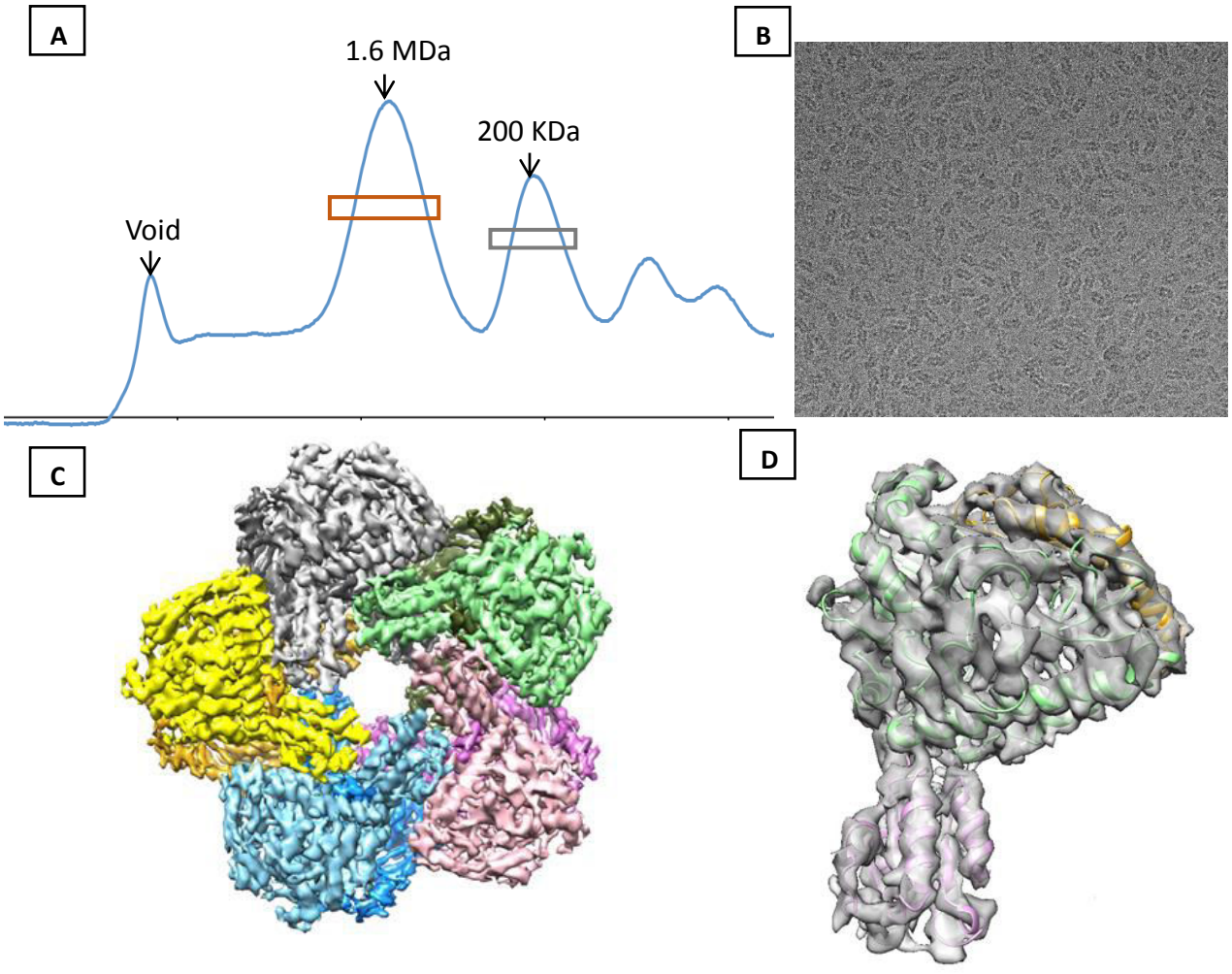

Figure 58. Structure of LdcA from *P. aeruginosa*. A) Size exclusion chromatography of recombinant his-tagged LdcA. The protein appears in two oligomeric species: decamer (1.6MDa) and dimer (200KDa). **B)** Cryo EM image of recombinant LdcA **C)** & **D)** Preeliminary structure of LdcA decamer and dimer at 4.2 A of resolution.

10. LdcA forms a new group of LDC in bacteria

Biochemical and structural analysis of LdcA has revealed similarities with Arginine decarboxylases of *E. coli*. Indeed, enzymatic analysis has shown that ppGpp does not influence the activity of LdcA as it is the case for the ADC. This is coherent with bioinformatic analysis showing that important residues of the ppGpp binding pocket are not conserved in LdcA. Structural analysis of LdcA by cryo EM technique has revealed a protein forming decameric flower-like structures like the ones described for the lysine decarboxylases from *E. coli* LdcI and LdcC.

Molecular phylogeny of LDCs from the AAT-fold family in bacteria show that LdcA forms a new class in the family of Lysine Decarboxylases. This analysis also revealed that homologue Arginine Decarboxylase close to LdcA does not belong to a clearly defined monophyletic group. The presence of LDC from *Burkholderia sp.* Inside the ADC group suggest that ADC could arise from horizontal transfers, recombination events and mutations from other LDCs.

The results of our multidisciplinary characterization demonstrate that LdcA belongs to a new class of LDC from the AAT-fold family. It does not play a role in acid or oxidative stress response, unlike LdcI in *E. coli*. It confers a fitness advantage under certain growth conditions and modulates the amount of persister cells in liquid cultures of *P. aeruginosa*.

Our research open new horizons in the field of the Lysine decarboxylase, and we believe that the study of LdcA in *P. aeruginosa* should focalize in the advantages that LdcA could confer in the formation of biofilms and the role of cadaverine in persistence mechanisms.

Organism	*E. coli*		*P. aeruginosa*
Protein	**LdcI**	**LdcC**	**LdcA**
		Genetic environment	
In operon	Yes (*cadB*)	No	Yes (*ldcB*)
Proximal genes	*cadC, cadB*	*rnhB, dnaE*	*rnhA, dnaQ, ldcB*
		Expression	
Low pH	Yes	No	No
Oxidative stress	Yes	?	No
Amino acids	Lysine	No	Arginine
Growth phases	Stationnary phase	Stationnary phase	Stationnary phase
Anaerobiosis	Yes	?	Yes
Fluoroquinolones	?	Yes	?
Regulators	CadC, ArcA, GadE, GadX, H-NS	RpoS	ArgR, PqsR?
		Physiology	
Acid stress response	Yes	No	No
Oxidative stress response	Yes	Yes	No
Antibiotic resistance	?	Yes	No
Antibiotic persistance	?	?	Yes (carbenicillin)
Growth fitness	under acid stress conditions	?	MMP L-glutamate/aspartate
Virulence	Inhibition of T3SS Shiga-toxins,*	?	No inhibition of T3SS or toxins
Porin blockage	Yes (OmpC, OmpF)	Yes (OmpC, OmpF)	?
		Biochemistry	
pH optimum	5.5	7.4	8.5
ppGpp inhibition	Yes	Yes	No
Structure	Decameric (pH dependent)	Decameric (pH dependent)	Decameric (pH dependent)
RavA interaction	Yes	No	No evidence,**
		Phylogeny	
Monophyletic group	LdcI & LdcC clade	LdcI & LdcC clade	LdcA clade

Table 22. Resume of the different characteristics of LdcI and LdcC from *E. coli* and LdcA from *P. aeruginosa* from this study. *: LdcI homologue *in E. corrodens* is a toxin in biofilms; **: Electron microscopy assays of purified RavA homologue from *P. aeruginosa* didn't bind to purified LdcA.

Appendices

Appendix I

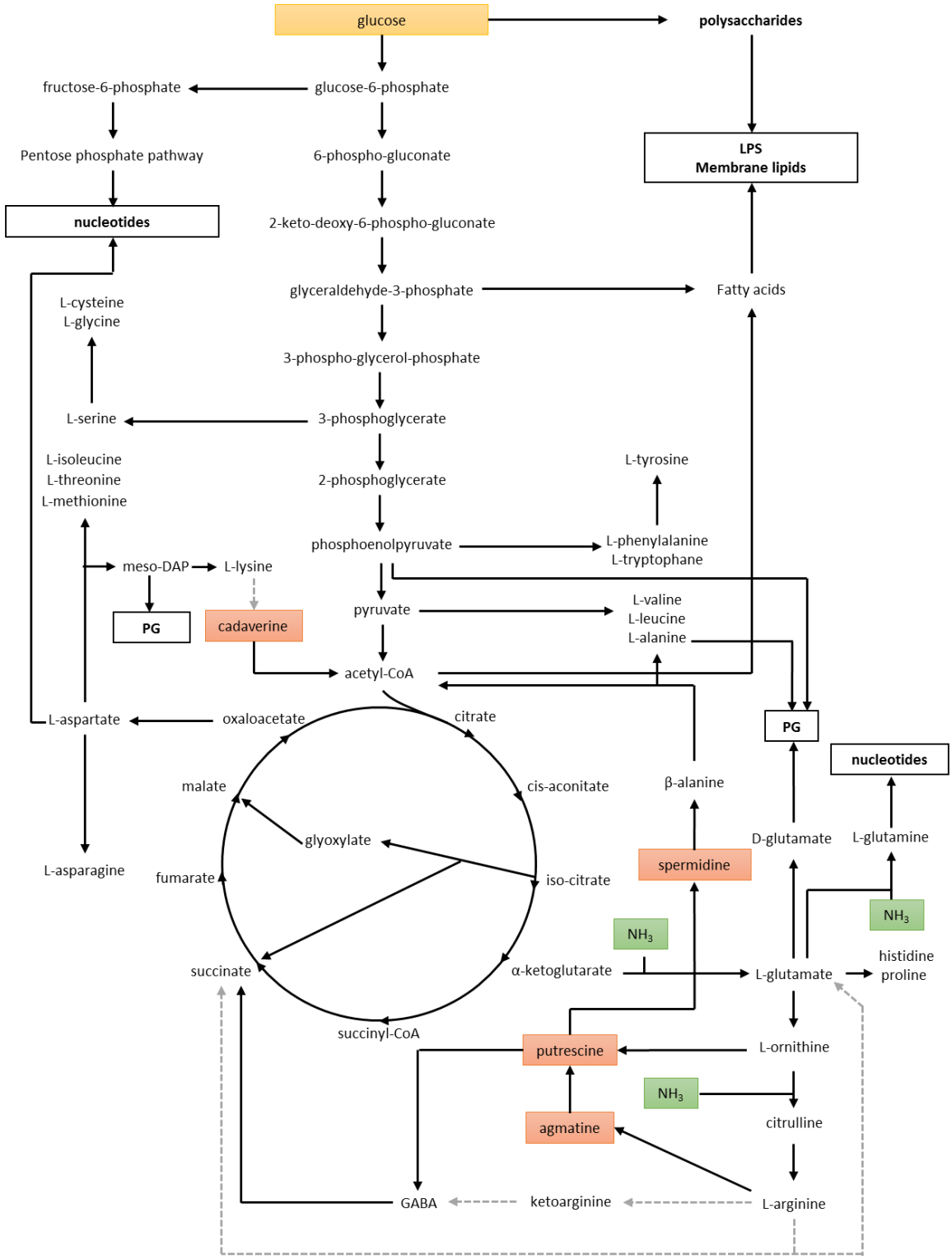

Figure 59: **Diagram of central carbon and nitrogen metabolism of *P. aeruginosa* when growing on glucose**. The glucose is integrated by the entner-doudoroff pathway into central carbon metabolism to produce energy via the Krebs Cycle but it is also used to synthetize the metabolites that will be necessary for the creation of LPS, membrane lipids, peptidoglycan, nucleotides, proteinogenic amino acids and polyamines (orange). The entrance of nitrogen in metabolism is represented by the arrows indicating NH_3 (green) entrance. The pathways in gray dotted lines represent the ones controlled by ArgR, as it is the case for LdcA. Built from KEGG and MetaCyc databases.

Appendix II

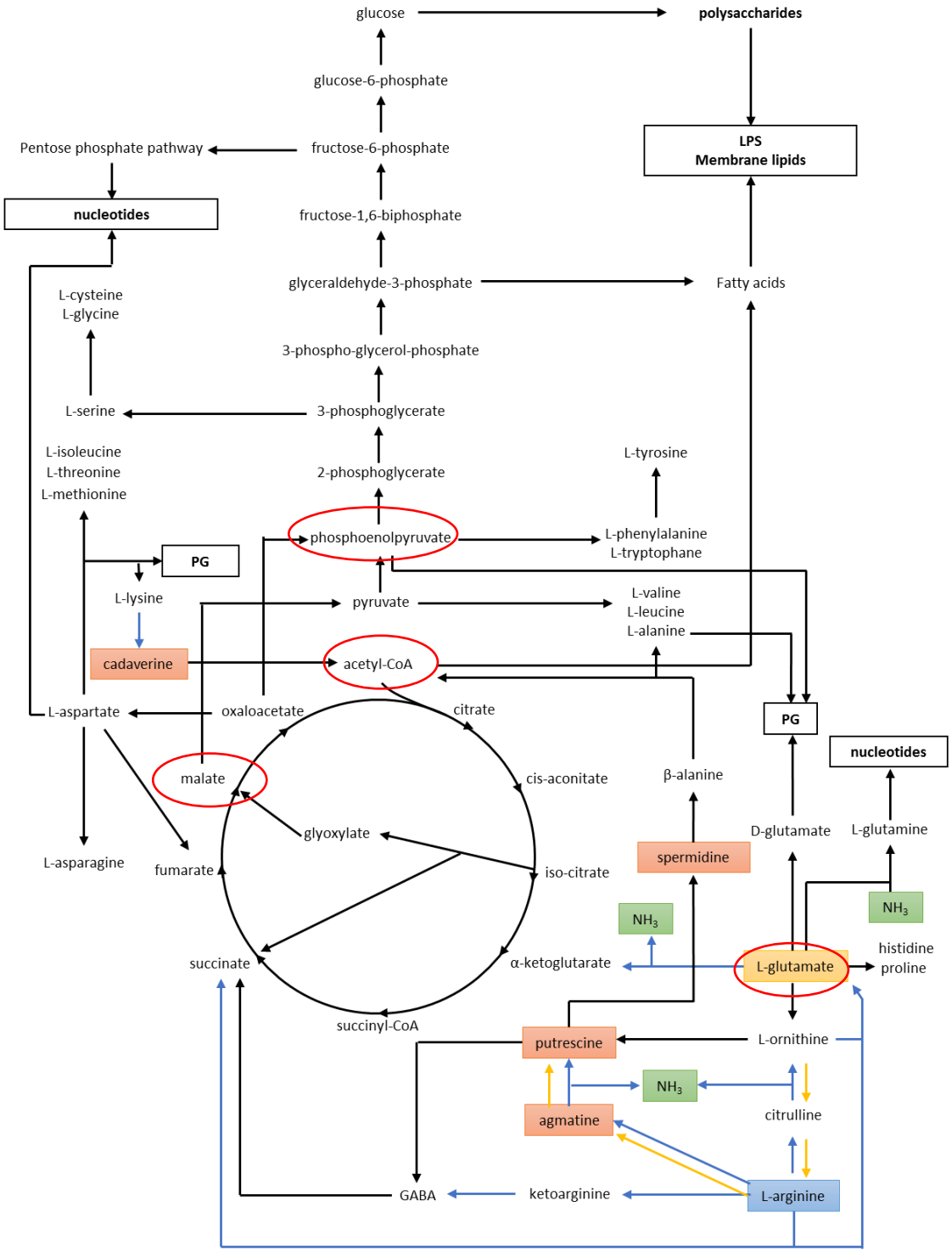

Figure 60: Diagram of central carbon and nitrogen metabolism when *P. aeruginosa* grows on glutamate. Glutamate is assimilated into the Krebs Cycle by the activity of the NADH-Glutamate dehydrogenase, or by the activity of transminases. The diagram also shows the effect of the activation of ArgR by the addition of L-arginine (blue), ArgR activation modifies the pathways in blue arrows, yellow arrows represent the pathways in absence of arginine. Red circles highlight metabolites that show different utilization compared to growth in glucose. Built from KEGG and MetaCyc databases.

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