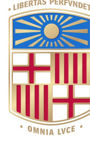

UNIVERSITAT DE BARCELONA

Comprehensive analysis of *Pseudomonas aeruginosa* oleate-diol synthase activity

Mónica Estupiñán Romero

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The discovery of new biotechnological applications of hydroxy fatty acids such as the oxylipins, has provided the basis to explore new mechanisms of oxylipin biosynthesis in prokaryotes, as no evidence on the occurrence of oxylipin production in bacteria existed for a long time. In 2010, a diol synthase activity was biochemically characterized in *Pseudomonas aeruginosa* 42A2, an environmental strain isolated from oily-waste water, which enables accumulation of oxylipins in the extracellular medium. This rare diol synthase activity has been described for the first time in bacteria, generating oxylipins with interesting properties by oxygenation of monounsaturated fatty acids, preferentially oleic acid. However, the genes involved in oxylipin biosynthesis and transport to the extracellular medium, and the biological relevance of these metabolites in this bacterium remained unknown. The current work reports the first outer membrane transporter of oxylipins in *P. aeruginosa*, the ExFadLO, and identifies the operon structure of the genes coding for two previously uncharacterized enzymes involved in oleate-diol synthase activity, acting sequentially as oleic acid dioxygenase (10*S*-DOX) and hydroperoxide isomerase or diol synthase (7,10-DS) activities. These proteins belong to a new subfamily of bacterial enzymes, designated as FadCCPs, only found among *P. aeruginosa* species. Insights into this unique oleate-diol synthase metabolic route evolutionary pathway, and the possible biological relevance of oxylipin biosynthesis and metabolism in *P. aeruginosa* are discussed. Moreover, a biotechnological approach to *P. aeruginosa* oleate-diol synthase activity for oxylipin production is proposed.

Comprehensive analysis of oleate-diol synthase system in *Pseudomonas aeruginosa*

Mónica Estupiñán Romero

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Comprehensive analysis of oleate-diol synthase system in *Pseudomonas aeruginosa*

Facultat de Farmàcia
Departament de Microbiologia i Parasitologia Sanitàries

Facultat de Biologia
Departament de Microbiologia

The cover illustration is an original watercolor with brushstrokes of blue-green pigments produced by characteristic strains of Pseudomonas aeruginosa.
Author: Mónica Estupiñán Romero.

UNIVERSITAT DE BARCELONA

COMPREHENSIVE ANALYSIS OF *Pseudomonas aeruginosa* OLEATE-DIOL SYNTHASE ACTIVITY

MÓNICA ESTUPIÑÁN ROMERO

2015

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Thesis presented by **Mónica Estupiñán Romero** to compete for the PhD degree by the University of Barcelona.

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"Satisfaction lies in the effort,
not in the attainment,
full effort is full victory".

M. Gandhi

"Entonces, me rio por dentro al pensar que las "colonias bacterianas" son como grandes ciudades y que en ellas puede haber, entre los millones y millones de microbichitos, uno haciendo el doctorado en biotecnología…"

Mónica Estupiñán Romero

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Mónica Estupiñán Romero.

SUMMARY

The discovery of new biotechnological applications of hydroxy fatty acids such as the oxylipins, has provided the basis to explore new mechanisms of oxylipin biosynthesis in prokaryotes, as no evidence on the occurrence of oxylipin production in bacteria existed for a long time. In 2010, a diol synthase activity was biochemically characterized in *Pseudomonas aeruginosa* 42A2, an environmental strain isolated from oily-waste water, which enables accumulation of oxylipins in the extracellular medium. This rare diol synthase activity has been described for the first time in bacteria, generating oxylipins with interesting properties by oxygenation of monounsaturated fatty acids, preferentially oleic acid. The catalytic mechanism proceeds through the dioxygenation of oleic acid to release (10*S*)-hydroperoxy-(8*E*)-octadecenoic acid (10*S*-HPOME), which is reduced to (10*S*)-hydroxy-(8*E*)-octadecenoic acid (10*S*-HOME) through a secondary non-enzymatic reaction, followed by conversion of the hydroperoxide intermediate into (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid (7,10-DiHOME). However, the genes involved in oxylipin biosynthesis and transport to the extracellular medium, and the biological relevance of these metabolites in this bacterium remained unknown. The current work reports the first outer membrane transporter of oxylipins in *P. aeruginosa*, the ExFadLO, and identifies the operon structure of the genes coding for two previously uncharacterized enzymes involved in oleate-diol synthase activity, acting sequentially as oleic acid dioxygenase (10*S*-DOX) and hydroperoxide isomerase or diol synthase (7,10-DS) activities. These proteins belong to a new subfamily of bacterial enzymes, designated as FadCCPs, only found among *P. aeruginosa* species. Insights into this unique oleate-diol synthase metabolic route evolutionary pathway, and the possible biological relevance of oxylipin biosynthesis and metabolism in *P. aeruginosa* are discussed. Moreover, a biotechnological approach to *P. aeruginosa* oleate-diol synthase activity for oxylipin production is proposed.

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1. INTRODUCTION

1.1. THE GENUS *Pseudomonas*

The genus *Pseudomonas* is one of the largest and most complex among Gram-negative, being the current number of recognized and validly published species 144, including 10 subspecies (Parte, 2014). *Pseudomonas* species are naturally widespread in the environment having been isolated worldwide. Because of its abundance and ubiquitoust distribution, the genus *Pseudomonas* was early described in the history of Microbiology long ago by Migula in 1894. The taxonomy of the genus has evolved simultaneously with the available methodologies since this first description, made according to morphological characteristics like its rod shape, negative Gram staining, non-sporulating and motility by means of polar flagella (Migula, 1900). For many years, the genus comprised many species that were not always well-characterized, being finally described as aerobic rods with alternative use of nitrate, nitrite or arginine as terminal electron acceptors under anaerobic conditions (Stanier *et al.*, 1966). More recently, an extensive genotypic characterization of strains allowed a revision of the genus, and a subdivision of five groups was implemented based on the DNA–DNA and rRNA-DNA hybridization (Palleroni, 1984). The five groups were assigned to the class *Proteobacteria* (Fig. 1) (De Vos and De Ley, 1983; De Vos *et al.*, 1985; De Ley, 1992; Palleroni, 2005) with members of the genus *Pseudomonas “sensu stricto”* belonging to the rRNA-DNA group I in the subclass *Gammaproteobacteria* (Kersters *et al.*, 1996; Anzai *et al.*, 2000; Peix *et al.*, 2009; Mulet *et al.*, 2010). Currently, multilocus sequence analysis of housekeeping genes and whole-genome sequence *in silico* tools allows to improve the specie classification, which is a prerequisite for the most diversity and evolutionary studies and provides an opportunity to further address the systematics of the genus (Gomila *et al.*, 2015).

Figure 1. Scheme of the distribution of the new genera of former *Pseudomonas* in the corresponding taxonomic hierarchies. Reproduced from Appendix 2, Volume 2, Second Edition of Bergey's Manual of Systematic Bacteriology (Palleroni, 2005). The roman numbers in bold and italics correspond to the groups described in the previous edition of the Manual.

Class I. Alphaproteobacteria
Order V. Caulobacterales
Family I. Caulobacteraceae
Genus III: *Brevundimonas* (**IV**)
Class II. Betaproteobacteria
Order I. Burkholderiales
Family I. Burkholderiaceae
Genus I: *Burkholderia* (**II**)
Genus VIII: *Ralstonia* (**II**)
Family IV. Comamonadaceae
Genus I: *Comamonas* (**III**)
Genus II: *Acidovax* (**III**)
Genus VI: *Delftia* (**III**)
Genus VIII: *Hydrogenophaga* (**III**)
Class III. Gammaproteobacteria
Order III. Xanthomonadales
Family I. Xanthomonadaceae
Genus X: *Stenotrophomonas* (**V**)
Order IX. Pseudomonadales
Family I. Pseudomonadaceae
Genus I: ***Pseudomonas*** (**I**)

1.1.1. Interest of the genus *Pseudomonas*

One of the most remarkable characteristics of the genus *Pseudomonas* is its metabolic and physiologic versatility, enabling colonization of diverse environmental niches such as terrestrial or aquatic habitats as well as plant, insect and animal tissues (Palleroni, 1992; Silby *et al.*, 2011). Although human pathogenesis constitutes a spotlight of the genus *Pseudomonas*, these bacteria display relevants features in agriculture, bioremediation and biotechnology due to their ability to tolerate, degrade or precipitate toxic compounds (Fig.2).

Many pseudomonads interact with plants and several species contributing to plant health by antagonizing plant-pathogenic microorganisms (biocontrol) and directly influencing plant disease resistance and growth (plant growth promotion) – both as plant endophytes (Ryan *et al.*, 2008) and as rhizosphere colonizers (Haas and Défago, 2005). As an example, *Pseudomonas fluorescens* has been shown to protect plants by producing antifungal agents (Péchy-Tarr *et al.*, 2008).

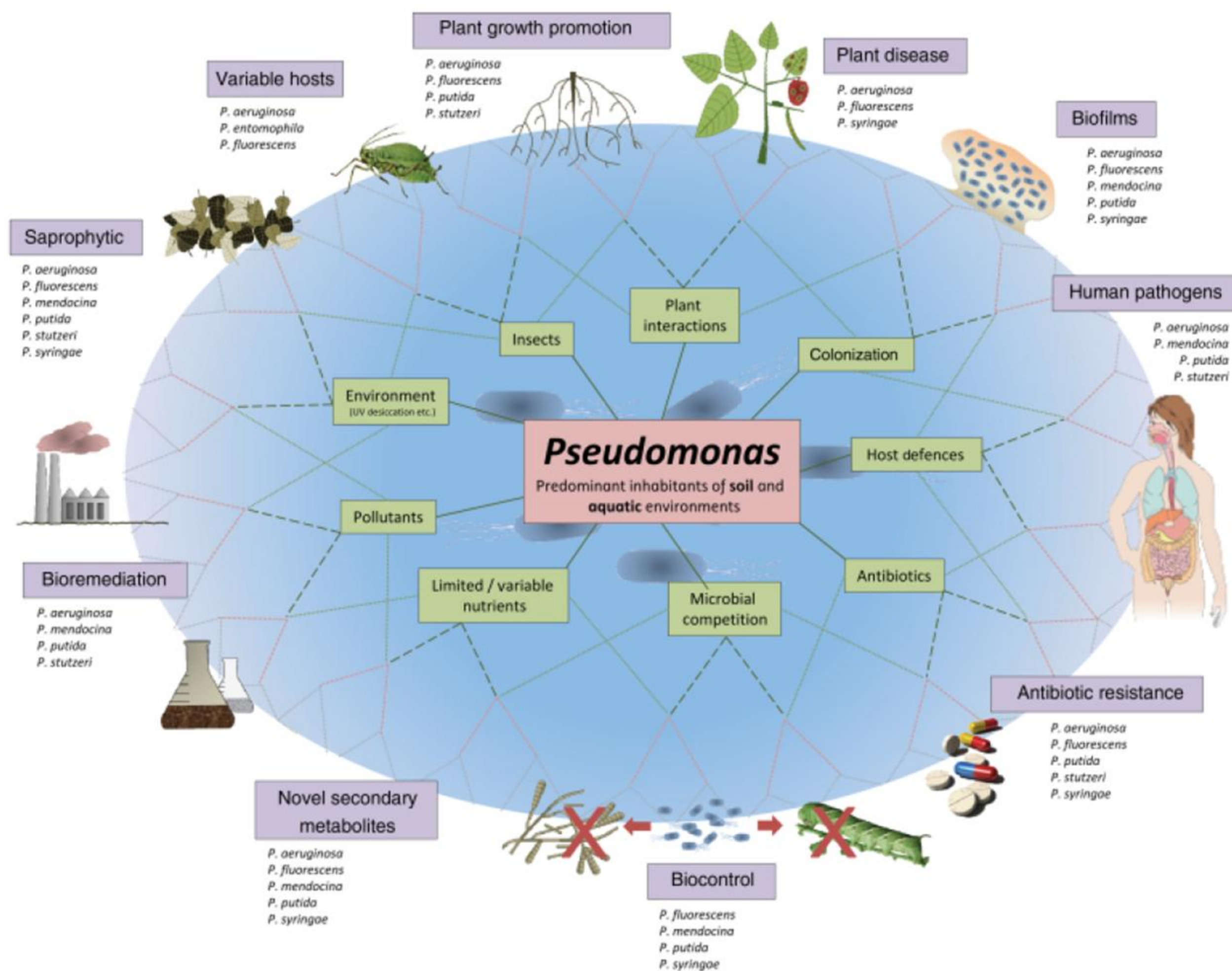

Figure 2. Functional and environmental range of *Pseudomonas* spp.
The *Pseudomonas* common ancestor has encountered a wide range of abiotic and biotic environments that has led to the evolution of a multitude of traits and lifestyles with significant overlap among species (From Silby *et al.*, 2011).

Moreover, the exceptional nutritional versatility of pseudomonads coupled with the production of biosurfactants that can mobilize hydrocarbons and nonaqueous phase liquids into an aqueous phase (Desai and Banat, 1997), makes them excellent candidates for bioremediation. In fact, *Pseudomonas aeruginosa,* which is frequently isolated from petroleum-contaminated soils and groundwaters (Ridgway *et al.*, 1990; Zhang *et al.*, 2012) and *Pseudomonas putida* has been extensively studied in environmental biotechnology because of its capabilities in the bioremediation of toxic organic wastes including aromatic hydrocarbon compounds (Loh and Cao, 2008).

Organic chemical industry needs to be replaced for more sustainable industrial processes known as “White Biotechnology”, a multi-disciplinary area which comprises the production of a variety of different chemical compounds using microorganisms (Fig. 3). The molecular

tools of White Biotechnology are enzymes that catalyze specific reactions inside living cells. Enzymes usually exhibit high substrate specificity and enantioselectivity, and, at the same time, work in aqueous environment and under mild reaction conditions. These properties suggest using them as biocatalysts in the chemical industry to (i) catalyze chemical reactions for which no suitable chemical catalysts are available, and (ii) conduct "green chemistry" by replacing chemical processes, which require high energy input and produce large amounts of toxic waste, by environmentally friendly bioprocesses (Drepper *et al.*, 2006).

The astonishing catabolic diversity of the genus *Pseudomonas*, which allows the ability to adapt to changing environmental conditions, provides a natural and powerful tool to transform a huge range of substrates into value-added compounds (Nikel *et al.*, 2014). Therefore, members of the *Pseudomonas* have an invaluable interest as microbial cell factories for the production or biotransformation of compounds that are not cost-effective or difficult or impossible to obtain by chemical synthesis due to the complexity of their structures and/or specific regio- and stereochemistry requirements (de Boer and Schmidt-Dannert, 2003).

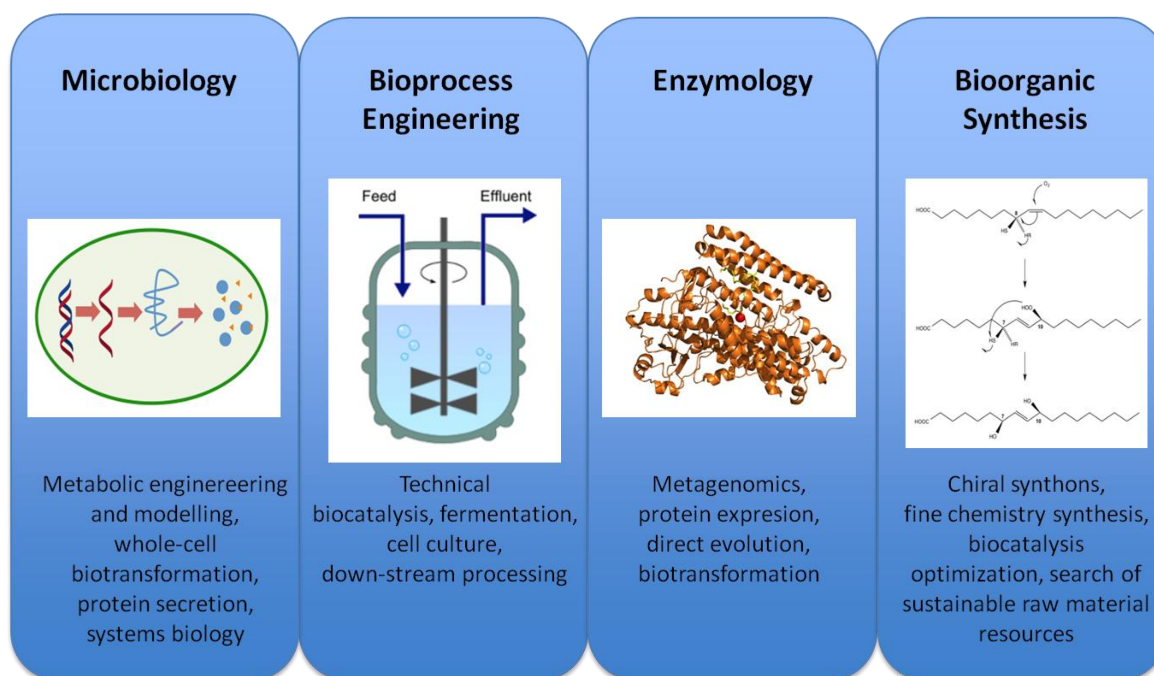

Figure 3. White biotechnology or industrial biotechnology research areas scheme.

1.1.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a free-living bacterium commonly found in soil and water. It displays an adaptable capacity to colonize and persist under a broad range of environmental conditions enabling infection in different hosts including humans, plants, nematodes and insects (Hardalo and Edberg, 1997; Tan *et al.*, 1999; Rahme *et al.*, 2000; Miyata *et al.*, 2003; Apidianakis and Rahme, 2009; Clatworthy *et al.*, 2009). It can grow at temperatures up to 42°C and most of the characterized phenotypes produce pigments like the yellow-green fluorescent siderophor pyoverdine (Meyer *et al.*, 2002) and the non-fluorescent blue-green pyocyanin, which is proposed to act as a virulence factor and as a signal molecule (Dietrich *et al.*, 2006). The colony morfology can be altered upon biofilm production, which protects the bacteria from antimicrobial agents and adverse environmental conditions, leading sometimes to chronic infections in human respiratory tract with eventually fatal development in immunocomprised patients, burn victims and cystic fibrosis patients (Davies, 2002; Driscoll *et al.*, 2007; Goldberg *et al.*, 2008). The ocurrance of three overlapping quorum sensing (QS) systems (Whiteley *et al.*, 1999; Girard and Bloemberg, 2008), a type III secretion system (Hauser, 2009), sigma factors (Potvin *et al.*, 2008) and two-component regulatory systems in controlling virulence and resistance in *P. aeruginosa* (Gooderham and Hancock, 2009) facilitate the establishment of infection. The emergence of increasing rates of antibiotic resistant *P. aeruginosa* strains makes the treatment a medical challenge (Hancock and Speert, 2000). The vast heterogeneity of compounds that can be used by *P. aeruginosa* strains for energy or nutrient uptake makes this bacterium a highly colonizer species and guaranties survival in most known habitats (Frimmersdorf *et al.*, 2010). This characteristic, along with the fact that *P. aeruginosa* can degrade polycyclic aromatic hydrocarbons, suggests the future uses of *P. aeruginosa* for environmental detoxification of synthetic chemicals and pesticides and for industrial purposes (Zhang *et al.*, 2011).

The most referenced strain is *P. aeruginosa* PAO1, a spontaneous chloramphenicol-resistant mutant of the original PAO strain (earlier called "*P. aeruginosa* strain 1") isolated in 1954 from a wound patient in Melbourne, Australia (Holloway, 1955; Holloway, 1975). It sequence and annotation are deposited at the National Center for Biotechnology Information (NCBI) genome database (Refseq. no. NC_002516) and in the *Pseudomonas* Genome Database (Winsor *et al.*, 2009) which also includes ongoing annotation updates.

This *P. aeruginosa* PAO1 strain has become the star for *Pseudomonas* genetics and functional analysis of its physiology and metabolism. As shown in Fig. 4, the number of publications per year including the topic of *P. aeruginosa* PAO1 has grown exponentially from 1970 to 2014 (Pubmed database from NCBI publications at U.S. National Library of Medicine).

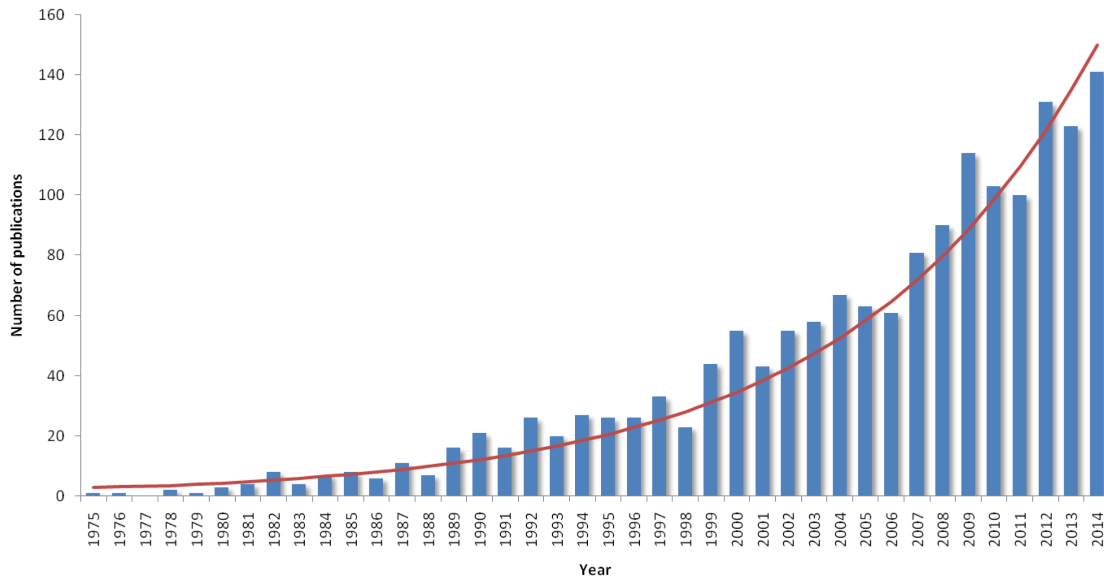

Figure 4. Number of publications per year of *P. aeruginosa* PAO1 extracted from Pubmed database. (Available athttp://www.ncbi.nlm.nih.gov/pubmed/).

1.1.3. *Pseudomonas aeruginosa* genome plasticity

The genetic repertoire of *Pseudomonas aeruginosa* reflects the life style of this ubiquitous bacterial species. The G+C rich *P. aeruginosa* genome (65-67%) consists in a single circular chromosome and a variable number of plasmids. The chromosome consists of a conserved core and a variable accessory part which results in variations of genome size between 5.2 and 7 Mbp (Schmidt *et al.*, 1996; Lee *et al.*, 2006). The core genomes of *P. aeruginosa* strains are mostly co-linear, exhibit a low rate of sequence polymorphism and contain few loci of high sequence diversity (Klockgether *et al.*, 2011). Each locus is present in all strains, but the genes in each one are highly divergent between strains. This phenomenon presumably results from a diversifying selection, a type of evolution system that maintains multiple alleles in the population, providing adaptability strategies (Smith *et al.*, 2005). The accessory genome consists of extrachromosomal elements like plasmids, repetitive extragenic palindromic elements and blocks of DNA inserted into the chromosome at various loci, constituting strain-specific or clone-specific genomic islets and genomic islands of DNA

elements within the range of a few hundred bases to more than 200 Kbp. The accesory genome is formed also by two of the three known large island types of tRNA genes that constitute the three hypervariable regions of *P. aeruginosa* genome (Römling *et al.*, 1997). Within the chromosomally integrated islands, very often phages, transposons, or insertion sequence elements are found, indicating that the majority of the accessory genome originates from mobile DNA elements which have been acquired and kept by the host strain (Klockgether *et al.*, 2011). Therefore, the individual composition of the accessory genome accounts for most intra- and interclonal genome diversity in *P. aeruginosa* (Klockgether *et al.*, 2010; Klockgether *et al.*, 2011).

The large size and genetic complexity of *P. aeruginosa* genome provide abitility for evolutionary adaptations to become successful in diverse ecological niches. This includes a large number of genes encoding outer membrane proteins, transport systems, and enzymes involved in nutrient uptake and metabolism, as well as one of the largest proportions of regulatory genes (8.4%) among bacterial genomes. In addition, *P. aeruginosa* is able to customize its genome to fit the needs for survival in specific environments by acquisition of blocks of genes by horizontal gene transfer from different sources including other species or genera or by discarding unused genetic information (Mathee *et al.*, 2008). Additionally and specifically, more than 500 regulatory genes were identified in the genome of strain PAO1, which create a complex regulatory networks and modulate the biochemical capabilities of this bacterium in fluctuating environmental conditions (Stover *et al.*, 2000; Goodman and Lory, 2004; Balasubramanian and Mathee, 2009; Galán-Vásquez *et al.*, 2011).

1.1.4. *P. aeruginosa* PAO1 functional genomics

Of the 5570 open reading frames (ORFs) of *P. aeruginosa* PAO1 genome, 32% of the genes show no homology to any previously reported sequences, 28.5% of the genes have been assigned a putative function based on the presence of conserved amino acid motifs, structural features, or limited homology, and 13.8% of the genes are homologous to previously reported genes of unknown function. In terms of characterized genes, only 19% have a function based upon a strongly homologous gene experimentally demonstrated in other organism, whereas only 6.7% of the genes have a function experimentally demonstrated in *P. aeruginosa* (Levesque, 2006) (Fig. 5).

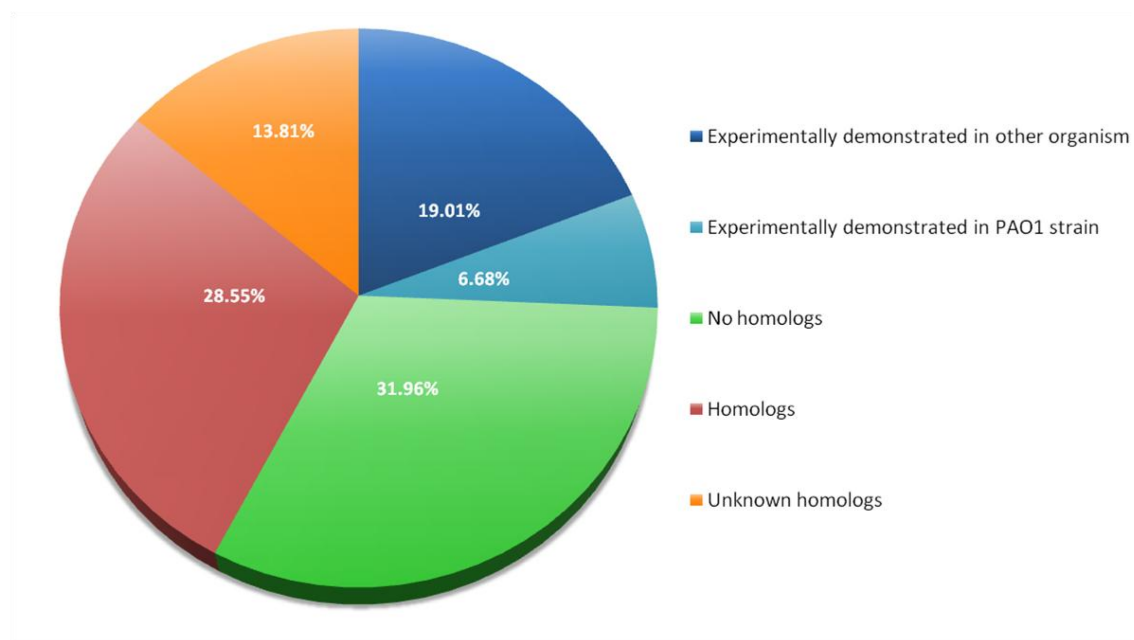

Figure 5. Circle charts of *P. aeruginosa* PAO1 functional genomics
(adapted from Levesque *et al.*, 2006).

One of the pillars of bacterial functional genomics research is transposon mutagenesis. Nowadays, in combination with complete genome sequences, additional has been gained by enabling the precise mapping of numerous insertion points, and their relative positions in the genome (Silby *et al.*, 2011). In 2003 Jacobs and collaborators constructed a saturated transposon mutant library in *P. aeruginosa* PAO1 strain and after sequencing and mapping the insertion points, a defined library of 30100 unique insertions was obtained, resulting in a very useful tool for new gene functional characterization in *P. aeruginosa* strains (Jacobs *et al.*, 2003; Held *et al.*, 2012).

1.1.5. Fatty acid metabolism in *P. aeruginosa*

One of the most remarkable aspects of *P. aeruginosa* strains is their capability to live and to adapt to a vast array of habitats due to its metabolic versatility. This versatility is made possible by a large number of enzymes that allow *P. aeruginosa* metabolize all known natural organic compounds, including toxic organic chemicals such as aromatic or aliphatic hydrocarbons (Hancock and Speert, 2000; Wongsa *et al.*, 2004; Zhang *et al.*, 2011). Fatty acids are aliphatic monocarboxylic acids required as nutrients by cells, which can undergo a set of processes addressed to either cell survival or to production of derivative compounds which may be useful for the producing cell, and in some instances can be of biotechnological interest. The major role of fatty acids in cell metabolism is energy production. In fact, when compared to other nutrient classes like carbohydrates or proteins, fatty acids release far more ATP on an energy per gram basis. This ATP is mostly acquired through the fatty acid β-oxidation pathway, a mechanism widespread among all organisms. In addition, fatty acids are important for energy storage, phospholipid membrane formation, or signaling pathways (Berg *et al.*, 2002). Many microorganisms display specific routes that produce biologically and biotechnologically important molecules from fatty acids (Hou, 2008), which can be used as building blocks for other complex molecules like secondary metabolites or can act as signalling molecules to trigger physiological changes (Andreou *et al.*, 2009).

P. aeruginosa can degrade and transform a set of short, medium and long chain fatty acids, which are used for ATP production through catabolic pathways when they are supplied as a sole carbon sources, or converted into derivative compounds (Kang *et al.*, 2008; Torrego-Solana *et al.*, 2012). Long-chain fatty acids (LCFAs) are simple and indispensable molecules to any living cell. Incorporated in phospholipids, they make up the major part of the plasma membrane and besides structural role, they also function as a carbon or energy source. *P. aeruginosa* displays also the ability to perform bioconversion of unsaturated long chain fatty acids like oleic, linoleic or mixtures of waste oils into polyhydroxyalkanoates (PHAs), rhamnolipids, or a class of oxygenated fatty acids known as oxylipins (Fernández *et al.*, 2005; Bassas *et al.*, 2006; Martínez *et al.*, 2010) which can be further polymerized into estolides (Peláez *et al.*, 2003). PHAs accumulate as carbon storage granules within the cell, whereas rhamnolipids, estolides and oxylipins are usually found in the culture medium (Mercadé *et al.*, 1988; Peláez *et al.*, 2003; Bassas *et al.*, 2008). Up to date, except for the carbon and energy storage role of PHAs, the physiological function of the extracellular fatty acid derivatives produced by *P. aeruginosa* is still not well understood.

1.2. OXYLIPINS AND OTHER HYDROXY FATTY ACIDS

Oxylipins are a class of hydroxylated fatty acids (HFAs) which are formed from fatty acids by pathways involving at least one step, a mono- or dioxygenase (DOX) activity (Gerwick *et al.*, 1991; Bleé, 1995). Oxygenated fatty acids can be formed enzymatically or by autoxidation (Porter *et al.*, 1995; Yin and Porter, 2005; Schneider, 2009). The non-enzymatic pathway of oxylipin synthesis derives from spontaneous formation of reactive oxygen species (ROS) such as hydroxyl radical, which may initiate lipid peroxidation (Brodhun and Feussner, 2011).

Oxygenation of fatty acids is one of the main reactions in lipid metabolism and oxylipins are among the starting points for the synthesis of a great variety of metabolites or are themselves biologically significant compounds such as the leukotrienes and the prostanoids in animals (Funk, 2001), jasmonic acid and its derivates in plants (Mosblech *et al.*, 2009) or fungal sporulation regulatory oxylipins (Brodhun and Feussner, 2011). Therefore, it has been proposed that oxylipins might display a wide range of biological functions involved in inflammation, signaling, plant pest defense, germination or fungal reproduction (Noverr *et al.*, 2002; Mosblech *et al.*, 2009; Brodhun and Feussner, 2011). Oxylipins can also be involved in numerous cell signaling processes acting as integral components of both, prokaryotic and eukaryotic cell elements (Feussner and Wasternack, 2002; Andreou *et al.*, 2009).

1.2.1. Hydroxy fatty acids in biotechnology

Fatty acids from plant and animal origin, or from agricultural waste, constitute a vast renewable resource, which can be used to supplement the declining natural petroleum reserves, allowing production of new and "greener" oleochemicals and oil-based industrial products (Schörken and Kempers, 2009). HFAs have a higher reactivity, solvent miscibility, stability, and viscosity, compared to non-hydroxylated fatty acids (Metzger and Bornscheuer, 2006; Hou, 2008; Hou, 2009; Bajpai *et al.*, 2009). Biotechnological applications of HFAs have extensively been studied, constituting important emulsifying agents in food and cosmetics industries (Peláez *et al.*, 2003; Alewijn *et al.*, 2007; Martín-Arjol *et al.*, 2010). Moreover, hydroxylated LCFA products have been described as biologically active antibacterial or antifungal substances (Kim *et al.*, 2000; Shin *et al.*, 2004; Shin *et al.*, 2007; Hak-Ryul Kim *et al.*, 2008; Hou, 2008; Martín-Arjol *et al.*, 2010; Sohn *et al.*, 2013). However, the most relevant properties of HFAs is their role as useful intermediates in the synthesis of fine

chemicals and pharmaceuticals due to their higher reactivity (Hou, 2008; Paul *et al.*, 2010; Ellamar *et al.*, 2011).

Modification of the alkyl chain of fatty acids for introduction of new functionalities can be performed through chemical or biological systems. The chemical synthetic pathways for HFAs functionalization suffers from several drawbacks like poor selectivity and harsh reaction conditions, as catalysts for the specific hydroxylation reaction on the carbon atom in the fatty acyl chain are limit (Liu *et al.*, 2011). On the contrary, the microbial enzymatic-catalyzed reactions have the advantage of stereo and regio-specificity, lower energy demand, and decreased by-product and waste generation under mild conditions, with a concominant decrease of environmental concerns (Burton, 2003; Wackett *et al.*, 2004; Cao *et al.*, 2013; Adrio and Demain, 2014). Therefore, taking advantage of high microbial potential which enables *P. aeruginosa* to transform residual products specifically by an arsenal of enzymes, research projects have been developed and focused in obtaining basic and applied knowledge on this topic by screening environmentally relevant microorganisms and decode their key biodegradative pathways (Mercadé *et al.*, 1988; Torrego-Solana *et al.*, 2012), through bioremediation of oily contaminated environments and isolation of recalcitrant compound degrader microorganisms (Benincasa *et al.*, 2010; Fernández-Luqueño *et al.*, 2010), by new microbial product biotransformation characterization, kinetic analysis and use of resisual carbon substrates (De Andrés *et al.*, 1994; Chang *et al.*, 2008; Wadekar *et al.*, 2012; Martín-Arjol *et al.*, 2014), for production and characterization of products derived from industrial oily residues in biorreactor fermentations such as the production of PHAs, rhamnolipids and estolides, represented in Fig. 6 (Haba *et al.*, 2000; Bassas *et al.*, 2006; Martín-Arjol *et al.*, 2013; Gong *et al.*, 2015), and through genome searches and heterologous expression of enzymes involved in the production of hydroxylated fatty acids (Busquets *et al.*, 2004; Vidal-Mas *et al.*, 2005; Hansen *et al.*, 2013; Garreta *et al.*, 2013). Thus, industrial uses of hydroxy fatty acids could constitute an environment-friendly and sustainable technology in the near future (Kim and Oh, 2013).

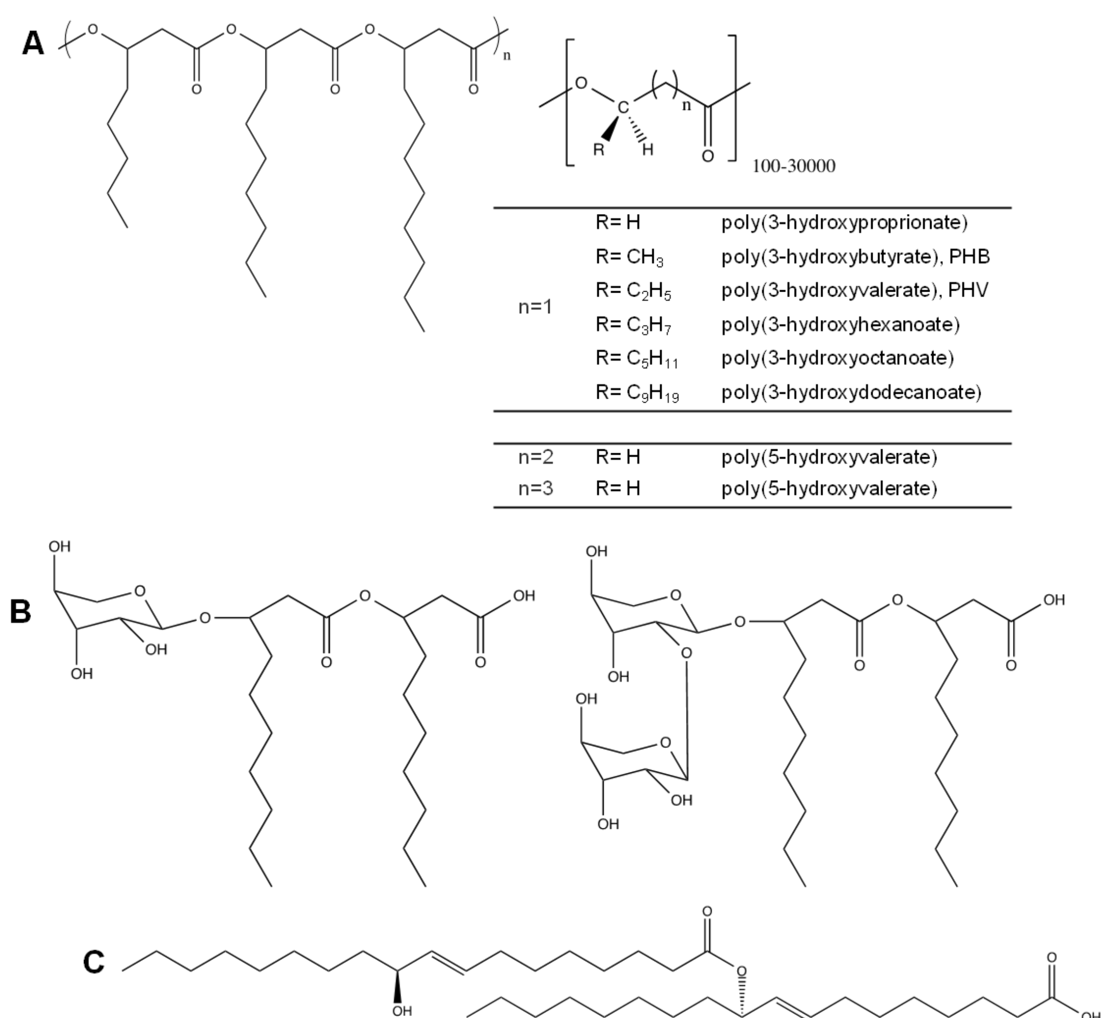

Figure 6. Schematic representation of A) PHAs (Tan *et al.*, 2014). **B) Mono-rhamnolipid and di-rhamnolipid** (Lang and Wullbrandt, 1999). **C) Estolides** (Martín-Arjol *et al.*, 2013).

1.2.2. Oxylipin and hydroxy fatty acid derivatives production by *Pseudomonas aeruginosa*

An environmental screening performed in 1988 aimed at using waste products for potential value-added biotransformations led to the isolation of biotensioactive microbial producers from oil-contaminated water samples, used as highly selective environments for microbial adaptation. As a result, several strains of *P. aeruginosa* were isolated due to their ability to transform LCFA in biotensoactives (Mercadé *et al.*, 1988; Guerrero *et al.*, 1997). Among them, *P. aeruginosa* 42A2 (NCIMB 40045) was selected as a potential study-target because it exhibited great capacity to accumulate in the culture supernatant different species of HFAs when grown on mineral medium supplemented with oleic acid as the sole carbon source (Mercadé *et al.*, 1988; Robert, 1989).

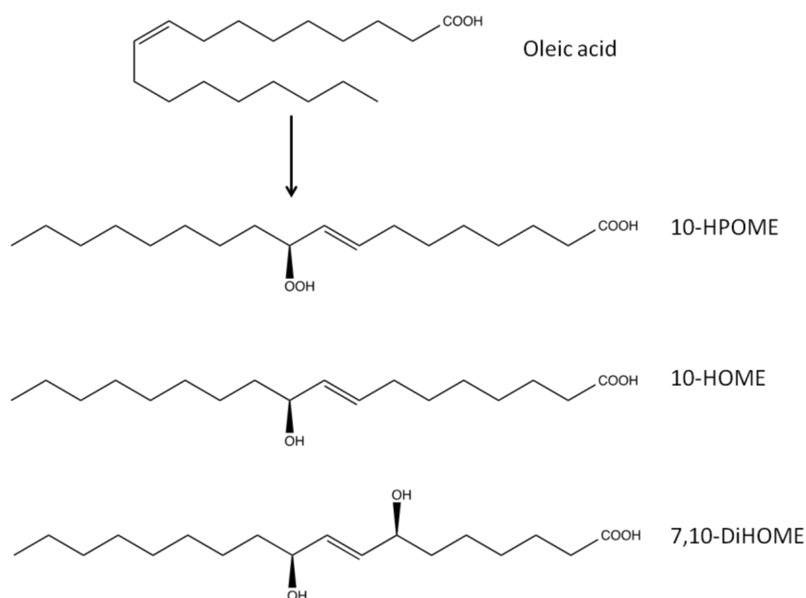

Figure 7. Oxylipins produced from oleic acid by *P. aeruginosa* 42A2*.*
(Mercadé *et al.*, 1988; Guerrero *et al.*, 1997).

As shown in Fig. 7, when strain *P. aeruginosa* 42A2 grows on oleic acid (OA) releases the compounds (E)-10-hydroperoxy-8-octadecenoic acid (10-HPOME), which is reduced to (E)-10-hydroxy-8-octadecenoic acid (Guerrero *et al.*, 1997; Bastida *et al.*, 1999) and the product (E)-7,10-dihydroxy-8-octadecenoic acid (7,10-DiHOME) (Mercadé *et al.*, 1988) as a result of the hydroxylation of the substrate with migration of the double bond. Accumulation of oxygenated LCFAs has been reported also for *P. aeruginosa* PR3 strain, *P. aeruginosa* 2HS and *P. aeruginosa* T1, with unknown genome sequence so far (Hou *et al.*, 1991; Keudell *et al.*, 2000; Hasanuzzaman *et al.*, 2004).

For more than ten years, production of oleic acid derived-oxylipins seemed to be restricted to these strains. Nevertheless, more recent studies revealed that production of 7,10-DiHOME is a general feature of all *P. aeruginosa* strains (Kuo and Nakamura, 2004), including strains PAO1. The development of analytical techniques have allowed the identification of the stereoconfiguration of the hydroxyl groups in the released oxylipins (10*S*)-hydroperoxy-8(*E*)-octadecenoic acid (10*S*-HPOME), (10*S*)-hydroxy-(8*E*)-octadecenoic acid (10*S*-HOME) and (7*S*, 10*S*)-dihydroxy-8(*E*)-octadecenoic acid (7,10-DiHOME) (Hou *et al.*, 1991; Nilsson *et al.*, 2010; Martínez *et al.*, 2010).

Fatty acids	Diols
16:01	
(9*Z*)-16:1	(7*S* ,10*S*)-Dihydroxy-(8*E*)-hexadecenoic acid[a]
18:01	
(8*Z*)-18:1	(6*S* ,9*S*)-Dihydroxy-(7*E*)-octadecenoic acid[b]
(9*Z*)-18:1	(7*S* ,10*S*)-Dihydroxy-(8*E*)-octadecenoic acid
(9*E*)-18:1	ND[c]
(11*Z*)-81:1	(11*S* ,14*S*)-Dihydroxy-(12*E*)-octadecenoic acid
(13*Z*)-18:1	ND
Ricinoleic acid	(7*S* ,10*S* ,12*R*)-Trihydroxy-(8*E*)-octadecenoic acid[d]
18:02	
(9*Z* ,12*Z*)-18:2	(7*S* ,10*S*)-Dihydroxy-(8*E* ,12*Z*)-octadecadienoic acid[e]
(5*Z* ,9*Z*)-18:2	ND
C20-C24	
(9*Z*)-20:1	(7*S* ,10*S*)-Dihydroxy-(8*E*)-eicosenoic acid
(13*Z*)-22:1	ND
(15*Z*)-24:1	ND

[a] (Bae *et al.* , 2007)
[b] Structure confirmed by GC-MS analysis of the trimethylsilyl ether methyl ester derivative (data not shown).
[c] ND, significant transformation could not be detected.
[d] (Kuo *et al.* , 2001)
[e] Structure confirmed by GC-MS analysis.
The other metabolites were identified by LC-MS/MS analysis.

Table 1. Summary of the oxygenation of fatty acids to diols by the diol synthase activity of the extract of *P. aeruginosa* 42A2 (Martínez *et al.*, 2010).

P. aeruginosa also produces the more hydrophilic compound 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (7,10,12-TriHOME) as a derivative of ricinoleic acid (Kim *et al.*, 2000; Kuo *et al.*, 2001; Martínez *et al.*, 2010), and the compound 7,10-dihydroxy-8(*E*)-hexadecenoic acid from palmitoleic acid (Bae *et al.*, 2007; Martínez *et al.*, 2010). The bioconversion of linoleic acid by *P. aeruginosa* PR3 into 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD) and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD) has also been reported (Bae *et al.*, 2010; Bae *et al.*, 2011; Seo *et al.*, 2014). Furthermore, production of 9,12-dihydroxy-10(*E*)-eicosenoic acid from ecosanoic acid (Back *et al.*, 2011) and the conversion of arachidonic acid to 15-hydroxy-5,8,11,13-(*E*)-eicosatetraenoic acid, (15-HETE) (Vance *et al.*, 2004) by *P. aeruginosa* has been described.

Although the biological function of these oxylipins is still unknown as mentioned above, they have been shown to display biotechnological interest through the years not only as biosurfactants but also for their antibacterial, antifungal and pharmaceutical properties (Culleré *et al.*, 2001; Bajpai *et al.*, 2006; Bajpai *et al.*, 2009; Martín-Arjol *et al.*, 2010). In addition to these oxylipins, production of natural estolides by *P. aeruginosa* has also been observed in strain 42A2 (Peláez *et al.*, 2003). It was suggested that the released hydroxy

fatty acids would be polymerized in the culture medium by one or more lipases produced by the strain, resulting in a mixture of estolides (Peláez *et al.*, 2003; Martín-Arjol *et al.*, 2013). Estolides are polymeric molecules mostly formed by hydroxy fatty acids that constitute a group of fatty acid polyesters resulting from ester bond formation between a hydroxyl or olefinic group of one hydroxy fatty acid and the terminal carboxyl group of a second one (Hayes *et al.*, 1995; Erhan *et al.*, 1996). The resulting estolide compounds are of high biotechnological interest owing to their high viscosity, thermal stability and biodegradability, which make them suitable to replace certain components of wax polyesters, lubricants, coating agents, inks, cosmetics or surfactants (Erhan and Kleiman, 1997; Isbell, 2011).

1.3. OXYLIPIN-FORMING ENZYMES

As stated above, oxylipins are not only important metabolites but also potential biotechnological molecules. However, the bottleneck of HFAs chemical production is due to the low reactivity of the fatty acid hydrophobic chain (Cao *et al.*, 2013). This problem is solved in nature through several enzymatic strategies involving fatty acid oxygenases. The discovery of enzymatic pathways capable to synthetize oxylipins goes back to the early 1930s. Since then, most research projects have foccused on the identification of this biologically active products (von Euler, 1936; Baker, 1990) in different cell or extracellular media from different biological origin.

Oxygenases are ubiquitous in nature and play an important role in the metabolism of a broad range of compounds (Burton, 2003; Wackett *et al.*, 2004; Ullrich and Hofrichter, 2007).

Fatty acid oxygenases can be divided into mono- and dioxygenases, which catalyze the regio- and stereospecific insertion of one or two atoms of molecular oxygen into a fatty acid, respectively. Dioxygenases include lipoxygenases (LOX), heme-containing fatty acid dioxygenases (DOX), cyclo-oxygenases (COX), diol synthases (DS) and α-dioxygenases (α-DOX), which produces fatty acid hydroperoxides or endoperoxides (Hamberg *et al.*, 1994; Funk, 2001). Monoxygenases belong to the cytochrome P450 superfamily (CYPs) and can oxidize double bonds with epoxide formation or saturated carbons forming alcohols (Guengerich, 2008).

These oxylipin-forming enzymes have extensively been described in eukaryotes, mostly plants and mammals (Liavonchanka and Feussner, 2006; Schneider *et al.*, 2007; Christensen

and Kolomiets, 2011) or fungi (Tsitsigiannis and Keller, 2007; Brodhun *et al.*, 2009; Christensen and Kolomiets, 2011; Brodhun and Feussner, 2011) but little information is available about bacterial oxylipin-forming enzymes.

1.3.1. Lipoxygenases

Lipoxygenases (LOX) are metallooxygenases that catalyze the stereoselective dioxygenation of polyunsaturated fatty acids with one or more 1Z,4Z-pentadiene units to hydroperoxy fatty acids (Andreou and Feussner, 2009). By the abstraction of the hydrogen on the carbon between the double bonds, a free radical is generated. Molecular oxygen is inserted in anantarafacial way and regio- and stereospecifically at C1 or C5 of the pentadieneand a peroxyl radical is formed. The latter is reduced by the catalytic metal and becomes a peroxyl anion, which after protonation yields the endproduct, a *cis-trans* conjugated hydroperoxide (Brash, 1999) (Fig. 9A).

Lipoxygenases are described as non-heme iron containing dioxygenases consisting of one polypeptide chain folded into two domains, the N-terminal domain and the catalytic moiety β-barrel domain which contains the catalytic center with the catalytic metal (Brash, 1999; Schneider *et al.*, 2007).

As versatile biocatalysts, lipoxygenases are involved in different types of reactions: dioxygenation of lipid substrates, formation of hydroperoxide lipids, and synthesis of leukotrienes (Liavonchanka and Feussner, 2006).

Lipoxygenases occur widely in the eukaryotic kingdom and have been studied for years in animals (Kühn and Thiele, 1999), plants (Liavonchanka and Feussner, 2006), or fungal organisms (Oliw, 2002; Wennman *et al.*, 2014) and for a long time, they seemed to be restricted to these kingdoms with versatile biological functions and anti-inflamatory properties among others (Vance *et al.*, 2004).

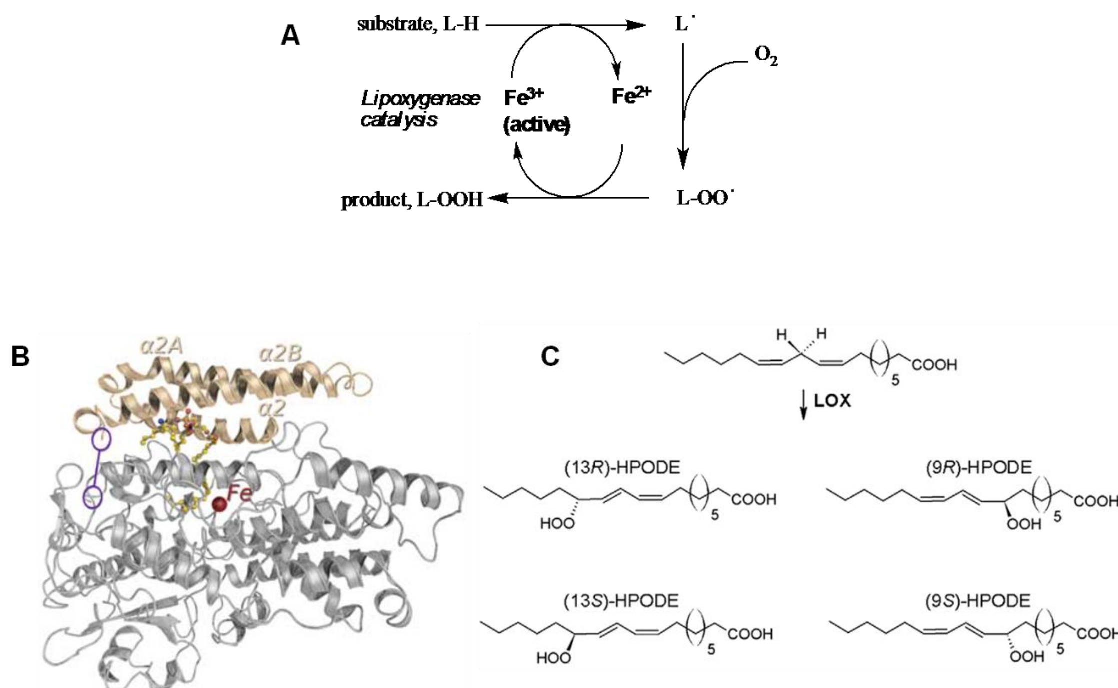

Figure 8. A) Lipoxygenase catalysis scheme. The enzymes are usually in the ferrous (inactive) form when isolated. Oxidation to the active ferric enzyme is required for catalysis (Brash, 1999). **B) Overall structure of the prokaryotic PaLOX determined for crystal form (PDB code: 4G32).** The phospholipid found in the substrate binding pocket is shown as balls and sticks and the catalytic iron is shown as a red sphere. The insertion of ~100 residues in *Pa*_LOX with respect to eukaryotic LOXs (beige) corresponds mainly to a remodeled helix α2 and to the new helices α2A and α2B (Garreta *et al.*, 2013). **C) The LOX reaction and the stereochemistry of the reaction mechanism** (Andreou and Feussner, 2009).

However, the recent characterization and cristallyzation of the periplasmic *P. aeruginosa* LOX (PaLOX; PA1169) has modified the phylogeny of these enzymes, generating a new subfamily of bacterial lipoxygenases (Garreta *et al.*, 2011; Hansen *et al.*, 2013) (Fig. 9B). Furthermore, PaLOX is active on arachidonic acid, producing 15-hydroxyeicosatetraenoic acid (15-HETE) (Vance *et al.*, 2004) and is also able to convert linoleic acid producing the oxylipins 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPODE) and 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9*S*-HPODE), in a catalytic pathway coupled by a phospholipid-specific union (Garreta *et al.*, 2013) (Fig. 9B, 9C).

1.3.2. Heme-containing dioxygenases (DOX)

Heme is a prosthetic group consisting on a protoporphyrin IX with a ligated iron coordinated to four nitrogen atoms of the planar organic ring structure (Fig. 10) (Layer *et al.*, 2010). Besides the four nitrogen ligands of the heme, it usually binds two additional axial ligands, a

fifth lower iron and a sixth upper axial iron ligand. The nature of these ligands, of which at least one is usually a functional group of an amino acid of the protein to which it is bound, greatly influences the reactivity of the heme group (Rydberg *et al.*, 2004; Frey and Hegeman, 2007).

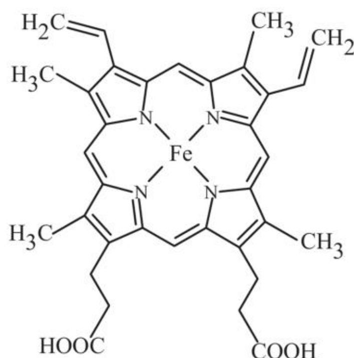

Figure 9. Chemical structure of heme group.

Heme-containing DOX can be divided into several groups of oxylipin-forming enzymes. Among them, the prostaglandin H synthases (PGHS) of vertebrates, the linoleate diol synthases (LDS) and 10*R*-DOX of filamentous fungi, and the α-DOX of plants (Sanz *et al.*, 1998; Hörnsten *et al.*, 1999; Garscha and Oliw, 2009) have been described and experimentally characterized. Fatty acid heme dioxygenases occur in eukaryotes, often associated with a cytochrome P450 that transforms the released peroxide product (Brash *et al.*, 2014). Moreover, other examples of heme-containing enzymes are P450, peroxidases, or catalases (Hersleth *et al.*, 2006).

1.3.3. Diol Synthases

Nowadays, diol synthases constitutes an increasingly heterogeneous group of enzymes studied in detail in the eukaryotic kingdom. Diol synthases (DS) activity comprise the sequential dioxygenation of unsaturated fatty acids to hydroperoxy fatty acids and their isomerization to fatty acid diols. In fungi, dihydroxy fatty acids are derived from the most abundant fatty acids like linoleic or α-linoleic acids, common precursors of fungal oxylipins (Stahl and Klug, 1996; Nemec *et al.*, 2006). In 1989, 8*R*-hydroxylinoleic (8R-HODE) and 5*S*,8*R*-dihydroxyoctadeca-9*Z*,12*Z*-dienoic acid (5,8-DiHODE) were the first oxylipins described produced by *Aspergillus nidulans*, and were reported to possess hormone-like activities by acting as precocious sexual inducers (psi) of sporulation, involved in the regulation of the sexual or asexual phases of sporulation (Champe and El-Zayat, 1989; Mazur *et al.*, 1990).

Moreover, psi factors were described to be also involved in host-pathogen communication in promoting fungal infection of plants (Tsitsigiannis and Keller, 2006; Christensen and Kolomiets, 2011; Brodhun and Feussner, 2011).

Linoleate diol synthases are bifunctional proteins as a result of the fusion of N-terminal DOX and C-terminal P450 domains, where the latter accounts for hydroperoxide isomerase activity.

In 1992, Brodowsky and coworkers identified other oxylipins derived from linoleic acid from the fungus *Gaeumannomyces graminis*, which performed the bioconversion of linoleic acid into 8R-HPODE, and its subsequent isomerization to (7*S*,8*S*)-dihydroxylinoleic acid (7,8-DiHODE) (Brodowsky *et al.*, 1992; Brodowsky *et al.*, 1994). The responsible native enzyme was purified and characterized, being known as 7,8-LDS and constituting the diol synthase prototype (Su *et al.*, 1995). In addition to the latter reaction, 8*R*-hydroperoxylinoleic (8R-HPODE) can be transformed to its corresponding alcohol, 8R-HODE, by chemical reduction. The N-terminal DOX domain shows catalytic and sequence homology to prostaglandin H synthases (PGHS) (Garscha and Oliw, 2008a; Garscha and Oliw, 2008b) and its catalityc mechanism starts with the oxidation of the heme, which forms a tyrosyl radical. This radical abstracts the pro-S hydrogen at C8 from linoleic acid and molecular oxygen is then inserted in an anterafacial way leading to formation of 8R-HPODE. Abstraction of the pro-*S* hydrogen at C7 and suprafacial oxygen insertion yields the end product 7,8-DiHODE (Brodowsky *et al.*, 1992; Hamberg *et al.*, 1994) (Fig. 10).

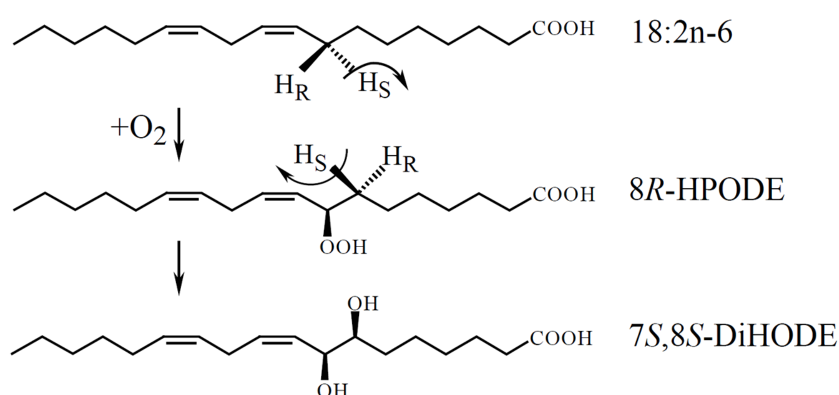

Figure 10. Catalytic reaction mechanism of 7,8-LDS of *G. graminis*. The enzyme is bifunctional as the first step is catalyzed by its DOX activity, while the second reaction is catalyzed by an hydroperoxyde isomerase activity (Hamberg *et al.*, 1994).

Over recent years, *Gaeumannomyces graminis* 7,8-LDS has been considered functionally and structurally similar to the mammalian prostaglandin H2-synthase, and genes encoding 7,8-LDS have been also detected in several pathogenic and non-pathogenic fungi such as: *Cercosporazeae-maydis* (Shim and Dunkle, 2003), *Magnaporthe grisea* (Cristea *et al.*, 2003), *Magnaporthe oryzae* (Jernerén *et al.*, 2010), or *Ustilago maydis* (Huber *et al.*, 2002).

Homologous 7,8-LDS coding genes were latter reported in *Aspergillus* strains, including *Aspergillus clavatus, A. fumigatus, A. nidulans, and A. niger*, which can also produce 5,8- and 8,11-DiHODE from linoleic acid (Garscha *et al.*, 2007; Wadman *et al.*, 2009; Jernerén *et al.*, 2010). This indicates the presence of three ortholog genes to *Gaeumannomyces graminis* 7,8-LDS in the genomes of these fungi, being designated *psi producing oxygenases (ppo) A*, *ppoB*, and *ppoC* since it was shown that the corresponding enzymes affected the biosynthesis of psi-factors and the development of the fungus (Tsitsigiannis *et al.*, 2005; Horowitz *et al.*, 2008). Recombinant expression of ppoA from *A. nidulans* and ppoC of *A. fumigatus* and *A. nidulans* demonstrated that these genes could be linked to formation of 8-HPODE and 5,8-DiHODE and to 10*R*-HPODE, respectively (Brodhun *et al.*, 2009; Garscha and Oliw, 2009; Brodhun *et al.*, 2010), being the corresponding enzymes designated as 5,8-LDS and 10*R*-dioxygenase (10*R*-DOX), respectively (Hoffmann *et al.*, 2010). However, up to date, the function of the *ppoB* gene remains still unknown although a recent report shows that formation of 8,11-DiHODE is not related to *ppoB* (Jernerén *et al.*, 2012).

The hydroperoxide isomerase activity of 7,8-LDS was suggested to be a P450 cytochrome by Lee et al. in 2008, a fact that was confirmed one year later by Brodhun and coworkers (Lewinson *et al.*, 2008; Brodhun *et al.*, 2009). Using spectroscopic analysis of the heme-carbon monoxide complex these authors showed that the C-terminal domain of 5,8-LDS of *A. nidulans* is a P450, showing indeed the bifunctional catalysis mode of action of LDS (Brodhun *et al.*, 2009).

1.3.4. Cytochromes P450

Most fatty acid monooxygenases belong to the P450 superfamily, which are present in virtually all organisms and catalyze a vast array of reactions involved in the degradation and biosynthesis of exo- and endogenous compounds (Meunier *et al.*, 2004; Guengerich, 2008). P450 (CYP) are hemoproteins that were named due to their absorption of light at 450 nm in the reduced state upon binding of carbon monoxide.

Over 11000 P450 genes are included in the internationally used nomenclature system, and probably even more extensive number of non-classified genes are still non assigned from the emerging pile of genome sequencing data. Besides being so nomerous, the P450 superfamily is also highly divergent, which is further illustrated by the huge number of families (977) and subfamilies (2519) described up to date (Van Bogaert *et al.*, 2011).

The prototype P450 reaction proceeds through hydroxylation, which occurs as illustrated in Fig. 11. A water molecule is replaced when the substrate binds to the enzyme. Then, the ferric heme is reduced to the ferrous state by an electron supplied by NADPH via a P450 reductase before binding of molecular oxygen, which yields a ferrous P450 dioxygen complex. After another one-electron reduction and protonation of the Fe(III)-hydroperoxy complex, the (P450[Fe^{III}-O-OH]) intermediate is obtained. A sequential protonation and heterolytic cleavage of the dioxygen bond with elimination of H_2O forms the highly reactive iron-oxo intermediate (P450[Fe^{IV}=O]$^{\bullet+}$), designated *Compound I.* The latter intermediate performs hydrogen abstraction from the substrate, yielding P450[Fe^{IV}-OH], also referred to as *Compound II*, and a substrate radical. Finally, the oxygenated product is obtained which dissociates from the heme, that returns to its resting state (Brash, 2009)

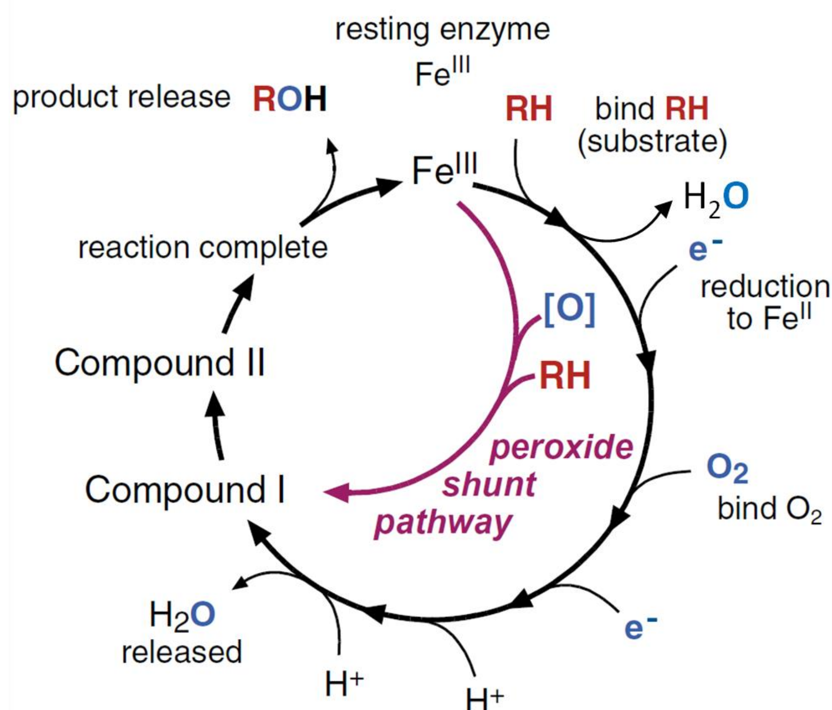

Figure 11. The catalytic cycle of cytochrome P450s. The typical hydroxylation or epoxidation pathway follows the complete circular route (black). In these typical hydroxylase and epoxidase P450s, the cycle can be short-circuited via the peroxide shunt pathway when an oxygen donor oxidizes the ferric enzyme by two equivalents directly to *Compound I* (magenta route). If H_2O_2 is used as oxygen donor it short-circuits the cycle to a Fe[O2] intermediate prior to forming *Compound I* (adapted from Brash, 2009).

Bacterial P450s are often soluble, in contrast to their commom membrane-bound eukaryotic counterparts (Nelson, 1999). The first bacterial CYP crystallized was P450cam from *Pseudomonas putida*, which catalizes the selective hydroxylation of the 5-methylen carbon of D(+)-camphor to form the exo-5-alcohol (Poulos *et al.*, 1985). Since that date, the number of bacterial CYPs identified has increased exponentially, diversifying both the catalytic activity and the structure. For example, the CYP BM-3 from *Bacillus megaterium* is unique among others as the P450 and reductase conforms a single polypeptide chain (Warman *et al.*, 2005). This enzyme catalizes the NADPH-dependent hydroxylation of several LCFAs at the ω-1 through ω-3 positions (McLean *et al.*, 2005).

The huge range of CYP substrates and the versatility of these enzymes highlights their applications as industrial biocatalysts, but a recent review article by Kumar in 2010 also includes various characteristics of CYPs in terms of low activity, limited stability, and their need for an expensive cofactor (NADPH) to reduce the heme iron, which make them less promising for industrial applications (Kumar, 2010).

1.3.5. Enzymatic oxylipin formation in *P. aeruginosa* 42A2

Although biotechnological production of different HFAs has been extensively studied and optimized in the last years for microbial bioconversion of different raw materials (Kuo *et al.*, 1998; Kuo *et al.*, 2003; Kuo and Lanser, 2003; Hou, 2008; Chang *et al.*, 2008; Joo *et al.*, 2012; Seo *et al.*, 2014; Seo *et al.*, 2015), the true metabolic pathway of OA oxygenation was determined much later following the discovery of new fungal enzymatic activities producing oxidation of octadecenoic acids to hydroperoxides and diols (e.g., *Gaeumannomyces graminis*, aspergilli), providing new insights on its catalytic nature (Martínez *et al.*, 2010).

According to preliminary results, an initial hypothesis was generated to understand the enzymatic conversion of oleic acid by *P. aeruginosa* 42A2, where hydroxylation of OA would occur by migration of the double bond to release the dihydroxylated fatty acid 7,10-DiHOME. Production of a monounsaturated hydroperoxyde intermediate or 10-HPOME and its chemical reduction to 10-HOME by the strain was also reported (Guerrero *et al.*, 1997). The initial catalityc mechanism proposed two enzymes for the synthesis of the diol: first a lipoxygenase-like enzyme for oleic acid conversion into 10-HOME, as these enzymes have been described in most animals, plants and microbial biotransformations of unsaturated LCFAs (Busquets *et al.*, 2004; Vidal-Mas *et al.*, 2005; Hansen *et al.*, 2013), and a second

isomerase/hydroxylase enzyme for 7,10-DiHOME formation (Kim *et al.*, 2000) (Fig.12). Nowadays, it has been demonstrated that monounsaturated fatty acids are poor substrates for LOX enzymes (Clapp *et al.*, 2006) and, as was mentioned before, diol formation has been observed in other microorganisms like fungi, suggesting that a revision of the catalytic mechanism and the nature of the enzyme/s involved in oleate-derived oxylipins formation is required.

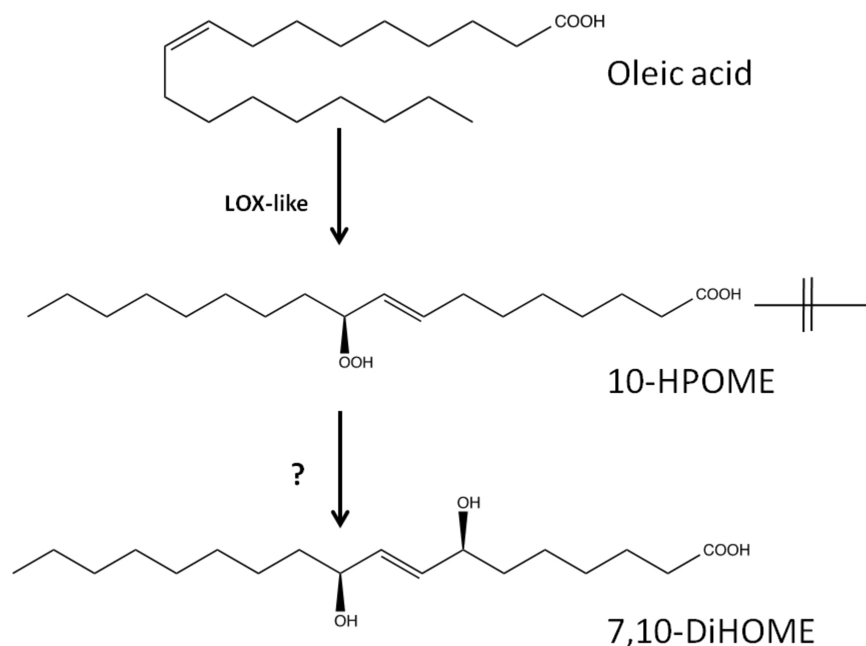

Figure 12. Hypothesis for oleic acid bioconversion in *P. aeruginosa* 42A2
(Guerrero *et al.*, 1997; Busquets *et al.*, 2004; Vidal-Mas *et al.*, 2005; Kim *et al.*, 2000).

The conversion pathway of oleic acid by *P. aeruginosa* 42A2 was accurately described in 2010 as a diol synthase activity (DS), which proceeds through the dioxygenation of OA to release hydroperoxide (10*S*)-hydroperoxy-(8*E*)-octadecenoic acid (10-HPOME), which is reduced to 10-HOME (10*S*)-hydroxy-(8*E*)-octadecenoic acid through a secondary non-enzymatic reaction, followed by conversion of the hydroperoxide intermediate into (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid (7,10-DiHOME), both of which accumulate in the culture supernatant (Martínez *et al.*, 2010). In comparison with fungal LDS, the nature of the (10*S*)-DOX activity, responsible for the first step of the reaction is unknown, whereas the reaction mechanism of the hydroperoxide isomerase is consistent with P450 catalysis (Martínez *et al.*, 2010) (Fig. 13). The importance of *P. aeruginosa* 42A2 oleate-diol synthase is mostly due to the fact that it constitutes the first described diol synthase (DS) among bacteria.

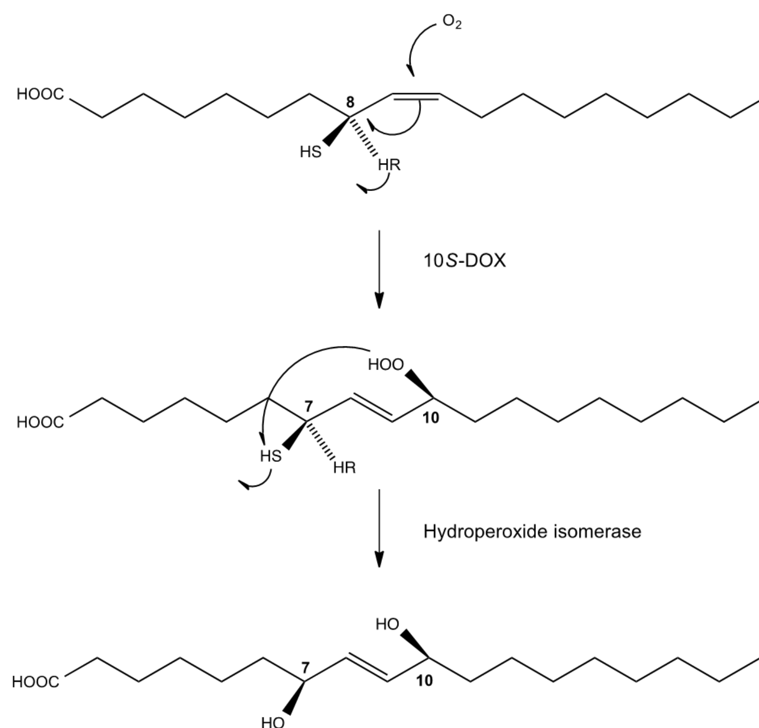

Figure 13. Proposed mechanism of the oleic acid diol synthase of *P. aeruginosa* 42A2 (Martínez *et al.*, 2010).

According to the new enzymatic mechanism elucidated, oleic acid is the preferred but not the only substrate of *P. aeruginosa* diol synthase (Martínez *et al.*, 2010). As previously shown in Table 1, the range of substrates susceptible of conversion by *P. aeruginosa* 42A2 diol synthase semipurified extracts, was shown to be specific for the position of the double bond in the substrate molecule, being vaccenic acid the exception. The common denominator of converted substrates was an unsaturated carbon chain from the carboxyl group to the *cis* double bond at position 9,10, whereas a double bond at position 8,9 resulted in slow but significant conversion. Therefore oleic acid, palmitoleic acid and ricinoleic acid (regioisomeric 18:1 substrates) were properly bioconverted. However, additional double bonds between the carboxyl group and the 9*Z* double bond inhibited transformation. Moreover, 18:2*n*-6, 20:1*n*-9, 20:1*n*-11, and 20:2*n*-6, with the double bond at 18:2*n*-6 and 20:1*n*-11 were oxidized. Whereas the methyl ester of oleic acid was not a substrate. The results suggested that substrate might bind the active site of the corresponding enzyme 'head' first because the distance from the carboxyl group to the first double bond appeared to be critical. Whereas, the distance from the **ω**-end to the double bond does not appear to be so determinant, being the anomalous oxygenation of vaccenic acid probably due to a possible reverse orientation at the active site of the enzyme when binding this substrate (Martínez *et al.*, 2010) (Fig. 14).

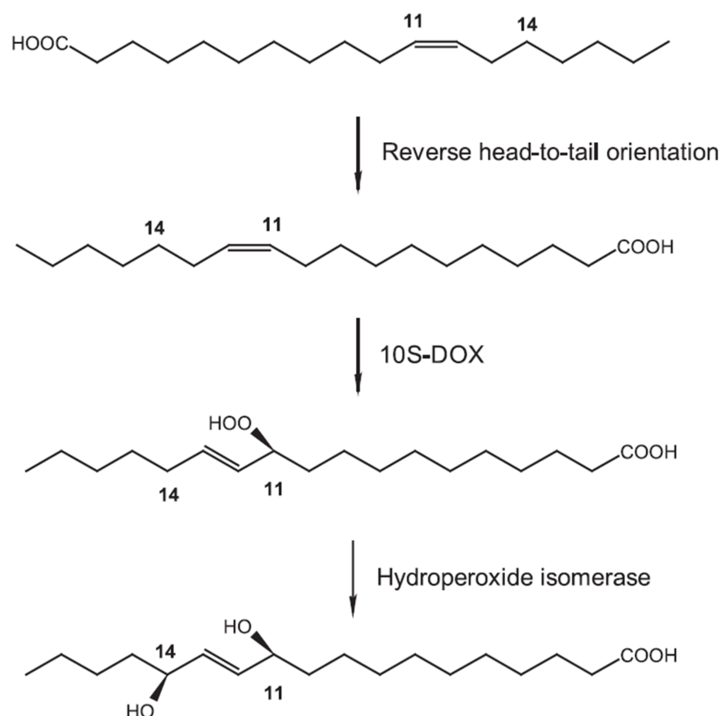

Figure 14. Schematic view of the oxidation of vaccenic acid ((11*Z*)-18:1) by *P. aeruginosa.* The 11,14-diol can be visualized as formed from (11*S*)-HPOME by reverse orientation of the fatty acid in the two active sites compared with the orientation of oleic acid. 12-HPOME was also formed, but this compound was apparently not isomerized to a diol (Martínez *et al.*, 2010).

1.3.6. Oleate-diol synthase of *P. aeruginosa* 42A2

The nature of *P. aeruginosa* oleate-diol synthase activity has been discussed thoroughly. Martínez and coworkers observed that that (10*S*)-dioxygenase and diol synthase activities co-eluted on ion exchange and gel filtration chromatography with an apparent molecular size of ~50 kDa, suggesting that both activities were linked in a soluble bifunctional enzyme, like those of fungi, associated to crude bacterial cell extracts. The observation that 10*S*-HPOME intermediate was transformed to the 7,10-diol less efficiently than its precursor oleic acid, also suggested that both activities were functionally coupled. Moreover, the authors discuss that (10*S*)-DOX activity probably involves hydrogen abstraction at C-8 by a catalytic metal so that the double bond migrates (9*Z*→8*E*), and the carbon radical centered at C-10 reacts with molecular oxygen and forms a hydroperoxide in analogy with (10*R*)-DOX of aspergilli (Garscha and Oliw, 2009). But unfortunately, the genome of *P. aeruginosa* PAO1 contains no obvious homologes to heme-containing fatty acid dioxygenases (cyclooxygenases, linoleate diol synthases, 10-DOX or α-DOX). The finding of two genes homologous to cytochrome P450 (corresponding to ORFs PA2475 and PA3331) in the

genome of *P. aeruginosa*, suggested that the corresponding enzymes could be responsible for the studied activities. However, analysis of the inhibition on the formation of 7,10-DiHOME by using 1 and 5 mM of 1-aminobenzotriazole (a nonspecific and irreversible P450 inhibitor), resulted in a 30 and 50% reduction of diol formation respectively, whereas neither CO nor the P450 inhibitor piperonyl butoxide (5mM) appeared to inhibit the diol synthase activity (Martínez *et al.*, 2010), suggesting that P450 was not indeed involved in oleic acid bioconversion. Therefore, the enzyme or enzymes responsible for the conversion of oleic acid in *P. aeruginosa* strains remains still undiscovered, and the complex background described here clearly shows that it deserves further investigation to unveil the nature of oleate-diol synthase activity in *P. aeruginosa*, including the discovery of the responsible gene or genes for further cloning and expression.

1.4. LONG-CHAIN FATTY ACID TRANSPORT IN BACTERIA

Bacterial fatty acids constitute major and important components of lipid membranes and they are also used as a source of energy (DiRusso *et al.*, 1999). Exogenous long-chain fatty acids influence a myriad of cellular processes including intracellular signaling and gene expression patterns (Dirusso and Black, 2004). Given the energetic cost of fatty acid synthesis and the necessity for obtaining essential fatty acids from the environment for catabolism, the transport of LCFAs across the outer and inner membranes represents a fundamental biological process.

The genetic and biochemical foundations of LCFA transport were first elucidated in the Gram-negative bacterium *Escherichia coli.* The key of this system is the coupled transport and activation of exogenous LCFAs, which in turn leads to changes in transcription patterns of the genes encoding the proteins required for fatty acid biosynthesis and degradation (Dirusso and Black, 2004). The three central components of this system include FadL, an outer membrane-bound fatty acid transport protein, FadD, an inner membrane-associated long chain acyl-CoA synthetase involved in uptake of medium and long-chain fatty acids, and FadR a long-chain acyl-CoA-related transcription factor. FadR controls the expression of nine genes primarily involved in fatty acid degradation and biosynthesis (DiRusso *et al.*, 1998; DiRusso *et al.*, 1999).

It is known nowadays that fatty acid degradation (fad) pathways may play critical roles in *P. aeruginosa* pathogenesis as they may have important implications in nutrient acquisition

and virulence within the cystic fibrosis lung (Son *et al.*, 2007), through the chemotaxis to LCFAs and phosphatyldiletanolamine (PE) (Miller *et al.*, 2008) and the degradation of an essential lung surfactant component like phosphatidylcholine (PC) (Fig.15).

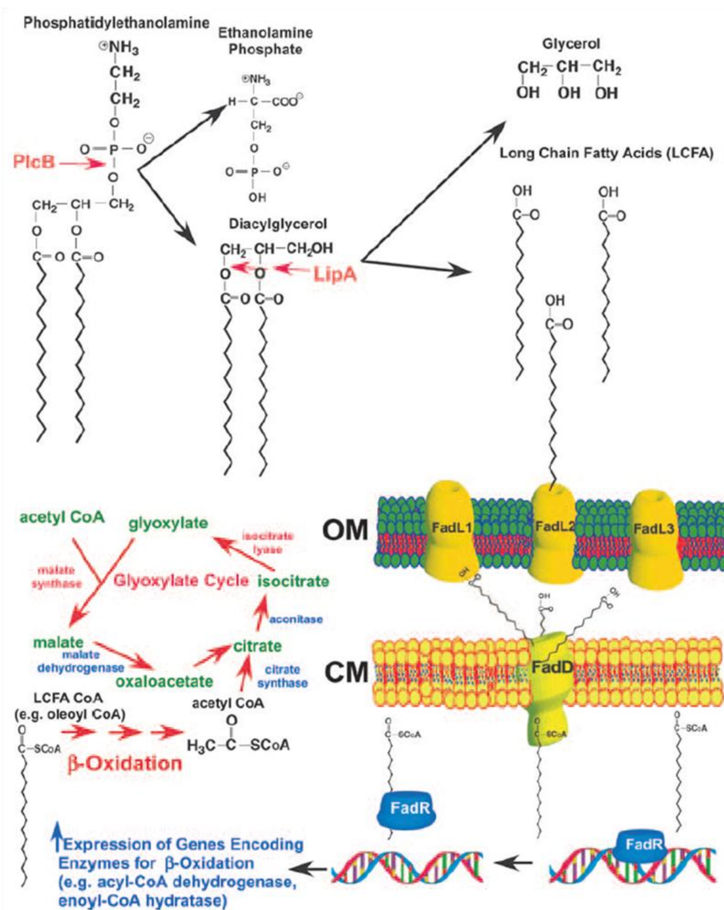

Figure 15. Proposed fatty acid degradation pathway of *P. aeruginosa* based *on E. coli* β-oxidation.
i) PE is degraded by PlcB and lipases to generate LCFAs, ii) LCFAs are transported through the inner (CM) and outer membrane (OM) of *P. aeruginosa*, iii) LCFAs are metabolized via β-oxidation to acetyl-CoA, and iv) acetyl-CoA is processed by the glyoxylate shunt to generate energy (Miller *et al.*, 2008).

The comprehensive analysis of transcriptional regulators as PrsA, modulated by the environmental concentration of LCFAs, which controls expression of *fad* genes, strongly supports the hypothesis that lipids within the lung may be important nutrient sources for *P. aeruginosa* (Kang *et al.*, 2008; Kang *et al.*, 2009; Kang *et al.*, 2010).

1.4.1. The outer membrane of Gram-negative bacteria

The uptake or export of LCFAs in bacteria depends on the basic characteristics of the most outsite layer, the outer membrane (OM). The OM of Gram-negative bacteria provides an

effective barrier to their often-harsh extracellular milieu (Nikaido, 2003). In particular, the outer leaflet of the OM is not a canonical monolayer of phospholipids but an assymetrycal construction (Beveridge, 2001). It is composed of lipopolysaccharide (LPS), a molecule generally consisting of a core of Lipid A decorated with inner and outer core oligosaccharides. The oligosaccharides extend ~30 Å above the plane of the lipid headgroups of the outer leaflet, which make up to 75% of the total membrane surface and create specific contacts with integral OM proteins (Alexander and Rietschel, 2001; Bos and Tommassen, 2004) (Fig. 16). As such, it is an effective permeability barrier against potentially harmful compounds like hydrophobic anitibiotics, dyes and detergents (Nikaido and Vaara, 1985). Additionally, bacterial lipoproteins anchor the OM in the periplasmic peptidoglican layer (Bos and Tommassen, 2004) and divalent cations are tighly associated with the anionic membrane-proximal regions of the LPS, increasing the strength of the structure (Vaara, 1992).

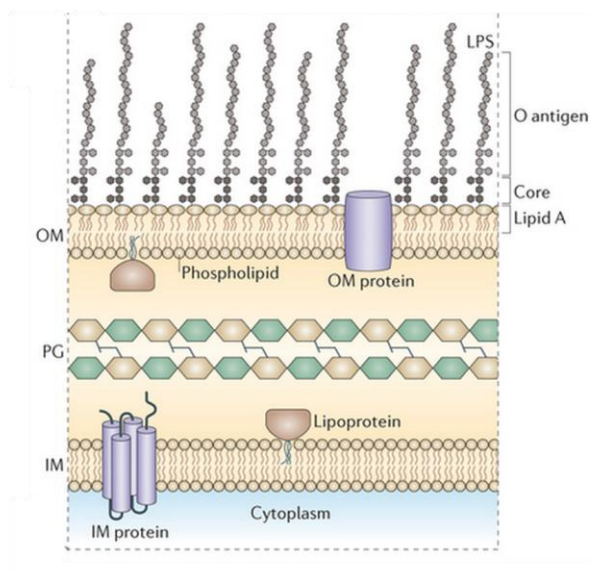

Figure 16. Schematic structure of the Gram-negative cell envelope. The typical inner (IM) and outer bilayers (OM) that are separated by the periplasm, which contains peptidoglycan (PG). The outer leaflet of the outer membrane contains lipopolysaccharide (LPS), which is anchored to the membrane by the LPS lipid A domain. The inner leaflet of the outer membrane and also the entire inner membrane are composed of phospholipids only, and both bilayers can contain a range of different types of membrane protein (Needham and Trent, 2013).

However, membrane permeability is required for bacterial survival as, among others, the uptake of nutrients is essential, and OM transport proteins are required to conduct this function. With some exceptions, virtually all OM proteins are β-barrels, consisting of an even number of eight to twenty-four β-strands forming a pore-like structure. Many of these OM pore-like β-barrels are classified as porins, which function passively, and most nutrients with relatively small molecular weight (< 600 Da) uptake is accomplished by this way, setting them into the periplasm across a concentration gradient. However, these water-filled pores generally exclude entry of hydrophobic substances (Nikaido, 2003). Among the transport proteins characterized to date, only the *E. coli* LCFA transport protein FadL fulfills the criteria

that defines an integral OM transporter of hydrophobic compounds (Wiener and Horanyi, 2011; Lepore *et al.*, 2011).

1.4.2. The long-chain fatty acid transport protein FadL

E. coli FadL is localized in the outer membrane forming a β-barrel specific for the transmembrane movement of long-chain fatty acids (van den Berg, 2010). Bacterial strains with a deletion of the *fadL* gene cannot grow on LCFAs as the sole carbon and energy source and cannot transport long-chain fatty acids across the cell envelope; yet they retain their ability to β-oxidize long-chain fatty acids *in vitro* (Mangroo and Gerber; Nunn and Simons, 1978; Black *et al.*, 1987). Research works of Black *et al.*, localized FadL in the OM of *E.coli* (EcFadL), and its function was described as along-chain fatty acid transport protein in concert to inner membrane-associated fatty-acyl CoA synthase (FadD) in a vectorial acylation way since the imported fatty acid becomes metabolically trapped by esterification with Co A (Black and DiRusso, 2003). The crystal structure of *E. coli* FadL was determined ten years ago and revealed to be a monomeric transport protein formed by a 14-stranded β-barrel with a luminal N-terminal hatch domain occluding the barrel (van den Berg, 2005). A significant feature of the first FadL structure described is an inward-facing kink in the third β-strand (S3) of the barrel that creates an unusual lateral opening in the transmembrane area (Fig. 17) (van den Berg *et al.*, 2004). Latter on, the uptake mechanism of LCFAs was confirmed by site-specific mutagenesis, showing that it was ocurring by lateral ligated-gate diffusion through an opening in the barrel wall that allows the LCFA movement into de OM and final diffusion into the periplasmic space (van den Berg, 2005; Lepore *et al.*, 2011).

Although transport of LCFAs in other bacteria, including *P. aeruginosa*, is under studied, it has been suggested that a FadL-based transport mechanism similar to that of *E. coli* could occur in other Gram-negative bacteria (Black and DiRusso, 2003).

In silico analysis of the sequenced genome of *P. aeruginosa* strain PAO1 genome (Stover *et al.*, 2000) revealed the presence of three genes annotated as *PA1288*, *PA1764* and *PA4589,* encoding for proteins similar to EcFadL transporters. The product of ORF PA4589 shows low (23%) sequence identity to EcFadL (Hearn *et al.*, 2009). However, crystallization of this protein overexpressed in *E. coli,* displayed high structural similarity to EcFadL, where the characteristic lateral opening is conserved. Therefore, lateral diffusion has been proposed as

a general feature of FadL channelsin bacterial fatty acid transporters (Hearn *et al.*, 2009; Touw *et al.*, 2010) (Fig. 17).

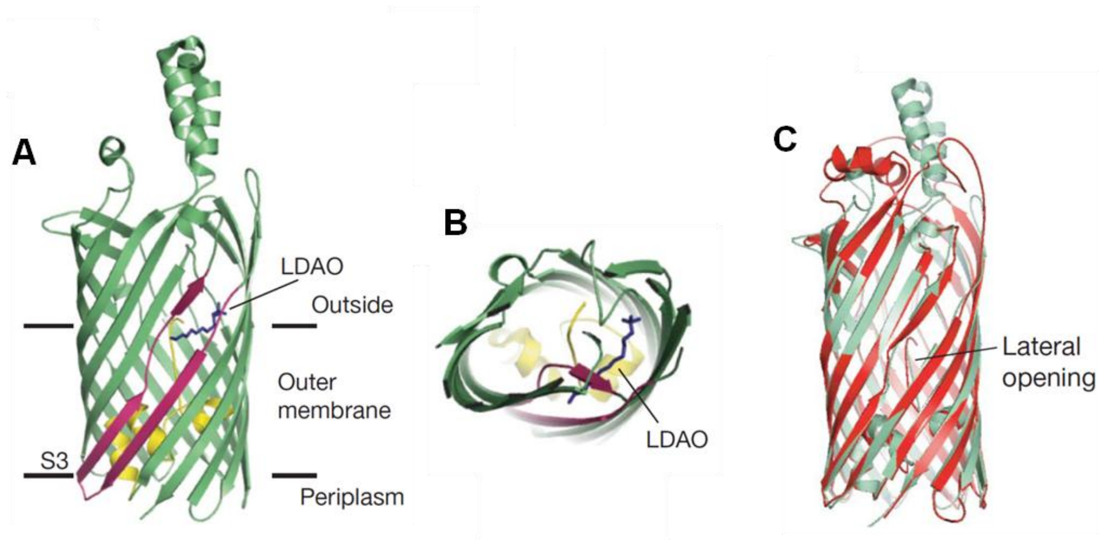

Figure 17. Crystal structure of *E. coli* FadL transporter. A) Viewed laterally. B) Viewed from the extracellular side. An lauryldimethylamine-oxide (LDAO) molecule (dark blue) protrudes through the opening in the barrel wall between strands S2 and S3 (dark pink). The hatch domain is coloured yellow. C) Superposition of EcFadL (green) and PaFadL (red), showing the conservation of the lateral opening, the hydrophobic passageway for substrate diffusion in PaFadL (Hearn *et al.*, 2009).

The products encoded by *P. aeruginosa* ORFs PA1288 and PA1764 show 25% and 23% sequence identity to EcFadL, respectively. None of these putative transporters have been functionallycharacterized yet but in a rec ent study, single, double andtriple mutants of the three ORFs (PA1288, PA1764 and PA4589) were constructed and tested in a twitching-mediated chemotaxis assay towards a gradient of LCFAs. Interestingly, all FadL mutants, including the triple mutant, showed the same ability to migrate towards a phosphatidylethanolamine (PE) gradient as the wild-type parent strain (Miller *et al.*, 2008). These results suggest that there must be an alternative pathway by which LCFAs are able to go across the OM in *P. aeruginosa*. Although the three FadL-like proteins found in *P. aeruginosa* genome contain computationally predicted signal peptides, only that encoded by *PA1288* has been shown to be located in the OM, as revealed by proteomic studies (Nouwens *et al.*, 2002). A recent study performed using various strains of *P. aeruginosa*, including PAO1, two clinical isolates and an environmental sample, revealed that the product of *PA1288* is a major protein in the bacterial OM and vesicles derived from these strains (Bauman and Kuehn, 2006).

Basic research on LCFA transport could be applied to improve uptake and concentration of the LCFA substrates or to facilite the release of oxylipins thus improving their downstream purification procedures.

2. AIMS OF THE WORK

The huge potential shown by *P. aeruginosa* 42A2 for biotensoactive and oxylipin production, their biotechnological exploitation and the possibility to get closer knowledge to their biological effects, motivated the study of oleate-diol synthase activity in *P. aeruginosa* 42A2 and the type strain PAO1.

This research project has been focused on the study of *P. aeruginosa* oxylipin synthesis, genetics and transport, as it covers not only biological but also biotechnological interests. As mentioned in the introduction, when this work began, the bacterial outer membrane transport of the oleate-diol synthase-derived oxylipins and the nature and evolutionary pathway of the enzyme/s involved in oleic acid bioconversion by *P. aeruginosa* 42A2 or PAO1 remained unknown.

In order to solve these questions, the following specific objectives of the work were defined:

1. The study of the outer membrane transport system of oxylipins like 7,10-DiHOME and related products in *P. aeruginosa (Paper I)*
2. Gene identification and functional characterization of the enzyme/s involved in oleate-diol synthase activity in *P. aeruginosa (Paper II)*
3. Phylogenetic analysis for enzyme classification and functional studies *(Paper III)*
4. Insights into the evolutionary pathway of the elements involved in oleate-diol synthase activity *(Paper III)*
5. A functional and biotechnological approach to *P. aeruginosa* oleate-diol synthase activity *(Additional results)*

3. RESULTS

3.1. LIST OF PUBLICATIONS

1. **Functional characterization of ExFadLO, an outer membrane protein required for exporting oxygenated long-chain fatty acids in *Pseudomonas aeruginosa*.** Eriel Martínez*, **Mónica Estupiñán***, F.I. Javier Pastor, Montserrat Busquets, Pilar Diaz, Àngels Manresa. 2013.

Biochimie, 95:290-298.

Impact Factor (2013): 3.123. Quartile: Q2

*: As stated in the paper, both authors contributed equally to this publication

2. **Unveiling the genes responsible for the unique *Pseudomonas aeruginosa* oleate-diol synthase activity.**

Mónica Estupiñán, Pilar Diaz, Àngels Manresa. 2014.

Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids, 1841:1360-1371.

Impact Factor (2014): 5.126. Quartile: Q1

3. ***In silico/in vivo* insights into the functional and evolutionary pathway of *Pseudomonas aeruginosa* oleate-diol synthase. Discovery of a new bacterial di-heme cytochrome c peroxidase subfamily.**

Mónica Estupiñán, Daniel Álvarez-García, Xavier Barril, Pilar Diaz, Àngels Manresa. 2015.

PLOS ONE, 10(7): e0131462.

Impact Factor (2014): 3.234. Quartile: Q1

4. **Functional and biotechnological properties approach to *P. aeruginosa* oleate-diol synthase activity**.

Mónica Estupiñán, Pilar Diaz and Àngels Manresa.

In progress.

3.2. PAPER I

Functional characterization of ExFadLO, an outer membrane protein required for exporting oxygenated long-chain fatty acids in *Pseudomonas aeruginosa*

3.2.1. Resumen

Caracterización funcional de ExFadLO, una proteína de membrana externa necesaria para la exportación de ácidos grasos de cadena larga en *Pseudomonas aeruginosa*

Eriel Martínez, **Mónica Estupiñán***, F.I. Javier Pastor, Montserrat Busquets, Pilar Diaz, Àngels Manresa*

Las proteínas bacterianas de la familia FadL se han asociado frecuentemente a la asimilación de sustratos hidrofóbicos exógenos. Sin embargo, su ubicación en la membrana externa y su participación en la captación de sustratos se han deducido principalmente por su similaridad de secuencia con la proteína FadL de *Escherichia coli*, los primeros transportadores de ácidos grasos de cadena larga de membrana externa caracterizados en bacterias. Aquí se presenta la caracterización funcional de una proteína de membrana externa de *Pseudomonas aeruginosa* PAO1 (ORF PA1288) que muestra similitud con los miembros de la familia FadL, y es el motivo por el que proponemos el nombre de ExFadLO. Se demuestra experimentalmente que se requiere esta proteína para exportar los ácidos grasos de cadena larga 10-HOME y 7,10-DiHOME, derivados de la actividad oleato diol sintasa, desde el compartimento periplasmático al medio extracelular. La acumulación de los derivados oxigenados 10-HOME y 7,10-DiHOME en el medio extracelular de *P. aeruginosa* resultó inhibida por una mutación generada por la inserción de un transposón en el gen ExFadLO (*exFadLO*$^{-}$ mutante). Sin embargo, la actividad oleato diol sintasa se encuentra intacta en el periplasma de este mutante, lo que indica que ExFadLO participa exclusivamente en la exportación a través de la membrana externa de estos ácidos grasos de cadena larga oxigenados. La capacidad de ExFadLO^{-} mutante para exportar 10-HOME y 7,10-DiHOME se recuperó después de su complementación mediante la expresión de la proteína ExFadLO en un plásmido, restituyendo el fenotipo salvaje. Además, un ensayo de *western blot* con una variante de ExFadLO fusionada al epítopo V5 confirmó la ubicación de ExFadLO en la membrana externa bacteriana en las condiciones experimentales ensayadas. Estos resultados proporcionan la primera evidencia de que proteínas de la familia FadL, involucradas en la asimilación de sustratos hidrófobos

desde el entorno extracelular, también funcionan como elementos de secreción de metabolitos de relevancia biológica.

Biochimie, 95 (2013):290-298

*****: Los dos autores participaron en la misma medida en el desarrollo del este trabajo

Biochimie 95 (2013) 290–298

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Research paper

Functional characterization of ExFadLO, an outer membrane protein required for exporting oxygenated long-chain fatty acids in *Pseudomonas aeruginosa*

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ABSTRACT

Bacterial proteins of the FadL family have frequently been associated to the uptake of exogenous hydrophobic substrates. However, their outer membrane location and involvement in substrate uptake have been inferred mainly from sequence similarity to *Escherichia coli* FadL, the first well-characterized outer membrane transporters of Long-Chain Fatty Acids (LCFAs) in bacteria. Here we report the functional characterization of a *Pseudomonas aeruginosa* outer membrane protein (ORF PA1288) showing similarities to the members of the FadL family, for which we propose the name ExFadLO. We demonstrate herein that this protein is required to export LCFAs 10-HOME and 7,10-DiHOME, derived from a diol synthase oxygenation activity on oleic acid, from the periplasm to the extracellular medium. Accumulation of 10-HOME and 7,10-DiHOME in the extracellular medium of *P. aeruginosa* was abolished by a transposon insertion mutation in *exFadLO* (ExFadLO mutant). However, intact periplasm diol synthase activity was found in this mutant, indicating that ExFadLO participates in the export of these oxygenated LCFAs across the outer membrane. The capacity of ExFadLO mutant to export 10-HOME and 7,10-DiHOME was recovered after complementation with a wild-type, plasmid-expressed ExFadLO protein. A western blot assay with a variant of ExFadLO tagged with a V5 epitope confirmed the location of ExFadLO in the bacterial outer membrane under the experimental conditions tested. Our results provide the first evidence that FadL family proteins, known to be involved in the uptake of hydrophobic substrates from the extracellular environment, also function as secretion elements for metabolites of biological relevance.

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1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium of ubiquitous distribution, known for its metabolic versatility and genetic plasticity [1]. It is also an opportunistic pathogen in immunocompromised patients [2] and has a remarkable ability to metabolize an extensive number of substrates, including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons [1]. Some strains have been confirmed to produce metabolites that stimulate plant growth or inhibit plant pests [3,4].

We recently reported that *P. aeruginosa* causes oxygenation of oleic acid into (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid (7,10-DiHOME) by a diol synthase activity. The enzyme responsible for this bioconversion has not yet been identified, but it was described as a soluble protein associated to crude bacterial cell extracts [5]. Other long-chain oxygenated fatty acids like (10*S*)-hydro(per)oxy-(8*E*)-octadecenoic acid (10-HPOME) and (10*S*)-hydroxy-(8*E*)-octadecenoic acid (10-HOME), are also produced by the strain [6–9]. All these oxygenated derivatives of long-chain fatty acids mainly accumulate in the extracellular medium, suggesting that although oxygenation occurs inside the cell, the released products should subsequently cross the outer membrane (OM) and accumulate in the culture supernatant.

Abbreviations: LCFAs, long-chain fatty acids; 10-HOME, (10*S*)-hydroxy-(8*E*)-octadecenoic acid; 7,10-DiHOME, (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid; 7,10,12-TriHOME, (7*S*,10*S*,12*R*)-trihydroxy-(8*E*)-octadecenoic acid; OM, outer membrane; IM, inner membrane; HPLC, high performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; RP-HPLC, reversed phase-HPLC; DS, diol synthase; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; AP, alkaline phosphathase.

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The OM of Gram-negative bacteria acts as a barrier, preventing free passage of hydrophobic and hydrophilic molecules, in part due to the outermost layer of lipopolysaccharide and to the phospholipidic bilayer [10]. Thus, uptake or secretion of long-chain fatty acids must proceed through specific transporter pathways. Up to date, the OM FadL protein of *Escherichia coli* (EcFadL) is the only one known to function as a true LCFAs transporter [11]. *E. coli* FadL has been characterized [12,13] and was recently crystallized, showing a monomeric structure constituted by a 14-stranded β-barrel. An opening in the lateral wall of the barrel would mediate diffusion of LCFAs from the extracellular environment to the periplasm [11,14]. It has been demonstrated the ligand-gate character of this protein with a high affinity binding site in the lumen of the barrel [15].

Although transport of LCFAs in other bacteria, including *P. aeruginosa*, is understudied, it has been suggested that a FadL-based transport mechanism similar to that of *E. coli* could occur in other Gram-negative bacteria [16].

In silico analysis of the sequenced genome of *P. aeruginosa* strain PAO1 genome [17] revealed the presence of three genes −PA1288, PA1764 and PA4589− annotated as encoding for proteins similar to *E. coli* FadL transporters. The product of ORF PA4589 shows low (23%) sequence identity to *E. coli* FadL [18]. However, crystallization of this protein overexpressed in *E. coli* displayed high structural similarity to *E. coli* FadL [11]. The products encoded by ORFs PA1288 and PA1764 have 25% and 23% sequence identity to *E. coli* FadL, respectively. None of these putative transporters has been functionally characterized yet but in a recent study, single, double and triple mutants of the three ORFs (PA1288, PA1764 and PA4589) were constructed and tested in a twitching-mediated chemotaxis assay towards a gradient of LCFAs. Interestingly, all FadL mutants, including the triple mutant, showed the same ability to migrate towards a phosphatidylethanolamine gradient as the wild-type parent strain [19]. These results suggest that there must be an alternative pathway by which LCFAs are able to go across the OM in *P. aeruginosa*.

Although the three FadL-like proteins found in *P. aeruginosa* genome contain computationally predicted signal peptides, only that encoded by PA1288 has been shown to be located in the OM, as revealed by proteomic studies [20]. A recent study performed using various strains of *P. aeruginosa*, including PAO1, two clinical isolates and an environmental sample, revealed that the product of PA1288 is a major protein in the bacterial OM and also in vesicle membranes derived from these strains [21]. Here we studied the possible role of PA1288, a FadL-like product, in the export of LCFAs and their oxygenated derivatives across the bacterial OM.

2. Materials and methods

2.1. Materials

Oleic acid (99%) and ricinoleic acid (99%), used in bioconversion assays were supplied by Sigma. Oleic acid (90%) Fluka was used in culture media. Solvents for organic extractions and HPLC were from Panreac.

2.2. Bacterial strains, media and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 wild-type strain was used as a control and all PAO1 transposon insertion mutants used were purchased from the comprehensive *P. aeruginosa* transposon mutant library (www.genome.washington.edu/UWGC/pseudomonas). Presence of the transposon was confirmed by sequencing the PCR products obtained after amplification with transposon internal and flanking primers. *E. coli* DH5α was used as host for plasmid constructions. All strains were routinely grown in TSB (17 g casein peptone, 3 g soymeal peptone, 2.5 g glucose, 5 g NaCL, and 2.5 g KH_2PO_4 at 37 °C on a rotary shaker operated at 200 rpm). Antibiotics were added, when necessary, at the following concentrations: for *E. coli*, ampicillin 100 μg/ml and chloramphenicol, 50 μg/ml; for *P. aeruginosa*, chloramphenicol 200 μg/ml was used; and for *P. aeruginosa* transposon insertion mutants, tetracycline 5 μg/ml was added. When fatty acids were used as a carbon source, Tween 80 (Sigma) at 1% was used as emulsifier. When necessary, alkaline phosphatase, used as a periplasmic enzyme marker, was induced by growing *P. aeruginosa* PAO1 at 37 °C for 20 h in a phosphate-deficient medium [22]. For induction of diol synthase activity, the same media were supplemented with 1% oleic acid.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (80 *lacZ*ΔM15) *hsdR1 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
P. aeruginosa strains		
PAO1	Wild type, reference strain	Jacobs et al., 2003
PW3330	Tet^r, PA1288-G02::ISphoA/hah (*fadLO*$^-$ transposon mutant)	UWGS[a]
PW4109	Tet^r, PA1764-A03::ISlacZ/hah	UWGS[a]
PW8729	Tet^r, PA4589-E10::ISphoA/hah	UWGS[a]
Plasmids		
pGEMT®-T Easy	Amp^r, cloning vector	Promega
pET101/D-TOPO®	Amp^r; vector for production of recombinant protein with a C-terminal V5 epitope tag	Invitrogen
pBBR1-MCS1	Cm^r, *lacPOZ′ mob*$^+$, broad-host-range plasmid	[28]
pGEM-*fadLO*	Amp^r, harbouring a 1551 bp fragment which corresponds to *fadLO* with its native promoter	This study
pGEM-*fadLO-Sac*I	Amp^r, harbouring *fadLO* gen and *Sac*I restriction site in 3′ extreme	This study
pBB-*fadLO*	Cm^r, the *fadLO* from pGEM-*fadLO* inserted into the *Sph*I and *Spe*I site of pBBR1-MCS1 shuttle vector	This study
pBB-*fadLO-Sac*I	Cm^r, the *fadLO-V5* from pET101-*fadLO* inserted into the *Xba*I and *Cla*I site of pBBR1-MCS1 shuttle vector	This study
pET101-*fadLO*	Amp^r, the *fadLO-Sac*I from pGEM-*fadLO-Sac*I inserted into the *Eco*RI and *Sac*I site of pET101/D-TOPO	This study

[a] Strains obtained from the University of Washington Genome Center.

2.3. Purification of 10-HOME and 7,10-DiHOME for bioconversion assays

Culture supernatants of PAO1 strain grown in TSB supplemented with 1% oleic acid were used to purify the released oxygenated oleic acid derivatives. Cultures were centrifuged at 8000 × g for 5 min and the supernatants recovered. A vol/vol organic extraction of the supernatants was performed with ethyl acetate. The organic phase was then evaporated and the mixture of LCFAs derivatives was resolved in an open column of silica gel. 10-HOME was eluted with hexane:ether:acetic acid (75:15:10), and 7,10-DiHOME was subsequently eluted with methanol. Purity of these metabolites was checked by TLC followed by LC-MS (MS/MS analysis, Supplemental Fig. S1 and Fig. S2).

2.4. Cell fractionation

Cell fractionation was performed following the method of Wood [23] with modifications by Robles-Price et al. [24]. Briefly, cells

were collected by centrifugation (4000 × g, 10 min, 4 °C), washed twice in 30 mM Tris-HCl, 150 mM NaCl (pH 7.1), and kept in ice for no longer than 1 h. Spheroplasts were further obtained by cell suspension in 6 ml 30 mM TrisHCl, 20% sucrose, 4 mM EDTA, 0.5 mg/ml lysozyme, 1 mM PMSF (pH 8), and 60 min incubation at 30 °C with gentle shaking. $MgCl_2$ was added at 10 mM final concentration as soon as the suspension reached 30 °C. Finally, the suspension was centrifuged (11,000 × g, 15 min, 4 °C), and the supernatant containing the periplasmic fraction was collected. Precipitated spheroplasts were then suspended in 6 ml 30 mM TrisHCl, pH 7.1, and disrupted by sonication on ice for 3 cycles of 30 s, interrupted by 15 s cooling down intervals. Remaining intact cells and cell debris were discarded after centrifugation (5000 × g, 1 h, 4 °C), and the supernatant was recovered and ultra-centrifuged (40,000 × g, 2 h, 4 °C) to obtain the total cell membrane fraction. After ultracentrifugation, the supernatant fraction was discarded and the pelleted membranes were suspended in 1 volume of 10 mM HEPES containing 0.7% (w/v) N-lauroyl-sarcosine, and shaken for 2 h at room temperature to selectively solubilize the inner cytoplasmic membrane proteins. The suspension was centrifuged (40,000 × g, 2 h, 4 °C) and the purified outer membrane protein fraction (OM) was recovered from the sediment.

2.5. Activity assays

Diol synthase activity was assayed as previously described [5]. 10 µl aliquots of the different purified cell fractions or crude cell extracts of *P. aeruginosa* strains were assayed using 1–5 mM LCFAs as substrates in 50 mM Tris-HCL (pH 7.0) up to a final volume of 500 µl [5]. The reactions were incubated for 1–4 h at 37 °C and terminated by acidification to pH 2–3 with 0.5 M HCl. The released products were then extracted with ethyl acetate, analysed by TLC (hexane:diethyl ether:acetic acid; 75:15:10), and quantified by LC-MS/MS analysis. Alkaline phosphatase, isocitrate dehydrogenase, and lactate dehydrogenase activities in the different cell fractions were assayed as described elsewhere [25–27].

2.6. DNA isolation and manipulation

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit or QIAGEN plasmid Midiprep. DNA restriction, polymerization and modification enzymes were used according to the manufacturer's instructions (Roche). DNA fragments were analysed on 0.8% (wt/vol) agarose gels and visualized with ethidium bromide (1 µg/ml). The nucleotide sequences of both strands from all isolated genes were obtained using an ABI Prism dye-terminator ready reaction mix (Perkin Elmer), available at the Serveis Cientifico-Tècnics of the University of Barcelona.

2.7. Growth in minimal medium

TSB suspensions of strains PAO1, ExFadLO mutant and the ExFadLO complemented mutant, containing approximately 1×10^8 cfu/ml, were washed twice in bacterial ringer solution (Scharlau) and transferred to M9 minimal medium (Difco) supplemented with 1% oleic acid, ricinoleic acid, 10-HOME or 7,10-DiHOME as the sole carbon source. Cultures were incubated for 24 h at 37°C and growth was measured as total protein (Bio-Rad Lab. Richmond, Ca. USA). TSB suspensions of strains ExFadLO, PA4589 and PA1764 mutants where washed in ringer solution and transferred to M9 minimal medium supplemented with 1% oleic acid as the sole carbon source. For solid media cultures, a suspension of washed cells from the three strains containing approximately 5×10^6 cfu/ml, was plated on M9 minimal medium (Difco) with 1.5% (w/v) Noble agar (Difco), supplemented with 5 mM oleic acid, 10-HOME or 7,10-DiHOME, respectively, as the sole carbon source. After 48 h incubation at 37 °C, growth was observed in all media for the three strains.

2.8. Complementation of P. aeruginosa ExfadLO mutant

exfadLO gene from *P. aeruginosa* PAO1 strain was amplified by PCR using the High Fidelity System polymerase from Roche and the specific primers *exfadLO*-UP (5′-CGTCCGAAACAGACCAACCGG-3′) and *exfadLO*-DOWN (5′-AGAAGGCCATCGCCAGCCAG-3′). The isolated DNA fragment was inserted in pGEM-T Easy Vector (Promega) to obtain plasmid pGEM-*exfadLO*. The *exfadLO* gene was excised from pGEM-*exfadLO* with *Sph*I and *Spe*I and subsequently inserted in the same restriction sites in the *E. coli-P. aeruginosa* shuttle vector pBBR1-MCS [28], to obtain pBB-*exfadLO*. This construction was used to electroporate *P. aeruginosa* ExFadLO mutant for complementation.

2.9. Construction of an exfadLO-V5 genetic fusion

The *exfadLO* gene from *P. aeruginosa* PAO1 was amplified by PCR using the High Fidelity System of Roche and the oligonucleotides *exfadLO*-Up (see above) and *exfadLO*-Down-*Sac*I (5′-<u>GAGCTC</u>-GAAGCGATAGGTGACCTG-3′; *Sac*I restriction site underlined) for introduction of a *Sac*I site at the 3′end of the gene. The isolated DNA fragment was ligated to pGEM-T Easy vector (Promega) to obtain plasmid pGEM-*exfadLO-Sac*I. The *exfadLO* gene was then excised with *Eco*RI and *Sac*I, and the resulting fragment was inserted in the same sites of a re-ligated version of pET101/D-TOPO vector (Invitrogen) to produce plasmid pET101-*exfadLO*. In this construction, *exfadLO* is fused in frame with the sequences coding for the V5 epitope [29] at its 3′region. The V5-tagged gene was then excised from pET101-*exfadLO-V5* with *Xba*I and *Cla*I, and inserted in the same sites of the shuttle vector pBBR1-MCS to produce plasmid pBB-*exfadLO-V5*, which was used to express the V5-tagged protein in *P. aeruginosa ex*FadLO mutant.

2.10. Protein electrophoresis and western blotting

OM proteins, prepared as described above, were analysed by SDS-PAGE and by western blot, as described elsewhere [27]. An anti-V5 mouse monoclonal antibody 1:5000 (Invitrogen) was used to detect the ExFadLO-V5 fusion in *P. aeruginosa* ExFadLO mutant. A secondary antibody anti-mouse IgG, peroxidase-linked 1:5000 and the ECL Plus Western Blotting Detection Reagents (GE Healthcare) were used to develop the reaction in a LAS-3000 luminescent Image Analyser (Fujifilm).

2.11. LC-MS/MS analysis

Reversed-phase Liquid Chromatography coupled to MS/MS analysis was performed with a quaternary MS pump system (Alliance 2695, Waters) coupled with electrospray ionization in an ion mass spectrometer PE Sciex API 365 (Applied Biosystems, USA). An RP-HPLC column Tracer Excel 120C8 column (150 mm × 4.6 mm, 5 µm) (Teknokroma, Spain) was used, and optimum separation was achieved using a gradient elution of acetonitrile/water at 1 ml/min. The electrospray voltage was set at 3.5 kV and temperature of the heated capillary was set at 400 °C. Carbon values were estimated from the retention times of a known standard mixture of carbon source and mono-, di-and trihydroxylated fatty acid derivatives from oleic acid [4]. The LC-MS/MS fragmentation mechanism of these oxygenated products was recently elucidated [9]. 7,10-DiHOME yields a series of fragments in the lower mass range (*m/z* 141, *m/z* 155 and *m/z* 169) and in the in the upper mass range produces fragments at *m/z* 295, *m/z* 293 and *m/z* 251. The MS/MS

spectrum of 10-HOME yielded a characteristic signal at *m/z* 155, whereas the base-peak was *m/z* 279. 7,10,12-TriHOME yields fragments *m/z* 181, *m/z* 197 and *m/z* 215 in the lower mass range, and fragments *m/z* 311, and *m/z* 293 in the upper mass range.

2.12. Bioinformatics tools

The nucleotide and deduced amino acid sequence of ExFadLO were analysed for conserved motifs and homology by means of the complete non-redundant protein sequence databases EMBL/EBI and NCBI [30]. Signal peptide and transmembrane regions were searched through SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP) [31] and TMHMM at the CBS server (http://www.cbs.dtu.dk/services/TMHMM) [32], respectively, and prediction of protein subcellular localization was performed using PSORTb v. 2.0 for bacterial sequences (http://www.psort.org/) [33]. Sequence alignments were performed using ClustalW Multalign Software (http://www.ebi.ac.uk/clustalw/) [34]. Secondary structure prediction was performed using the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/) [35], whereas protein fold recognition using 1D and 3D sequence profiles coupled with secondary structure information was obtained from PHYRE web server (http://www.sbg.bio.ic.ac.uk/~phyre/) [36]. Automated comparative three-dimensional modelling of ExFadLO was obtained from Swiss Model server (http://www.expasy.org/Swissmod/SWISS-MODEL.html) [37], using the Swiss-PdbViewer v. 3.7 software (http://www.expasy.org/spdbv/) to visualize the protein models. Superfamily assignments and analysis of the structural domains of the amino acid sequences were performed using Prodom [38] and Search Pfam [39] (http://prodes.toulouse.inra.fr/prodom/current/html/home.php; http://www.sanger.ac.uk/Software/Pfam/search.html).

3. Results

3.1. Periplasmic production of oxygenated oleic acid derivatives

We recently reported that *P. aeruginosa* can accumulate oxygenated fatty acids in the external medium as a result of a diol synthase activity [5]. In order to understand the physiology of oxygenated fatty acid production in *P. aeruginosa*, we first investigated the cellular compartment where these compounds are produced. Thus, oxygenation activity was measured by assaying diol synthase activity in the purified subcellular fractions of *P. aeruginosa* PAO1. The results indicate that this activity was predominantly found in the periplasm (Fig. 1A). Subcellular marker enzymes (cytosolic isocitrate dehydrogenase, membrane-bound lactate dehydrogenase and periplasm-associated alkaline phosphatase) were used to assess the quality of the cellular fractionation performed (Fig. 1B).

3.2. ExFadLO negative mutant does not export oxygenated oleic acid derivates

As shown above, oxygenation of oleic acid by the diol synthase occurs in the periplasm. However, the oxygenated products –10-HOME and 7,10-DiHOME– accumulate mainly in the extracellular medium. Therefore, we tested if the FadL-like proteins (PA1288, PA1764 and PA4589) were involved in the transport of these oxygenated LCFAs across the OM from the periplasm to the extracellular medium. With this purpose we analysed the ability of three independent insertion mutants, one in each gene coding for the three FadL-like proteins, to accumulate oxygenated fatty acids in the culture supernatant. Interestingly, we found that PA1288 gene insertion completely abolished accumulation of oxygenated fatty acids in culture supernatants (Fig. 2A). In contrast, *PA4589* and *PA1764* insertion mutants accumulated similar amounts of extracellular oxygenated fatty acids as those of the wild type strain PAO1, indicating that their products are not involved in such a secretion

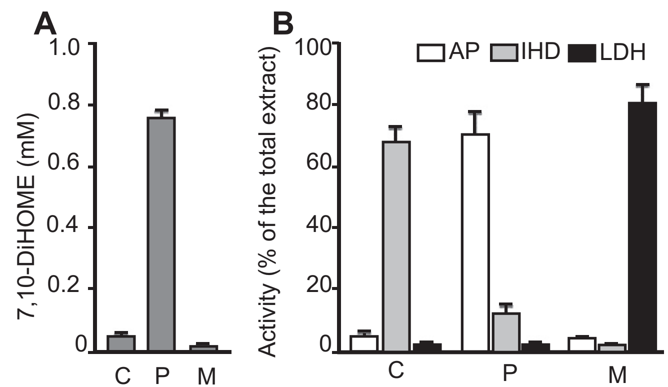

Fig. 1. Subcellular localization of oleate diol synthase (DS) activity in strain *P. aeruginosa* PAO1. Activity of specific subcellular maker enzymes, IDH, isocitrate dehydrogenase (cytosolic), LDH, lactate dehydrogenase (membrane) and AP, alkaline phosphatase (periplasmic) was also measured to confirm the accuracy of cell fractionation. Enzyme activity is expressed as a percentage of the total activity produced by crude cell extracts of the same cultures. Enzyme activity was measured spectrophotometrically for IDH, LDH and AP. In the case of DS by measuring the product by LC. Values are the mean (±SD) of three independent assays. Oleate diol synthase activity localizes in the periplasm of *P. aeruginosa* PAO1.

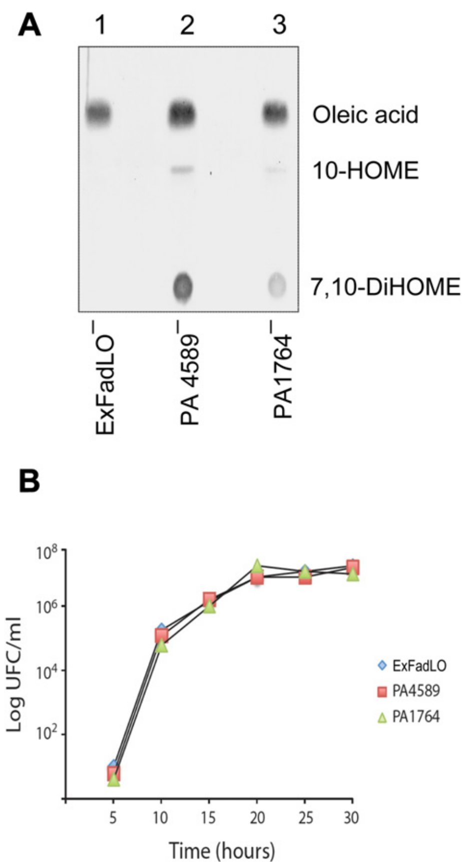

Fig. 2. A: TLC of oxygenated fatty acids accumulated in the extracellular media. Lane 1, ExFadLO mutant; Lane 2, PA4589 mutant; Lane 3, PA1764 mutant. B: Growth of the three FadL mutants in minimal media supplemented with oleic acid.

pathway. Accordingly, we designated PA1288 protein as ExFadLO due to its involvement in long-chain oxygenated fatty acid export. Interestingly, all three mutants grew at similar rates in minimal medium M9 supplemented with 1% of oleic acid (Fig. 2B). This indicates that none of the three FadL-like proteins is essential for the uptake of oleic acid.

To confirm that the lack of oxygenated oleic acid derivatives in the supernatant was really due to *exfadLO* gene interruption and not to polar or pleiotropic effects on others genes, mutant ExFadLO was complemented with the wild-type structural gene *exfadLO* (obtained from parental strain PAO1), carried by plasmid pBBR1-MCS (pBB-*exfadLO*). This construction was capable to completely restore accumulation of oxygenated fatty acids in the supernatant fraction, contrarily to what happened with ExFadLO mutant containing only vector pBBR1-MCS, used as a control (Fig. 3).

3.3. Diol synthase activity is intact in the periplasm of ExFadLO mutant

To verify if ExFadLO protein is indeed involved in transport but not in periplasmic production of oxygenated fatty acids, the presence of diol synthase activity in the periplasm of ExFadLO mutant was tested. As shown in Fig. 4, we found that diol synthase activity remained intact in the periplasm of ExFadLO mutant, that converted oleic acid into its oxygenated derivatives 10-HOME and 7,10-DiHOME to the same extent as parental *P. aeruginosa* PAO1 strain. These results confirm the role of ExFadLO in export of oxygenated oleic acid derivatives across the OM and indicate that there is not a direct relationship between ExFadLO and the diol synthase activity responsible for production of such compounds.

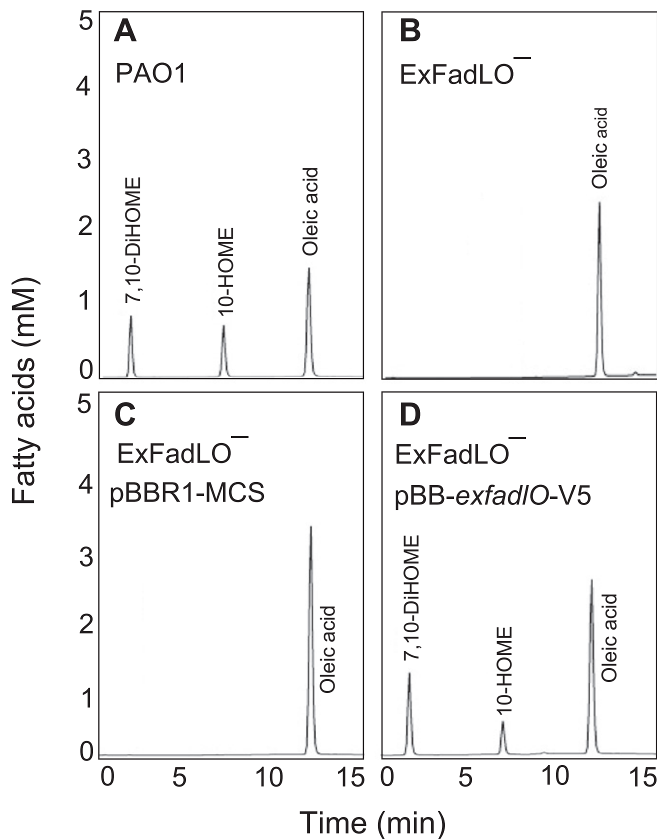

Fig. 3. LC analysis of oleic acid-derived products accumulated in the extracellular medium of *P. aeruginosa* grown in the presence of oleic acid, Initial concentration (I.C.) 1%. A: Supernatant from wild-type strain PAO1. B: Supernatant from ExFadLO mutant. C: Supernatant from control ExFadLO mutant transformed with plasmid pBBR1-MCS. D: Supernatant of ExFadLO mutant transformed and complemented with pBB-*exfadLO* construction.

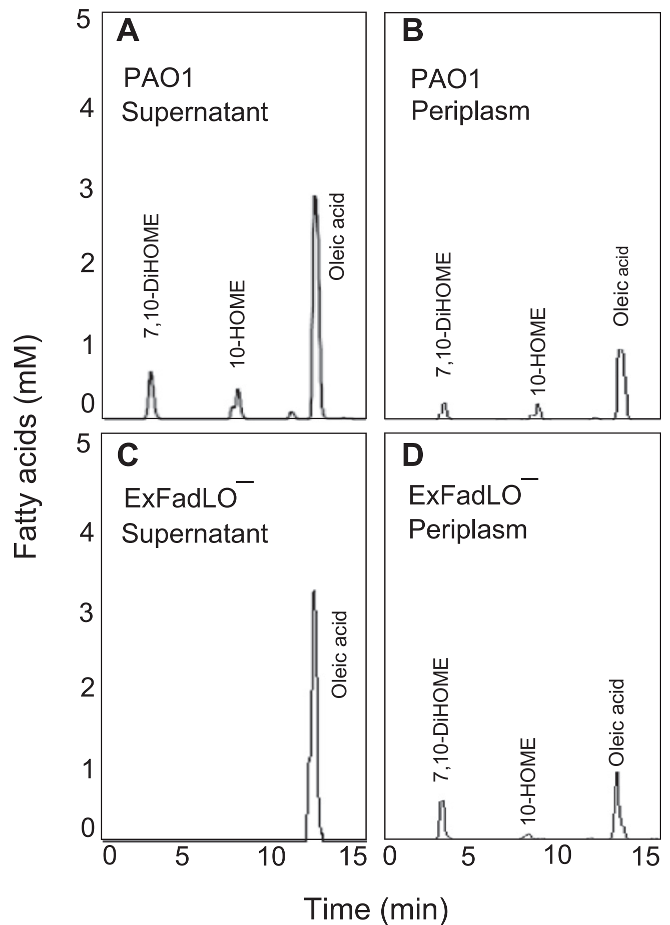

Fig. 4. LC analysis of oleic acid-derivatives produced by diol synthase activity. A: Supernatant from oleic acid bioconversion with *P. aeruginosa* PAO1 whole cells. B: *In vitro* bioconversion of oleic acid with the periplasmic fraction of strain PAO1. C: Supernatant from oleic acid bioconversion with ExFadLO mutant. D: *In vitro* bioconversion with the periplasmic fraction of ExFadLO mutant.

3.4. ExFadLO expression and location

Expression and location of protein ExFadLO in the OM was confirmed using a variant of ExFadLO carrying a V5 epitope tagged at the carboxy-terminal end of the protein (ExFadLO-V5). For this purpose, mutant ExFadLO was complemented with variant ExFadLO-V5 inserted in plasmid pBB-*exfadLO*-V5. Cell fractionation of such strain, followed by a western blot analysis using an anti-V5 antibody confirmed the location of ExFadLO in the OM under the experimental conditions (TSB, oleic acid 1%) used in this work. The faint ExFadLO-V5 band found at the inner membrane cell fraction (Fig. 5) indicates a slightly inefficient fractionation. The complementation assay performed with this construction also showed that ExFadLO-V5 restored oxygenated fatty acid accumulation in the culture supernatant (Fig. 3D), indicating that the carboxy-terminal fusion affected neither the correct directionaliation of ExFadLO nor the export function of the protein.

3.5. ExFadLO is not required for LCFAs uptake

Once known that ExFadLO is required to export the oxygenated fatty acids 10-HOME and 7,10-DiHOME to the culture supernatant,

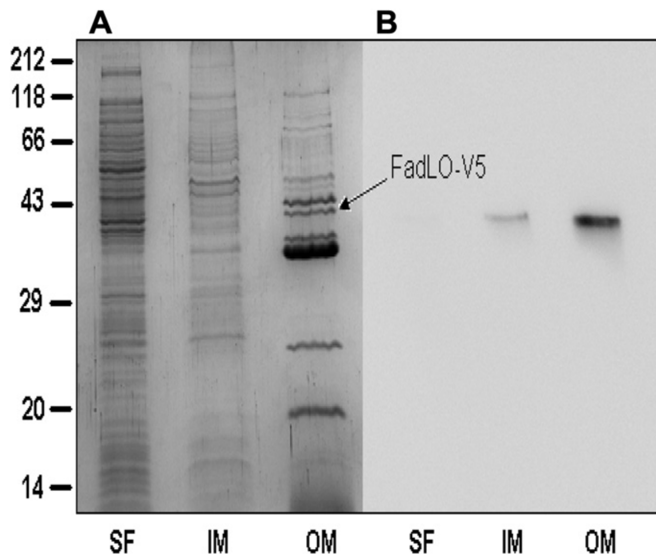

Fig. 5. Analysis of ExFadLO expression and location in ExFadLO mutant complemented with plasmid pBB-*exfadLO-V5*. A: 12% SDS-PAGE containing the following: Lane 1, soluble fraction (SF), periplasm plus cytosol. Lane 2, inner membrane proteins (IM). Lane 3, outer membrane proteins (OM). B: Western blot analysis of the same samples assayed with an anti-V5 mouse monoclonal antibody. The position of ExFadLO protein is indicated.

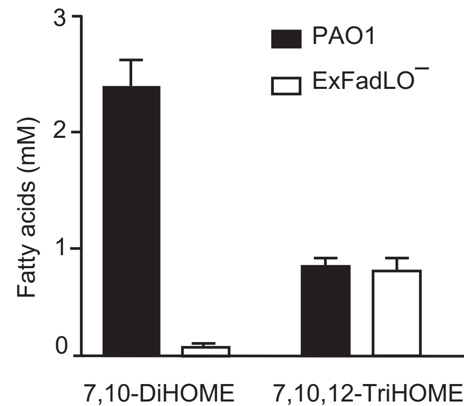

Fig. 6. Supernatant accumulation of 7,10-DiHOME and 7,10,12-TriHOME in parental strain PAO1 and in ExFadLO mutant, respectively. Abundance is expressed as a percentage of 7,10-DiHOME accumulated in supernatant of strain PAO1. The figure shows similar accumulation of 7,10,12-TriHOME in the supernatant of both strains. Values are the mean (±SD) of three independent assays.

the requirement of intact ExFadLO for uptake of LCFAs or their oxygenated derivatives was also investigated. It has previously been reported that *P. aeruginosa* can use oxygenated fatty acids 10-HOME and 7,10-DiHOME as carbon and energy sources [40]. The ability of ExFadLO mutant to grow on minimal medium supplemented with several LCFAs or oxygenated fatty acids as the sole carbon source was evaluated in solid (data not shown) and liquid cultures. Therefore, ExFadLO mutant was inoculated in M9 minimal medium containing alternatively oleic acid, ricinoleic acid, 10-HOME and 7,10-DiHOME as the sole carbon source. Interestingly after 24 h incubation similar growth, measured as total protein, was found. Mutant ExFadLO was able to use all carbon sources assayed (1.1 g/L when oleic acid or ricinoleic acid were supplied, and 1.6 g/L or 1.8 g/L when 10-HOME or 7,10-DiHOME were added). Similar growth was found for the complemented mutant strain (1.4 g/L and 1.3 g/L when oleic or ricinoleic acid were added, and 1.5 g/L and 1.8 g/L when 10-HOME or 7,10-DiHOME were supplied), or for parental strain PAO1 (1.5 g/L with oleic acid, 1.21 g/L when ricinoleic acid was supplied, and 1.2 g/L when 10-HOME or 7,10-DiHOME were added), indicating that protein ExFadLO is not required for the uptake of such fatty acids.

3.6. ExFadLO-independent export routes for more hydrophilic compounds

P. aeruginosa is able to grow on ricinoleic acid releasing the more hydrophilic compound (7*S*,10*S*,12*R*)-trihydroxy-(8*E*)-octadecenoic acid (7,10,12-TriHOME, C_{18}) [41]. This compound appears as result of the periplasmic diol synthase activity on ricinoleic acid, the same activity that releases 7,10-DiHOME and 10-HOME from oleic acid [5] or 7,10 dihydroxy-(8E)-hexadecenoic acid when grown on palmitic acid [42]. In order to determine whether ExFadLO is also involved in the export of 7,10,12-TriHOME across the OM, a bioconversion assay was performed with ExFadLO mutant, using ricinoleic acid as a substrate. Identification of the released products of this conversion was performed by LC-MS (Supplementary material Fig. S3). As shown in Fig. 6, ExFadLO mutant was able to accumulate 7,10,12-TriHOME in the supernatant to the same extent as PAO1 wild type strain, indicating that protein ExFadLO is not required for export of this more hydrophilic compound to the external medium. The lower apparent abundance of 7,10,12-TriHOME compared to that of 7,10-DiHOME in supernatant samples (Fig. 6) could be explained by the lower affinity of the diol synthase activity for ricinoleic acid with respect to that shown for oleic acid [5] or maybe also due to a less efficient export from the periplasm.

The results obtained here evidence that *P. aeruginosa* is capable to produce and export to the supernatant several oxygenated fatty acids derived from the corresponding enzymatic activities. However, not all oxygenated compounds produced and released by the strain use the same export route to go through the OM. According to our results, ExFadLO is required for secretion of oleic acid oxygenated fatty acids, whereas more hydrophilic oxygenated fatty acids would use a different, ExFadLO-independent route.

3.7. ExFadLO sequence and structure overview

A BLAST search was performed using the amino acid sequence of ExFadLO. Homology was found with known or putative *Pseudomonas* outer membrane proteins including the conserved domains of both, long-chain fatty acid transporters or aromatic hydrocarbon degradation proteins [43]. Pfam properly assigned ExFadLO to proteins of the FadL family, showing that they are ubiquitously distributed among *Pseudomonas* species [44]. This was confirmed by construction of a phylogenetic tree (Fig. 7), which clustered ExFadLO with the putative FadL transporters of *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Pseudomonas fluorescens*, *Pseudomonas syringae* or *Pseudomonas putida*, with identities ranging from 94 to 99%.

A 3D model structure of ExFadLO was obtained using the closest *3DWO* pdb derived from the known crystal structure of *P. aeruginosa* ORF PA4589 FadL homologue [18]. The resulting model revealed similar features and structural conformation as those described for previously crystallized FadL transporters, either from *E. coli* [11] or from *P. aeruginosa* [43,45], showing also a tubular 14-stranded β-barrel with an opening in the lateral wall. According to that, the carboxy-terminal end of ExFadLO would face the periplasmic space [14].

4. Discussion

LCFAs are an important source of carbon and energy and have a relevant functional and structural role in bacteria, as they are

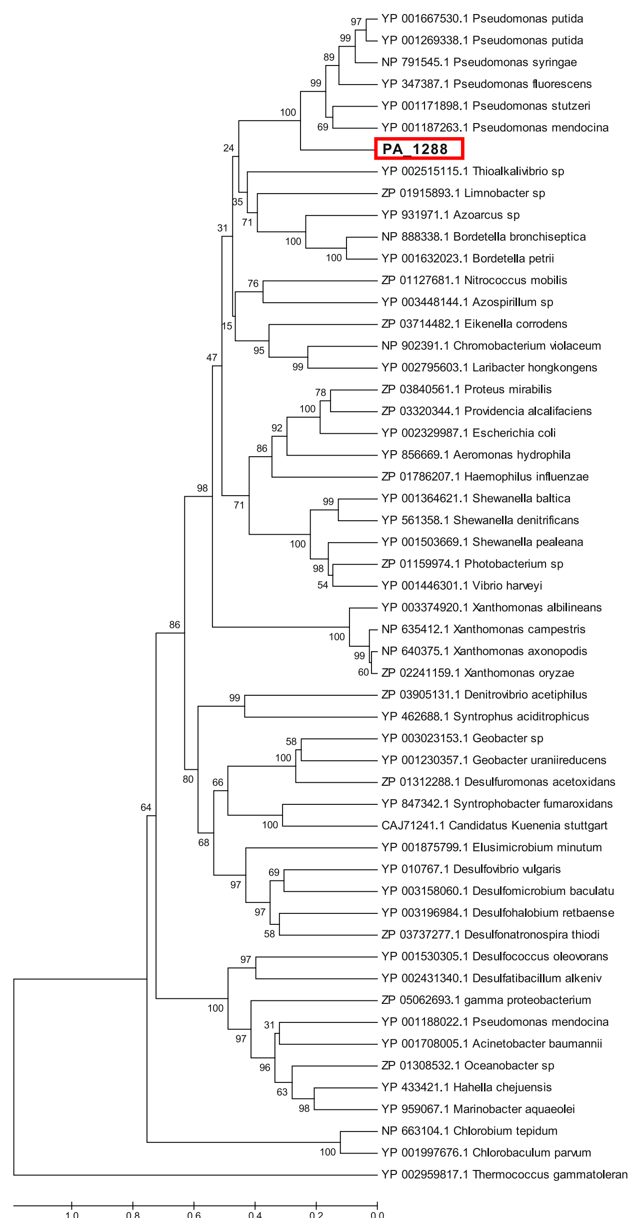

Fig. 7. Phylogenetic tree of ExfadLO (PA1288) family proteins.

essential structural components of cell membranes. Therefore, most of the literature related to the transport of lipids across membranes is devoted to the uptake of hydrophobic compounds such as LCFAs or xenobiotics such as toluene or benzene [43]. However, little is known about export of LCFAs or their derivatives to the external medium, an example being the efflux system for hydrophobic antimicrobial substances described in *N. gonorrhoea* [46].

Despite the fluid-like character of the cytoplasm membrane, which allows diffusion of hydrophobic molecules [47], the outer membrane of Gram-negative bacteria is highly asymmetric. For this reason, although LCFAs may cross the inner cell membrane due to their hydrophobic nature [48], transport of fatty acids into the cell requires a membrane bound protein involved in a process described as vectorial metabolism [16]. Thus, uptake of LCFAs in Gram-negative bacteria requires specialized channels, as demonstrated in a *fadL* knockout mutant of *E. coli*, which was not able to grow on LCFAs [13]. A coordinated action of such channels with an associated inner membrane FadD protein to drive the fatty acid into the cytoplasm has been described, which activates the fatty acids into the β-oxidation pathway [12,16].

A fairly common mechanism of export in Gram-negative bacteria is the release of outer membrane vesicles [49,50]. A proteomic analysis of such vesicles revealed that five proteins were abundant and common to all *Pseudomonas* strains studied. Among them, the putative outer membrane protein encoded by ORF PA1288 (*exFadLO*) was found [21]. Other attempts to identify OM lipid-transporters in Gram-negative bacteria have pointed to genes coding for multidrug efflux proteins involved in antibiotic resistance mechanisms [46] like alginate lyase or alginate transporter, but no conclusive results have been achieved [51].

In order to understand the production of extracellular (10*S*)-hydroxy-(8*E*)-octadecenoic and (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acids when *P. aeruginosa* is grown in the presence of oleic acid, we first characterized an oleate diol synthase activity as the enzyme responsible for such biotransformation [5]. In this study we have demonstrated that the diol synthase activity localizes in the periplasmic space of *P. aeruginosa* PAO1, suggesting that a transport mechanism responsible for the release of oxygenated oleic acid derivatives to the external medium should exist in this specie. Thus, in order to find a protein responsible for secretion of oxygenated oleic acid derivatives generated by the diol synthase in the periplasm, we studied the putative role of the products of three genes showing homology to FadL family of proteins in *P. aeruginosa*. Among the three putative FadL-like proteins found in the genome (ORFs PA1288, PA1764 and PA4589), only the product of ORF PA1288 was found to be a major protein in the bacterial OM and also in vesicle membranes after growth with oleic acid [21]. However, the ability of the three FadL-like proteins in oxygenated oleic acid derivative export was tested using the corresponding mutants. We found that only the product of PA1288, designated ExFadLO and abundantly expressed in the outer membrane of *P. aeruginosa* [20,21], has an essential role in oxygenated oleic acid derivatives export.

We also evidenced that periplasmic diol synthase activity remained intact in ExFadLO mutant, suggesting that production and release of oxygenated oleic acid derivatives are not coupled events. To confirm that ExFadLO was indeed responsible for oxygenated oleic acid derivatives export across the OM, a complementation assay of ExFadLO mutant was performed, resulting in the complete recovery of oxygenated fatty acid export to the extracellular medium. On the contrary, the insertion mutation in ExFadLO did not prevent release of the more hydrophilic compound 7,10,12-TriHOME, derived from the diol synthase activity on ricinoleic acid. Accordingly, secretion of 7,10,12-TriHOME across the OM in *P. aeruginosa* should proceed via an alternative transporter. Our observations are in agreement with previous reports suggesting that FadL family of proteins are substrate-specific transporters [18]. However, the fact that ExFadLO is also required for export of 10-HOME, a monooxygenated oleic acid derivative, suggests that such a specificity could rely also on the degree of hydrophobicity of the transported compound. Alternatively, the high specificity observed could be due to the requirement for a ligand-gating event as a prerequisite for ExFadLO channel formation, as described for EcFadL [15].

To find out if ExFadLO could also be involved in LCFAs uptake, growth of parental, ExFadLO mutant and complemented strains in minimal medium supplemented with several fatty acids and oxygenated derivatives as the sole carbon source was evaluated. The observation that the *exfadLO* insertion mutation did not affect growth of the mutant with respect to parental and complemented strains clearly indicates that alternative routes for uptake of such compounds must exist. Our results are thus in agreement with previous assays performed with single, double and triple mutants

of ORFs PA1288, PA1764 and PA4589, which did not abolish the twitching-mediated migration towards a gradient of LCFAs [19]. Therefore, *P. aeruginosa*, similarly to *P. putida*, bears an alternative pathway for uptake and degradation of LCFAs and their oxygenated derivatives from the extracellular medium into the cell [19,52].

To confirm the nature of ExFadLO as an OM-located LCFAs transporter, its expression and position under our experimental conditions was confirmed by western blot analysis. A 30 amino acid peptide containing the V5 epitope fused to the carboxy-terminal end of ExFadLO did not affect either directionalization of the transporter to the OM or secretion of oxygenated fatty acids. These results are in agreement with a recent report on EcFadL, showing the relevance of the amino-terminal end of EcFadL for correct LCFA uptake [15]. According to the predicted 3D model structure of ExFadLO, obtained using the crystal structure of *P. aeruginosa* gene *PA4589* product as a template ([11]; Swiss-Model), the V5 epitope bound to ExFadLO would constitute the last segment of the ultimate β-strand in the barrel, with a disposition that would allow close contact to the periplasmic space of the cell, thus allowing the correct assembly of ExFadLO to the OM.

Taking into consideration the previous results, a fundamental question arises: what is the benefit of having an oxygenated fatty acid efflux system for *P. aeruginosa*? Our results allow us to hypothesize that ExFadLO, acting in coordination with the periplasmic diol synthase activity [5], could work as an efflux system to expel the excess of free oxygenated LCFAs, which might be toxic to the cell. This efflux system could be advantageous for *P. aeruginosa*, a bacterium existing in many and diverse environments. Evidences have been provided that oxygenated fatty acids have an important role in plant defence reactions that take place as a consequence of infection by bacteria. In fact, a hydroxy-fatty acid-mediated protective effect of some *Pseudomonas* strains against fungal infection in plants has been found [53], and the biological activity of hydroxy-fatty acid derivatives against rice blast disease was also reported [54,55]. Supporting this hypothesis, the antibacterial and antifungal properties of microbial oxygenated fatty acids has recently been proposed [3,4].

Since a mutant of ExFadLO fails to export the products of the diol synthase activity on oleic acid, none of the efflux pumps of *P. aeruginosa* seems to be used to export these compounds from the periplasm. This is an interesting observation taking into consideration the narrow range of compounds that this transporter can handle. Our current studies are directed to understand the precise way ExFadLO transporter specifically recognizes and exports oxygenated fatty acid.

As stated above, attempts have been made to characterize OM lipid-transporters in Gram-negative bacteria but no conclusive evidences have been achieved [51,56]. To our knowledge the current study constitutes the first report on a membrane transporter required for export of hydrophobic oxygenated fatty acid derivatives across the OM in bacteria. These results extend the functional spectrum for the FadL-like family of proteins, described so far only for the uptake of hydrophobic substrates from the extracellular medium.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2012.09.032.

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SUPPLEMENTAL MATERIAL (Martínez *et al.* 2013)

Fig. S1.MS/MS spectra of 10-hydroxy-8(*E*)-octadecenoic acid (m/z 297→full scan).

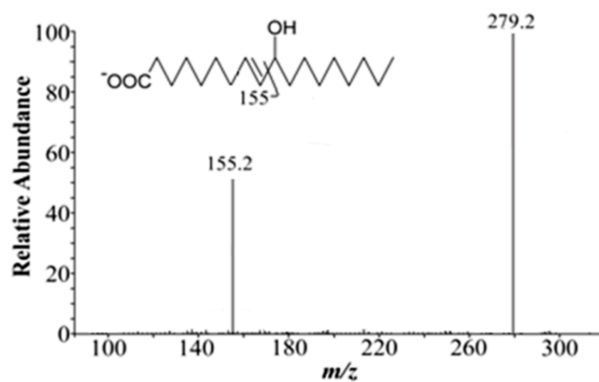
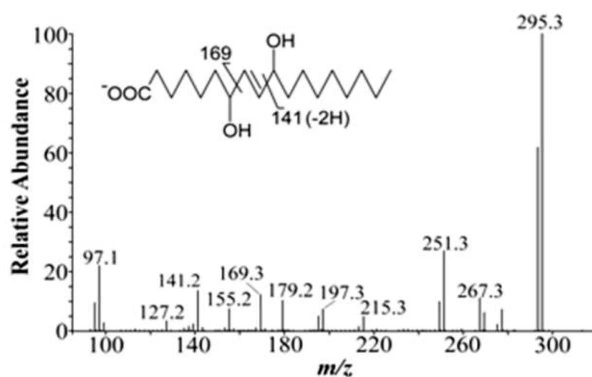


Fig. S2. MS/MS spectra of 7,10-dihydroxy-8(*E*)-octadecenoic acid (m/z 313→full scan).



3.3. PAPER II

Unveiling the genes responsible for the unique *Pseudomonas aeruginosa* oleate-diol synthase activity

3.3.1. Resumen

Desvelando los genes responsables de la exclusiva actividad oleato-diol sintasa de *Pseudomonas aeruginosa*

***Mónica Estupiñán**, Pilar Diaz, Àngels Manresa*

P. aeruginosa muestra la capacidad de realizar la bioconversión de ácido oleico en una clase de ácidos grasos hidroxilados conocidos como oxilipinas. La actividad diol sintasa es responsable de tal bioconversión, que tiene lugar a través de la dioxigenación de ácido oleico para liberar un intermediario hidroperóxido 10-H(P)OME (ácido (10*S*)-hidroxi-8*(E)*-octadecenoico), seguido por la conversión del hidroperóxido en 7,10-DiHOME (ácido (7*S*,10*S*)-dihidroxi-(8*E*)-octadecenoico), los cuales se acumulan en el sobrenadante del cultivo. Se seleccionaron varios mutantes de *P. aeruginosa* PAO1, analizando su capacidad para la producción de 10-H(P)OME y 7,10-DiHOME y sorprendentemente dos de ellos (ORFs PA2077 y PA2078), fueron incapaces de liberar ácidos grasos hidroxilados, eligiéndose para posteriores análisis. La participación de los ORFs PA2077 y PA2078 en la actividad oleato diol sintasa se confirmó, y su respectivo papel en la conversión de ácido oleico se determinó mediante la complementación de las mutaciones. La restauración de la actividad reveló que el gen *PA2077* codificaba para la actividad 10*S*-dioxigenasa (10*S*-DOX) responsable de la primera etapa de la reacción, mientras que el gen *PA2078* codificaba para la actividad (7*S*,10*S*) - hydroperoxido diol sintasa (7,10-DS), que permite la conversión de 10-H(P)OME en 7,10-DiHOME. La expresión heteróloga de ambas enzimas de manera independiente mostró que no se requiere la formación de hetero-complejos para que tenga lugar la actividad enzimática. Análisis bioinformáticos y de RT-PCR revelaron que ambos genes constituyen un nuevo operón oleato-diol sintasa finamente regulado, originado mediante un evento de duplicación de genes seguido por neo-funcionalización para la adaptación del medio ambiente, sin precedentes en procariotas.

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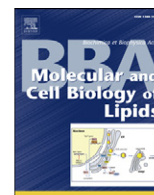

Unveiling the genes responsible for the unique *Pseudomonas aeruginosa* oleate-diol synthase activity

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ABSTRACT

Pseudomonas aeruginosa displays the ability to perform bioconversion of oleic acid into a class of hydroxylated fatty acids known as oxylipins. A diol synthase activity is responsible for such a conversion, which proceeds through the dioxygenation of oleic acid to release hydroperoxide 10-H(P)OME ((10*S*)-hydroxy-(8*E*)-octadecenoic acid), followed by conversion of the hydroperoxide intermediate into 7,10-DiHOME ((7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid), both of which accumulate in the culture supernatant. Several mutants of *P. aeruginosa* PAO1 were analyzed for the production of 10-H(P)OME and 7,10-DiHOME and two of them (ORFs PA2077 and PA2078), unable to release hydroxylated fatty acids, were detected and selected for further analysis. Involvement of ORFs PA2077 and PA2078 in oleate-diol synthase activity was confirmed, and their respective role in the conversion of oleic acid was analyzed by mutation complementation. Activity restoration revealed that gene *PA2077* codes for the 10*S*-dioxygenase activity (10*S*-DOX) responsible for the first step of the reaction, whereas *PA2078* encodes for the (7*S*,10*S*)-hydroperoxide diol synthase enzyme (7,10-DS) which allows the conversion of 10-H(P)OME into 7,10-DiHOME. Heterologous expression of both enzymes separately showed that no hetero-complex formation is required for enzymatic activity. Bioinformatics and RT-PCR analysis revealed that both genes constitute a new fine regulated oleate-diol synthase operon, originated by a gene duplication event followed by neofunctionalization for environmental adaptation, being unprecedented in prokaryotes.

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1. Introduction

Pseudomonas aeruginosa is a metabolically versatile gram-negative rod known for its adaptability, being associated with either free-living soil microorganisms or to pathogenic lifestyles in a wide range of hosts [1]. Its ability to metabolize an extensive number of substrates, including toxic organic chemicals, leads to a ubiquitous distribution [1]. *P. aeruginosa* can also use and transform unsaturated long-chain fatty acids (LCFAs), which can occasionally be converted into their hydroxylated derivatives (HFAs), also known as oxylipins [2,3]. Oxylipins display a wide range of biological functions as nutrients, signal molecules and precursors or integral components of both, prokaryotic and eukaryotic cell elements, being multifunctional molecules [4,5]. Moreover, HFAs have been described as antibacterial, antifungal, tensioactive or emulsifying agents, and are used as flavors in food industry, paintings or other value-added products [4,6,7] and as useful intermediates in the synthesis of fine chemicals and pharmaceuticals due to their high reactivity [8,9].

LCFAs can be oxygenated by lipoxygenases (LOX), heme-containing fatty acid dioxygenases (DOX), cyclo-oxygenases (COX) or cytochrome P_{450} (CYP450) [10–12]. These activities have extensively been described in eukaryotes, mostly mammals or plants, and more recently in algae [13–15]. Fungi can also oxidize unsaturated fatty acids to biological mediators [2,4], some of which are produced by bifunctional enzymes like 7,8-linoleate diol synthase (7,8-LDS) from *Gaeumannomyces graminis* [16], by psi-producing oxygenases PpoA (5,8-LDS), PpoB (10*R*-DOX) and 8,11-DS of aspergilli [17], or by 9*S*-DOX-AOS from *Fusarium oxysporum* [18].

The accumulation of oxygenated HFAs has been reported for several strains of *P. aeruginosa* (42A2, PR3, PAO1 among others), which can convert oleic acid (OA) into (10*S*)-hydroperoxy-(8*E*)-octadecenoic acid (10-H(P)OME), (10*S*)-hydroxy-(8*E*)-octadecenoic acid (10-HOME) and 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid (7,10-DiHOME) [19–22] through a metabolic pathway that was recently described for *P. aeruginosa* 42A2 [23]. The conversion of OA takes place by means of a unique oleate-diol synthase activity involving two sequential reactions: a first step where oleic acid is oxygenated by a dioxygenase — (10*S*)-DOX — to release hydroperoxide 10-H(P)OME, which is spontaneously reduced to 10-HOME, and a second reaction where 10-H(P)OME is converted into 7,10-DiHOME. OA is the preferred but not the only substrate of *P. aeruginosa* 42A2 oleate-diol synthase [23]. It was shown that the two activities co-eluted upon purification, suggesting that they are linked in a soluble bifunctional enzyme located in the periplasm [23,24].

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The enzymatic reaction mechanism of *P. aeruginosa* 42A2 oleate-diol synthase has already been elucidated and is described elsewhere [23], with an apparent Km of 1.7 mM and optimum pH and temperature of 7 and 35 °C, respectively. However, the genetic elements that constitute this activity are still unknown. Taking into consideration the particular properties of *P. aeruginosa* PAO1 genome and the biochemical knowledge of the diol synthase activity, a theoretical approach was used here for screening candidate genes of oleate-diol synthase activity. A collection of transposon insertion mutants of *P. aeruginosa* PAO1 selected genes [25] was analyzed for 10*S*-H(P)OME and 7,10-DiHOME production and two mutants, unable to release these HFAs, were found and selected for further identification and functional characterization.

2. Materials and methods

2.1. Materials

Oleic acid 99% (Sigma) was used either to induce diol synthase activity in *P. aeruginosa* strains, as a substrate for bioconversion assays or as autooxidation control in LC/MS analysis. 10(*S*)-hydroperoxy-8 (*E*)-octadecenoic acid (10*S*-H(P)OME), provided by Dr. E. Martínez, was purified from *P. aeruginosa* 42A2 culture supernatants as described previously [23] and used as a substrate for biotransformations. Fatty acids were dissolved in ethanol and stored in stock solutions (100 mM) at −20 °C. Solvents for organic extractions, HPLC and LC/MS analyses were from Panreac. Pre-coated TLC plates (0.25-mm Silica gel 60A, 20 × 20 or 5 × 20 cm) and molybdatophosphoric acid were from Fluka Analytical.

2.2. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this work are listed in Tables 1 and 2 and Supplementary material, Table S1. *P. aeruginosa* 42A2 and PAO1 strains were used as positive control for oleate-diol synthase activity. PAO1 insertion mutants were obtained from the transposon mutant library at the University of Washington (UWGS). The location and presence of the transposon were confirmed following the UWGS recommendations [26]. *Escherichia coli* DH5α was the host for plasmid constructions, and strain S17.1 was used for conjugation. All strains were routinely grown in TSB (17 g casein peptone, 3 g soymeal peptone, 2.5 g glucose, 5 g NaCl, and 2.5 g KH_2PO_4) at 37 °C on a rotary shaker operated at 200 rpm. Antibiotics were added when required at the following concentrations: for *E. coli*, ampicillin 100 μg ml^{-1} and chloramphenicol 50 μg ml^{-1}; for *P. aeruginosa*, chloramphenicol 200 μg ml^{-1} and carbenicillin 600 μg ml−1; and for *P. aeruginosa* transposon insertion mutants, tetracycline 5 μg ml^{-1} was added for propagation.

Table 1
Pseudomonas strains and transposon insertion mutants used in this work.

Strains[a]	Relevant characteristics — selection criteria
PAO1	Reference strain. Positive control for oleate-diol synthase activity
PW1950	Tet[r], PA0518-B04::ISphoA/hah — *i* ^ *ii* ^ *iii*
PW3110	Tet[r], PA1169-G08::ISlacZ/hah (Δ*Pa_LOX*) — *ii* ^ *iii* ^ *iv*
PW3795	Tet[r], PA1552-D11::ISlacZ/hah — *i* ^ *ii* ^ *iv*
PW3810	Tet[r], PA1560-E08::ISphoA/hah — *i* ^ *iv*
PW4578	Tet[r], PA2077-F05::ISlacZ/hah (ΔPA2077) — *i* ^ *iii* ^ *iv*
PW4581	Tet[r], PA2078-C02::ISlacZ/hah (ΔPA2078) — *i* ^ *iii* ^ *iv*
PW5155	Tet[r], PA2475-F12::ISlacZ/hah ($ΔP450_1$) — *ii* ^ *iv*
PW5168	Tet[r], PA2482-A04::ISlacZ/hah — *ii* ^ *iii* ^ *iv*
PW6607	Tet[r], PA3331-A10::ISlacZ/hah ($ΔP450_2$) — *ii* ^ *iv*
PW7224	Tet[r], PA3679- G11::ISphoA/hah — *ii* ^ *iv*
PW8191	Tet[r], PA4236-B07::ISphoA/hah — *ii* ^ *iii* ^*iv*
PW8519	Tet[r], PA4468-G12::ISlacZ/hah — *ii* ^ *iii*
PW8526	Tet[r], PA4471-H03::ISphoA/hah — *i* ^ *ii*
PW8693	Tet[r], PA4571-A06::ISphoA/hah — *i* ^ *ii* ^ *iii*
PW8725	Tet[r], PA4587-H04::ISphoA/hah — *i* ^ *ii* ^ *iii* ^ *iv*
Strains[a]	**Relevant characteristics — *Mutated Transcriptional Regulator***
PW2980	Tet[r], PA1097-C04::ISphoA/hah — *fleQ*
PW3598	Tet[r], PA1430-C01::ISlacZ/hah – *lasR*
PW4577	Tet[r], PA2076-D01::ISphoA/hah — *lysR-type*
PW6882	Tet[r], PA3477-B10::ISlacZ/hah — *rhlR*
PW7549	Tet[r], PA3879-A02::ISlacZ/hah — *narL*

[a] Strains obtained from the *Pseudomonas* transposon mutant collection, University of Washington Genome Centre (UWGC) [25]. *P. aeruginosa* PAO1 [27] was used as positive control for oleate-diol synthase activity. Antibiotic resistance is indicated: Tet[r], tetracycline resistance.

2.3. DNA isolation and manipulation

Genomic *P. aeruginosa* PAO1 and 42A2 DNA was isolated using Illustra-Prep bacteria genomic Prep Mini Spin Kit (GE Healthcare). Plasmid and PCR-amplified DNA (primers at Supplementary material, Table S2) were isolated using High Pure Plasmid Isolation Kit (Roche) and NucleoSpin and PCR Clean-up columns (Macherey-Nagel). DNA restriction, polymerization and modification enzymes were used according to the manufacturer's instructions (Roche, Spain). DNA fragments were analyzed on 0.8% (wt/vol) agarose gels and visualized with Gel Red (Biotium). Nucleotide sequences were obtained using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and a capillary sequencer ABI Prism 3730, available at the Serveis Cientifico-Tècnics of the University of Barcelona.

2.4. Complementation of ΔPA2078 and ΔPA2077

P. aeruginosa PAO1 mutant strains obtained from UWGC were complemented in *cis* and *trans* by one or both native genes. Genes *PA2077* and *PA2078* were individually amplified from PAO1 genomic DNA using specific primers (Supplementary material, Table S2). Recombination of ΔPA2078 was performed by conjugation with native *PA2078* cloned in pEX100Tlink (pEX-78), as previously described [28]. For *trans*-complementation, the amplified genes were cloned into pMMB207 under P*tac* promoter (Supplementary material, Table S1). The same protocol was used for amplification and cloning of the whole hypothetical operon *PA2078–PA2077* preceded or not by two different-length DNA fragments corresponding to upstream regions of *PA2078* including (DS1) or excluding (DS2) the whole intergenic *PA2079–PA2078* segment in pBBRMCS1 without transcriptional control of P*lac* promoter. Native RBSs were used for cloning each gene under the plasmids' promoter (Table 2; Table S1). All recombinant constructions were used to electroporate the mutants to evaluate their ability to restore the wild-type phenotype, or to transform *E. coli* DH5α, used as heterologous host.

2.5. RNA isolation and reverse transcription (RT-PCR) analysis

Total RNA from *P. aeruginosa* PAO1, ΔPA2077 and ΔPA2078 was isolated from cultures grown at mid exponential phase using High Pure RNA isolation kit (Roche, Spain). RNA integrity and concentration were verified and treated with DNase I using TURBO DNA Free™ Kit (Ambion). A 10 min heat shock at 95 °C and rapid ice cooling were performed to avoid RNA secondary structure formation. RT-PCR was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche), following the manufacturer's instructions. Specific-sequence primer RT-R was used for priming the RNA (Supplementary material, Table S2). cDNA amplification was performed using primers RT-F and RT-R, designed to obtain a 400 bp sequence including the intergenic region *PA2078–PA2077* (Fig. 2A; Supplementary material, Table S2). DNase I-treated RNA was used as negative control.

2.6. Bioconversion assays

Oleate-diol synthase activity was induced by growing *P. aeruginosa* strains in TSB supplemented with 1% (v/v) OA (18 h, 37 °C; 200 rpm). Crude cell extracts, culture media or whole-cells were obtained as

Table 2
Other relevant strains used in this work.

Strains	Relevant characteristics[a]	Reference
P. aeruginosa 42A2	Wild type. Positive control for oleate-diol synthase activity	[29]
E. coli S17.1	*recA pro* (*RP4-2Tet*:: *Mu Kan*::*Tn7*) Helper strain for conjugation	[28]
E. coli DH5α	*supE44 ΔlacU169* (φ 80 *lacZ*ΔM15) *hsdR1 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
Most significant complementant and recombinant strains		
ΔPA2077pBB	Tet^r, Cm^r ΔPA2077 mutant carrying pBBRMCS1, negative control	This study
ΔPA2078pBB	Tet^r, Cm^r ΔPA2078 mutant carrying pBBRMCS1, negative control	This study
ΔPA2078/DS1	Tet^r Cm^r ΔPA2078 mutant carrying pBBR-DS1	This study
ΔPA2077/DS1	Tet^r Cm^r ΔPA2077 mutant carrying pBBR-DS1	This study
ΔPA2078/DS2	Tet^r Cm^r ΔPA2078 mutant carrying pBBR-DS2	This study
ΔPA2077/DS2	Tet^r Cm^r ΔPA2077 mutant carrying pBBR-DS2	This study
ΔPA2077pMM	Tet^r Cm^r ΔPA2078 mutant carrying pMMB207, negative control	This study
ΔPA2078pMM	Tet^r Cm^r ΔPA2078 mutant carrying pMMB207, negative control	This study
ΔPA2077/77	Tet^r Cm^r ΔPA2077 mutant carrying pMMB-77	This study
ΔPA2077/78	Tet^r Cm^r ΔPA2077 mutant carrying pMMB-78	This study
ΔPA2078/77	Tet^r Cm^r ΔPA2078 mutant carrying pMMB-77	This study
ΔPA2078/78	Tet^r Cm^r ΔPA2078 mutant carrying pMMB-78	This study
ΔPA2077/DS	Tet^r Cm^r ΔPA2077 mutant carrying pMMB-DS	This study
ΔPA2078/DS	Tet^r Cm^r ΔPA2077 mutant carrying pMMB-DS	This study
ΔPA2078/pEX78	Tet^r Cb^r ΔPA2078 with pEX-78 integrated in chromosome	This study
Additional complementant strains		
ΔPA2077/Plac77	Tet^r Cm^r ΔPA2077 mutant carrying pBBR-77	This study
ΔPA2077/Plac78	Tet^r Cm^r ΔPA2077 mutant carrying pBBR-78	This study
ΔPA2078/Plac77	Tet^r Cm^r ΔPA2078 mutant carrying pBBR-77	This study
ΔPA2078/Plac78	Tet^r Cm^r ΔPA2078 mutant carrying pBBR-78	This study
ΔPA2078/Placrbs78	Tet^r Cm^r ΔPA2078 mutant carrying pBBR-rbs78	This study

[a] Plasmid construction specifications are available at Table S1. Antibiotic resistances are as follows: Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Cb^r carbenicillin resistance.

described before [24], and assayed as follows: 100 µl samples were tested using exogenous substrates (0.1% (v/v) OA or 10-HPOME) in 0.5 M Tris–HCl pH 7 up to a final volume of 300 µl [23]. Reactions were incubated for 1–2 h at 37 °C with occasional vortexing, and terminated by acidification to pH 2 with 0.5 M HCl. Released products were extracted with ethyl acetate (2:1; v:v) or on a C_{18} cartridge (SepPak/C_{18}, waters), routinely analyzed by TLC (hexane:diethyl ether:acetic acid; 75:15:10), and further identified by LC–MS/MS analysis.

2.7. LC–MS/MS analysis

LC–MS/MS fragmentation was used for the identification of the HFAs produced by oleate-diol synthase activity [6,20]. Reversed phase-HPLC coupled to MS/MS analysis was performed with a surveyor MS pump with electrospray ionization in an ion mass spectrometer (LTQ, ThermoFisher). A column containing octadecyl silica (5 µm, 150 × 2 mm) was used, and samples were eluted with methanol/water/acetic acid, 80/20/0.01, at 0.3 ml/min. The electrospray voltage was set at 4.5 kV and the temperature of the heated capillary was set at 315 °C.

2.8. Protein production

In order to obtain functional enzymes, *P. aeruginosa PA2078* and *PA2077* genes, cloned into pMMB207 with *tac* promoter, were expressed in ΔPA2078 and ΔPA2077 mutants (Supplementary material, Table S1). For enzyme purification, recovered cells from overnight 500 ml cultures of recombinant *E. coli* DH5α/pMMB-77 and DH5α/pMMB-78 (Supplementary material, Table S1) were suspended in 25 ml 0.05 M Tris–HCl (pH 7; 4 °C), and mechanically disrupted through French press (Thermo Spectronic). After ultracentrifugation (10,000 rpm; 20 min, 4 °C), supernatants were collected and 0.5 ml cell extract samples were purified by Q Sepharose High Performance strong anion exchange HiTrap Q HP™ 1 ml (Amersham Biosciences), and eluted with an ascendant NaCl gradient in 0.05 M Tris–HCl (pH 7) at a flow rate of 1 ml min^{-1}, using an ÄKTA™ FPLC™ system (GE Healthcare). 100 µl aliquots of each fraction were assayed for activity using OA and 10*S*-H(P)OME/10-HOME mix as substrates. After 30 min incubation, the released products were analyzed through TLC as described above. OA and 10*S*-H(P)OME were used as control substrates for LC/MS analysis.

2.9. Bioinformatics tools

P. aeruginosa genome annotation [26], Blast [30] and Orthologe [31] databases were used for *PA2077* and *PA2078* homology and orthologs search. Putative promoters were searched using Predictor for Prokaryotic Operons [32]. DNA folding was visualized at Mfold web server [33]. ProdoNet [34] was used to predict and visualize transcription regulators and DNA binding motifs. Vector NTI® Software (Invitrogen) was used for restriction pattern determination and primer design. BioEdit Sequence Alignment Editor v.7.0.1 was used for sequenced fragment analysis [35]. Sequence alignments were obtained using ClustalO Multalign Software [36]. Phylogenetic analysis of *PA2078* and *PA2077* was conducted using MEGA v5.2 software applying Maximum Likelihood (PALM) method (Tamura 3-parameter model). A bootstrap consensus tree was achieved after 1000 repeats, and results were obtained with a cut-off of 50% [37].

3. Results

3.1. P. aeruginosa rational genome screening for oxylipin-producing enzymes

The database www.pseudomonas.com containing the complete *P. aeruginosa* PAO1 genome [27] was used to search for putative oxylipin-related genes coding for lipoxygenases (LOX), heme-containing fatty acid dioxygenases (DOX, COX) or monooxygenases like cytochrome P_{450} (CYP450), the main enzymes responsible for oxygenation of unsaturated fatty acids [10–12]. No apparent homologs to COX, linoleate-diol synthases (DOX), (10*R*)-DOX, or α-DOX were found, whereas two *CYP450*-like genes, *PA2475* ($CYPP450_1$) and *PA3331* ($CYPP450_2$), and a lipoxygenase (LOX) were identified. *P. aeruginosa* 42A2 LOX gene has already been isolated and cloned, and the corresponding protein

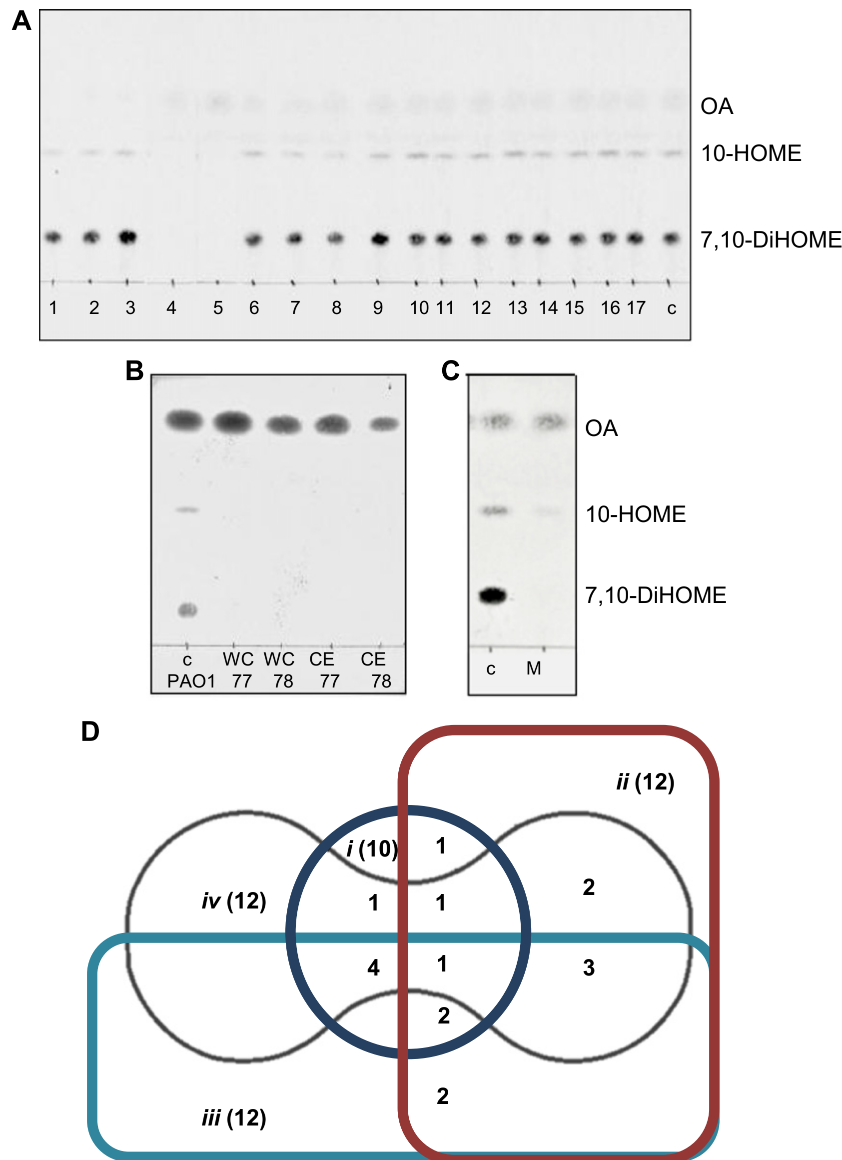


Fig. 1. A) TLC analysis of oxygenated fatty acids accumulated in the extracellular medium from 1% OA overnight-induced *P. aeruginosa* PAO1 transposon mutants. (1) ΔPA1169 (*Pa*_LOX); (2) ΔPA2475 ($P450_1$); (3) ΔPA3331 ($P450_2$); (4) ΔPA2077; (5) ΔPA2078; (6–17) other selected mutants (Table 1; rest of mutants, ascendant locus tag PA order); (c) wild-type PAO1 used as positive control. B) TLC analysis of the oxygenated fatty acids produced during in vitro OA bioconversion by whole cells (WC77–WC78) and cell extracts (CE77–CE78) from *P. aeruginosa* PAO1 mutants ΔPA2077 (WC77–CE77) and ΔPA2078 (WC78–CE78) with 0.1% OA-buffered solution; (c) PAO1 wild type whole cell bioconversion used as positive control. C) TLC analysis of the in vitro bioconversion of 0.1% OA by a mixture (1:1) of cell extracts from mutants ΔPA2077 and ΔPA2078 (M); (c) cell extract from wild type PAO1 was used as positive control. D) *P. aeruginosa* PAO1 mutant selection criteria (see text) represented in an Edward-Venn's diagram.

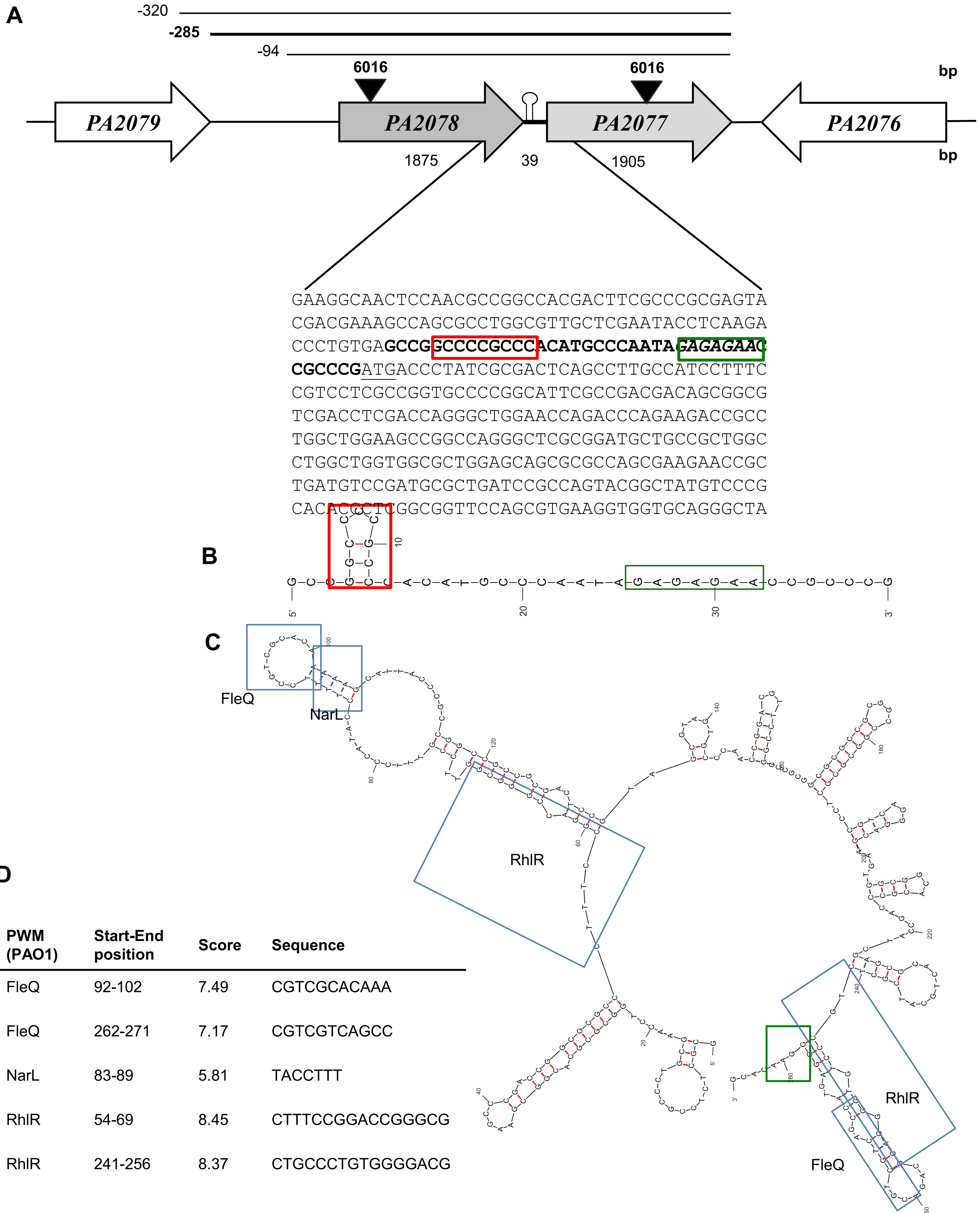
crystallized [38,39]. It shares 98.8% similarity with ORF PA1169 (*Pa_LOX*) from PAO1, involved in arachidonic and linoleic acid bioconversion [40]. Transposon insertion mutants of *PA1169* (*Pa*_LOX), *PA2475* and *PA3331* (CYP450) genes (Table 1) were tested through TLC for oxylipin accumulation in culture supernatants, but caused no reduction in oleate-diol synthase activity (Fig. 1A; lanes 1, 2, 3, respectively). Therefore, none of these enzymes is involved in OA conversion into 10*S*-H(P)OME and 7,10-DiHOME.

3.2. Search for new oleate-diol synthase candidate genes in P. aeruginosa PAO1

As previously reported, *P. aeruginosa* oleate-diol synthase activity occurs in the periplasm [24], whose sub-proteome has been extensively studied [41]. Results from a comparative transcriptomic analysis performed after OA induction, were used here for identification of the upregulated periplasmic proteins [42,43]. Therefore, a new collection of selected insertion mutants (Table 1) was analyzed by TLC for HFA accumulation in supernatants (Fig. 1A). The mutants tested were selected according to the following criteria: i) genes being over expressed during OA induction, ii) genes coding for enzymes that could be involved in oxygenation of LCFAs like copper/heme-containing proteins and other oxidoreductases [10], iii) genes with probable periplasmic location, or iv) proteins with a predicted protein size of 30–70 kDa [23]. Those genes exhibiting two or more of these criteria simultaneously are represented in a set intersection Edward-Venn's diagram in Fig. 1D.

Among all mutants tested (Table 1; Fig. 1A), only those corresponding to ORFs PA2077 (PA2077-F05::IS*lacZ*/hah; ΔPA2077) and PA2078 (PA2078-C02::IS*lacZ*/hah; ΔPA2078) were unable to accumulate HFAs in the extracellular medium of cells incubated with OA, suggesting a direct or indirect implication of these genes in oleate-diol synthase activity (Fig. 1A; lanes 4, 5). However, being a periplasmic activity, the absence of HFAs in culture supernatants might be due either to a

synthesis failure or to a lack of transport to the extracellular medium (Table 1; Fig. 1A), as occurred in mutant ΔExFadLO (ORF PA1288), recently shown to be involved in HFAs' export [24]. TLC analysis performed using whole cells and crude cell extracts of wild-type and mutants ΔPA2077 and ΔPA2078 incubated with OA (Fig. 1B) allowed the confirmation that no HFAs were produced by the two mutant strains



PWM (PAO1)	Start-End position	Score	Sequence
FleQ	92-102	7.49	CGTCGCACAAA
FleQ	262-271	7.17	CGTCGTCAGCC
NarL	83-89	5.81	TACCTTT
RhlR	54-69	8.45	CTTTCCGGACCGGGCG
RhlR	241-256	8.37	CTGCCCTGTGGGGACG

Table 3
Complementation phenotype of oleate-diol synthase mutants.

Strain[a]	Oleate-DS genes expressed	OA bioconversion to 10-H(P)OME/7,10-DiHOME[c]	Bioconversion of 10-H(P)OME/10-HOME mix to 7,10-DiHOME
PAO1	*PA2077/PA2078*	+/+	—[d]
ΔPA2077	PA2078	—	—[d]
ΔPA2077/77[b]	PA2077/PA2078	+/+	—[d]
ΔPA2077/78[b]	PA2078	—	+
ΔPA2077/DS[b]	PA2077/PA2078	+/+	+
ΔPA2078	—	—	—
ΔPA2078/77[b]	PA2077	+/−	—
ΔPA2078/78[b]	PA2078	—	+
ΔPA2078/DS[b]	PA2077/PA2078	+/D	+

[a] Cell extracts of *P. aeruginosa* PAO1 wild-type and transposon mutant strains (UWGC) without OA induction.
[b] Oleate-diol synthase (DS) tandem genes (*PA2078–PA2077*) cloned into multicopy plasmid pMMB207.
[c] Complete DS phenotype restoration is shown as +/+ for 10-H(P)OME/7,10-DiHOME production, respectively.
[d] Samples where gene *PA2078* is not expressed due to the lack of OA induction. D, activity detected in lower amount compared to wild-type phenotype under the same conditions.

(Fig. 1B), indicating that both ORFs are indeed involved in HFAs' formation.

3.3. Genomic organization of ORFs PA2077 and PA2078

The analysis of *P. aeruginosa* PAO1 genome revealed that ORFs PA2077 and PA2078 are annotated as 'hypothetical proteins'. The two genes are tandemly positioned within the chromosome, separated by an intergenic region of 39 bp (Fig. 2A, B), being transcribed from the minus strand, as does the flanking ORF PA2079 coding for a probable amino acid permease. In the plus strand, adjacent ORF PA2076 is annotated as a probable LysR-family transcriptional regulator, showing 44% similarity to *Pseudomonas putida* LysR (Fig. 2A).

The fact that two independent insertion mutants (ΔPA2077 and ΔPA2078), located in tandem within the genome, cause the loss of oleate-diol synthase activity suggests that both gene products are required for activity. To evaluate this possibility, a test was carried out using a mixture (1:1) of crude cell extracts from the two mutant strains. As shown in Fig. 1C, oleate-diol synthase activity was not restored, indicating that activity recovery is not achieved by independently expressed genes. Moreover, the tandem *PA2078–PA2077* (Fig. 2A) occurs in all known *P. aeruginosa* genomes, where these genes appear in the same order as a conserved set [27] with no potential promoter sites found in silico within the intergenic region (Fig. 1C) resembling a homology-deduced operon [44].

3.4. Expression in trans of the tandem PA2078–PA2077

We attempted to reproduce expression in *trans* of putative *PA2078–PA2077* operon under the native promoter in each mutant. For such purpose, two different-length DNA fragments corresponding to upstream regions of gene *PA2078* that included (pBBR-DS1) or excluded (pBBR-DS2) the whole 5′-untranslated region upstream *PA2078* were ligated to pBBRMCS1 (Table S1; Fig. 2A), electroporated into both mutants, and tested for OA bioconversion. However, no oleate-diol synthase activity was found (data not shown), suggesting an improper plasmid-driven expression of putative operon *PA2078–PA2077* due to the complex topology of predicted secondary structures [33] in this DNA region (Fig. 2C).

3.5. Screening for putative transcriptional regulators

In silico analysis of the most probable folded DNA structures predicted for this region revealed the presence of specific transcription factor-binding sites and possible metabolite-binding riboswitches at the intergenic sequences (Fig. 2B, C). To find out if such regulators could have a role in controlling diol synthase activity, a new set of transposon mutant strains of predicted transcription regulators (Table 1; Fig. 2D) was assayed for HFAs' production including the LysR-type transcriptional regulator (ORF PA2076) flanking ORF PA2077. Again, no activity depletion occurred (data not shown), indicating that such general transcription factors are not involved in the regulation of *PA2078–PA2077* in oleic acid-induced cells.

3.6. Unveiling the role of PA2078 and PA2077 genes

To test the operon nature of genes *PA2078–PA2077*, two hypotheses involving dependently expressed genes were approached: i) mono-enzymatic activity, where PA2077 would be responsible for the entire activity and PA2078 would act as a regulator, being transcribed initially, or ii) multi-enzymatic activity where PA2077 and PA2078 proteins act independently or as a complex. In order to unveil the role of each ORF in this metabolic pathway, activity recovery assays were performed through i) wild-type gene restoration in ΔPA2078 mutant, and ii) *trans*-complementation of each mutated gene under the control of *tac* promoter.

Cis-complementation was successfully achieved when *P. aeruginosa* ΔPA2078 mutant was reverted to wild-type genotype (not shown) by the replacement of the mutated *PA2078* gene by recombination and allelic exchange [28]. This confirmed the deleterious transposon-insertion effect over oleate-diol synthase activity in ΔPA2078. On the other hand, complementation in *trans* of mutant ΔPA2077 with gene *PA2077* produced the same pattern of HFAs as that of wild-type strain, with full oleate-diol synthase activity restoration (Table 3). Interestingly, when mutant ΔPA2078 was complemented in *trans* with gene *PA2077*, only

Fig. 2. A) Genomic environment and translation direction of ORFs PA2078 and PA2077, where the flanking ORFs PA2079 and PA2076 are also shown. Transposon insertion locations in ORFs PA2078 and PA2077 are represented as black triangles. A stem-loop covering the intergenic region between *PA2078* and *PA2077* is depicted. Straight lines above the genes indicate the length of the DNA fragments containing different native promoter regions used for cloning the complete region in *trans*-complementation assays (−320, −94), with the native intergenic region between *PA2079* and *PA2078* (−285) in bold-faced. The sequence box reveals the 400 bp DNA fragment expected from RT-PCR derived from a bicistronic mRNA including fragments from *PA2078* and *PA2077* plus their intergenic region (in bold); RBS (italics) is shown as a green box, and the ATG start codon of gene *PA2077* is underlined. B) Predicted secondary structure of the intergenic region *PA2078–PA2077* ($\Delta G = -1.23$ kcal·mol^{-1}) showing a putative loop that could serve as a Rho-independent transcription terminator to modulate the expression of *PA2077*; RBS is highlighted in a green box. C) Predicted structure of the intergenic region *PA2079–PA2078* showing putative stable secondary structures in the 5′-untranslated regions (5′-UTR) of *PA2078* ($\Delta G = -64.36$ kcal·mol^{-1}); RBS for *PA2078* is shown in a green box, and the predicted transcription factor (TF)-binding sites found at the 5′UTR *PA2078* sequence are enclosed in blue boxes. Red and blue lines in sequences indicate G–C and A–T complementary hydrogen bonds, respectively. D) Table of the predicted transcription factors and riboregulatory elements tested.

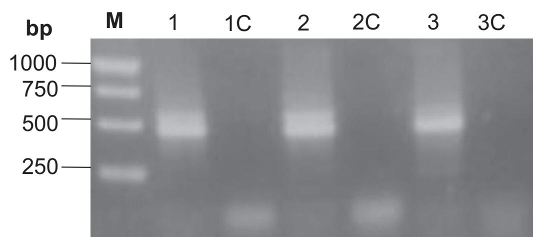


Fig. 3. Agarose gel electrophoresis of RT-PCR amplified DNA products of the diol synthase operon, obtained using primers covering a 400 bp DNA fragment, including the *PA2078*–*PA2077* intergenic region (see Fig. 2A, B). The same amplification pattern derived from total RNA of *P. aeruginosa* PAO1 (1) and mutants ΔPA2078 (2) and ΔPA2077 (3) appeared. Negative control reactions performed without RT to test the purity of PAO1, ΔPA2077 and ΔPA2078 RNA are shown in lanes 1C, 2C and 3C, respectively. (M) DNA ladder.

10-H(P)OME and 10-HOME were accumulated in culture supernatants (Table 3). It must be quoted that 10-HOME is a stable dead-end product of spontaneous reduction of 10*S*-H(P)OME to the alcohol [21,22], thus justifying the presence of 10-HOME in most analyzed samples.

The results obtained so far suggest that transposon insertion in *PA2078* causes a polar effect, abolishing the expression of *PA2077*. Moreover, they prove that PA2077 is responsible for the 10*S*-dioxygenation (10*S*-DOX) of OA, the first step of the pathway [23]. Since no oxygenated OA products were released upon *trans* complementation of both ΔPA2078 and ΔPA2077 mutants with *PA2078*, it can be concluded that PA2078 is not involved in the first step of oleate-diol synthase activity (Table 3).

Experiments aimed at deciphering the role of PA2078 showed that hydroperoxide 10-H(P)OME, resulting from bioconversion of OA by supernatant samples of mutant ΔPA2078 complemented in *trans* with *PA2077*, could be converted into 7,10-DiHOME when mixed with crude cell extracts of both mutants complemented in *trans* with gene *PA2078* (Table 3). However, no HFAs were released in the absence of PA2077 when OA was used as a substrate, even if *PA2078* was being expressed from the plasmid. These results indicate that 10-H(P)OME but not OA is a substrate for PA2078 and unambiguously demonstrate that PA2078 is responsible for the synthesis of the diol product (7,10-DS), converting 10*S*-H(P)OME into 7,10-DiHOME, the second step reported for *P. aeruginosa* 42A2 oleate-diol synthase activity [23].

Additional assays performed by complementation in *trans* of mutant ΔPA2077 with the putative operon *PA2078*–*PA2077* expressed under P*tac* promoter caused wild-type phenotype restoration, producing a complete pattern of HFAs from OA (not shown). However, when ΔPA2078 was complemented and tested in the same conditions, lower bioconversion rates compared to wild-type phenotype were observed (Table 3).

3.7. Oleate-diol synthase genes constitute indeed a bicistronic operon

To find out if genes *PA2078* and *PA2077* are co-transcribed, total RNA from strain PAO1 and the two mutants was extracted and RT-PCR was performed using specific primers (Supplementary material, Table S2) including the intergenic region (Fig. 2A). An expected 400-bp DNA product was obtained for all strains (Fig. 3), while no product appeared in amplification reactions carried out without RT, used as negative control (Fig. 3). These results confirm that a bicistronic mRNA is produced, and indicate that *PA2078*–*PA2077* genes are linked in a common transcript.

The observation that both genes are preceded by a ribosome-binding site (RBS) prompted us to study their functionality. Both RBSs appeared fully functional, even when the RBS from *PA2077* (located at the intergenic region; Fig. 2A, C) was artificially placed upstream *PA2078* (Table 2; primer RBS77-PA2078F 5′, Supplementary material, Table S2). Functional PA2078 was produced, showing the same activity as that of clones with native PA2078 RBS (Table 2). This novel oleate-diol synthase operon structure has never been reported before.

3.8. No hetero-complex formation is required for oleate-diol synthase activity

E. coli was used as oleate-diol synthase negative host to confirm the functional role of each gene product separately. Recombinant clones of *E. coli* expressing *PA2077* or *PA2078* cloned in pMMB207 were obtained and used for purification and bioconversion assays. In agreement with the previous observations [23], both enzymes eluted under similar conditions on ion-exchange chromatography. Purified enzyme fractions were assayed for OA or 10-H(P)OME bioconversion, and the released products were analyzed through TLC (Fig. 4A) and LC/MS–MS (Fig. 4B, C, D, E). The results obtained indicate that no hetero-complex formation is required for OA or 10-H(P)OME conversion. Moreover, the monomeric nature of both enzymes is assumed, as molecular exclusion chromatography rendered a single active fraction. Identical LC/MS–MS results were obtained when cell extracts of recombinant *E. coli* carrying non-inducible pGEMT-77 (Supplementary material, Fig. S1A, B) and pGEMT-78 (Supplementary material, Fig. S2A, B) were tested for bioconversion, indicating that minimal amounts of functional proteins allow the identification of PA2077 and PA2078 products.

3.9. Diol synthase-coding genes could have been originated by gene duplication followed by neofunctionalization

PA2078 and *PA2077* adjacent genes (Fig. 2A) share a significant 67.57% nucleotide sequence identity. However, this nucleotide homology is lower than that shown by *PA2078* and *PA2077* orthologs in other *P. aeruginosa* genomes (>98%). These properties suggest that the oleate-diol synthase conserved set could be the result of a gene duplication event inherited from a common ancestor, maintaining their adjacent location (Fig. 5). Duplications usually create redundancy (paralogs) derived either from an ancestral duplication with no orthologous relationships (outparalogs), or from a lineage-specific duplication, giving rise to co-orthologous relationships (inparalogs) [45]. As shown in Fig. 5, *PA2078* and *PA2077* paralogs derive from a lineage-specific duplication, being thus considered inparalogs. After a duplication event individual insertional mutations can have no effects on the phenotype if the duplicated genes are involved in identical function; alternatively, differential phenotypes will appear if one of the genes had evolved to adopt a new function [46]. In this particular case, two complementation phenotypes for *PA2077* and *PA2078* were found in the biochemical assays performed above, confirming the neofunctionalization hypothesis.

The search for orthologs of *PA2078* and *PA2077* revealed 19 and 5 putative orthologs, respectively, mostly annotated as 'hypothetical, uncharacterized proteins' [27]. Among these orthologs, inparalogs with similar relation as that shown by the couple *PA2078*–*PA2077* have also been predicted in *Azoarcus* sp. BH72 (*azo2594*–*azo2595*) and *Thauera* sp. MZ1T (*Tmz1t2187*–*Tmz1t2188*) (Fig. 5). However, no orthologs of the tandem *PA2078*–*PA2077* could be identified (Fig. 5) among sequenced bacterial genomes or even among other species of *Pseudomonas* [27]. This was experimentally confirmed here by the lack of OA bioconversion and absence of PCR amplification of these genes from other *Pseudomonas* species like *P. putida*, *Burkholderia cenocepacia* or *Pseudomonas fluorescens* (not shown). On the other hand, as strains 42A2 and PAO1 share the same catalytic mechanism of oleate-diol synthase, we amplified and sequenced the whole diol synthase operon of *P. aeruginosa* 42A2, which showed a 98% sequence identity and the same configuration as that of *P. aeruginosa* PAO1. The sequences obtained were submitted to NCBI database under accession number #GenBank KJ372239. Considering the high identity shown by PAO1 and 42A2 strains, we assume that those orthologs annotated in other *P. aeruginosa* genomes will also be involved in the same function.

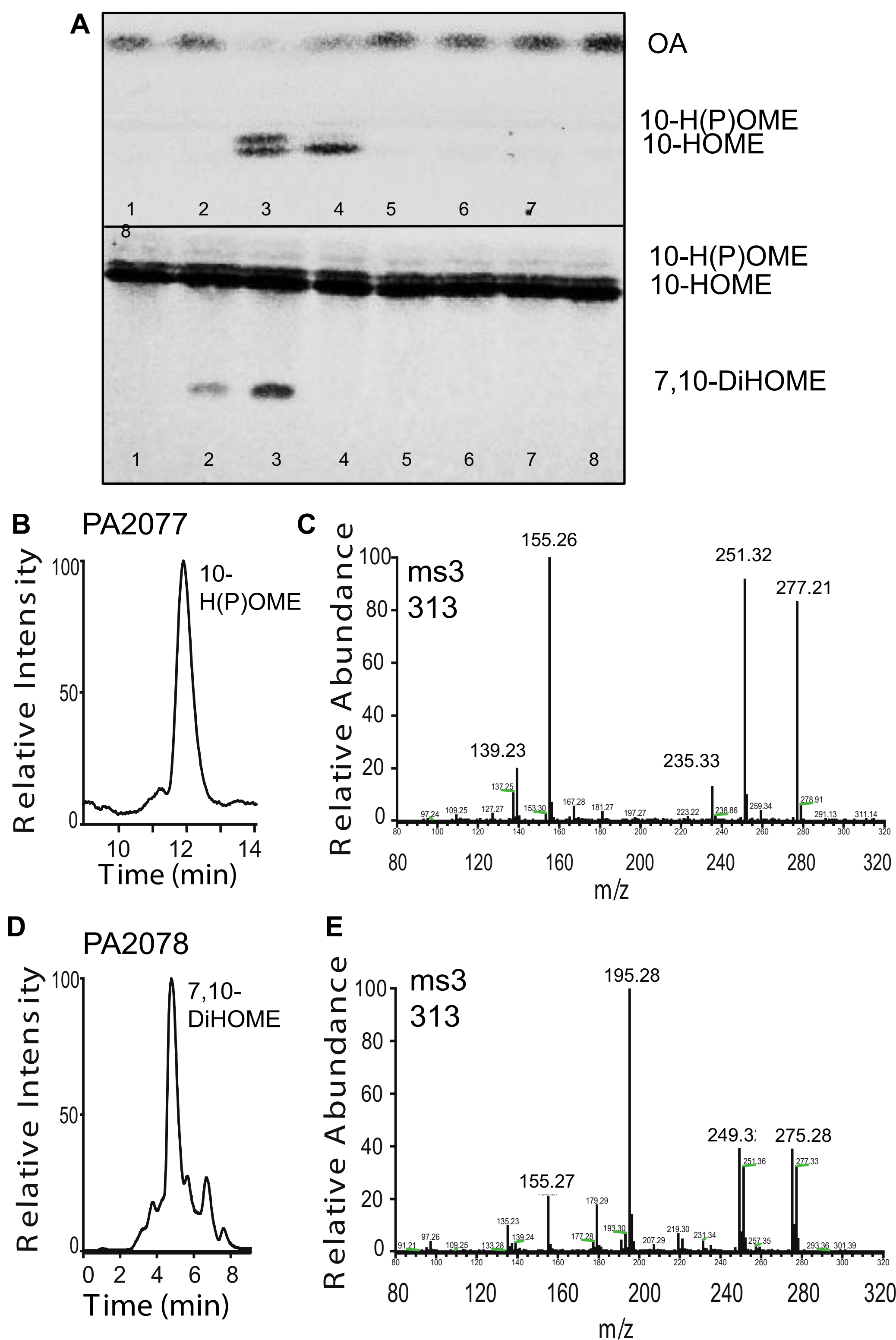


Fig. 4. A) TLC assay for in vitro monitoring the activity of eluted samples resulting from a Hitrap HP Q (anionic exchange) chromatography of recombinant *E. coli* DH5α/pMMB-77 (upper TLC) and DH5α/pMMB-78 (bottom TLC). Bioconversion of OA by *E. coli* DH5α/pMMB-77 to 10-H(P)OME was found in fractions 3–4 (maximum activity in sample 3), whereas bioconversion of 10-H(P)OME/10-HOME mix by *E. coli*/pMMB-78 to 7,10-DiHOME was mostly found in fractions 2–3 (maximum activity in sample 3). B) LC showing the elution of 10-H(P)OME resulting from bioconversion of OA by *E. coli* DH5α/pMMB-77 semi-purified sample. C) Identification of 10-H(P)OME after analysis of the MS^3 spectrum (m/z 313 full scan → 295), producing signals at m/z 277, 251, 155 and 139. D) LC showing the result of bioconversion of purified (10*S*)-H(P)OME by *E. coli* DH5α/pMMB-78 semi-purified sample. E) MS^3 spectrum of 7,10-DiHOME (m/z 313 full scan → 295) leaving predominant signals at m/z 251, 195 and 155. Both spectrographic analyses are equal to the data obtained previously for these compounds [20] (m/z data have been amplified for visualization).

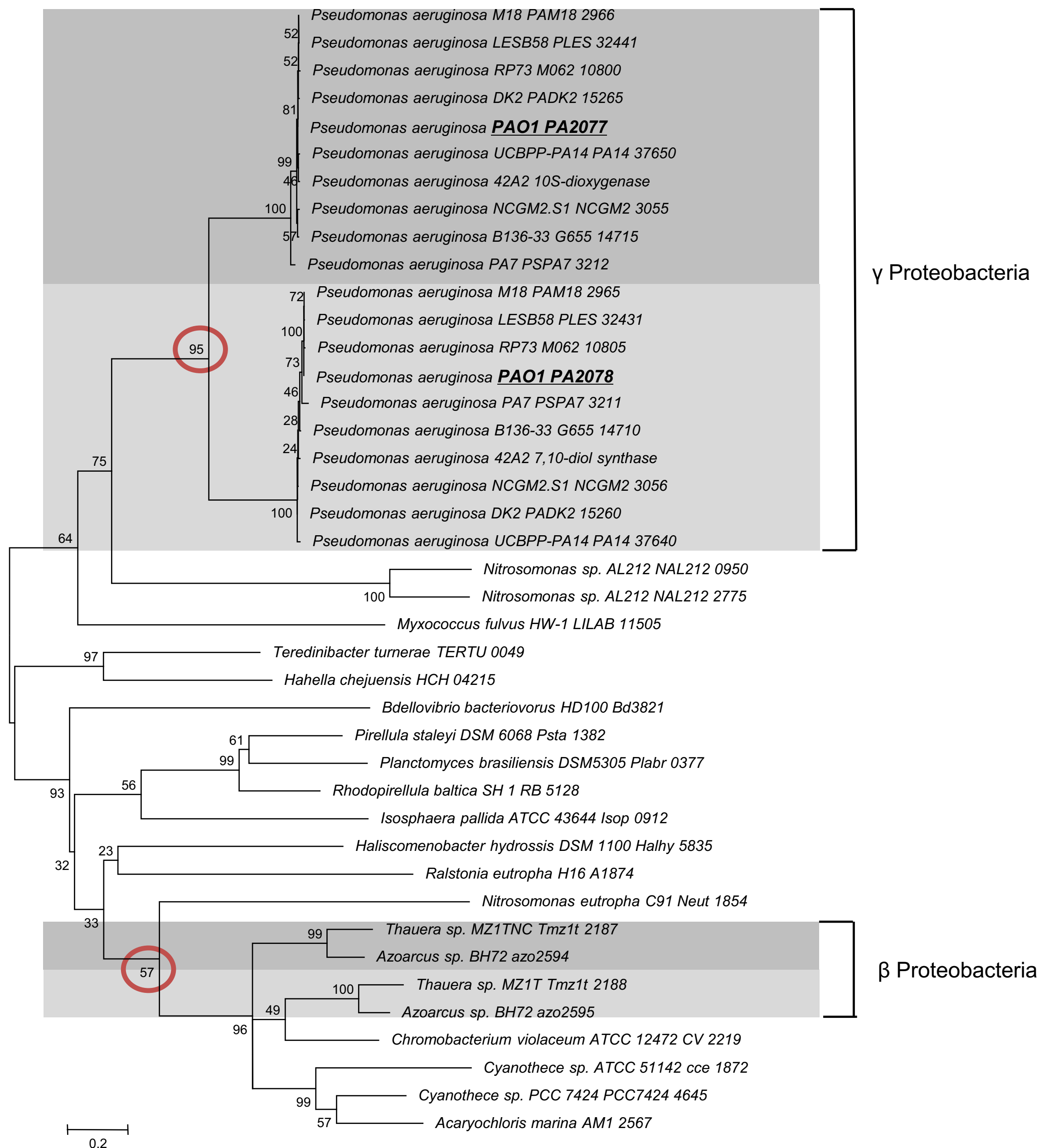


Fig. 5. Phylogenetic tree of orthologous diol synthase-coding genes found so far in the databases. Nodes (red circles) represent a duplication event that took place after speciation. Genes showing a tandem-duplicated inparalog relation (gray boxes) are exclusively found in γ-proteobacteria and β-proteobacteria. Paralog genes display more than 40% amino acid identity, with 60% length coverage. The most conserved gene set group corresponds to different strains of *P. aeruginosa* (strain and chromosomal location): PAM18 (*2966–2965*); PLES (*32441–32431*); RP73 M062 (*10800–10805*); PADK2 (*15265–15260*); PAO1 (*PA2077–PA2078*); UCBPP-PA14 PA14 (*37650–37640*); 42A2 (*10S-DOX–(7S,10S)-DS*); NCGM2.S1 (*3055–3066*); B135-33G655 (*14715–14710*), and PSPA7 (*3212–3211*).

3.10. Oleate-diol synthase metabolic pathway redefined

As shown here, ORFs PA2077 and PA2078 code for the two enzymes that are responsible for *P. aeruginosa* PAO1 oleic acid 10*S*-dioxygenation and 10*S*-H(P)OME conversion into 7,10-DiHOME. A working model of such an activity and the related transporter protein is presented in Fig. 6, where the nature of oleate-diol synthase activity is depicted in the cell context, including product formation and circulation in the bacterial periplasm and extracellular medium [23,24].

4. Discussion

A rational genome mining approach allowed the identification of two hypothetical proteins (ORFs PA2078 and PA2077) of *P. aeruginosa* causing conversion of oleic acid into 7,10-DiHOME, a product described for the first time twenty-five years ago in *P. aeruginosa* 42A2 [29]. Although the enzymatic mechanism of OA conversion into 7,10-diHOME was recently described [23] the genetic elements coding for this activity were still unknown.

Following a genome-based rational protocol, we performed a screening of genes coding for the main enzymes causing oxygenation of unsaturated fatty acids in eukaryotes [10–12], frequently described as bifunctional diol synthases encoded by a single gene [16] but never reported for prokaryotes. We demonstrate here that neither DOX, LOX, COX nor CYP450 is involved in *P. aeruginosa* oleate-diol synthase activity. These results are not in conflict with the previous assays showing that polyunsaturated fatty acids are the prokaryotic LOX major substrates [38], and support the previous evidence in which no

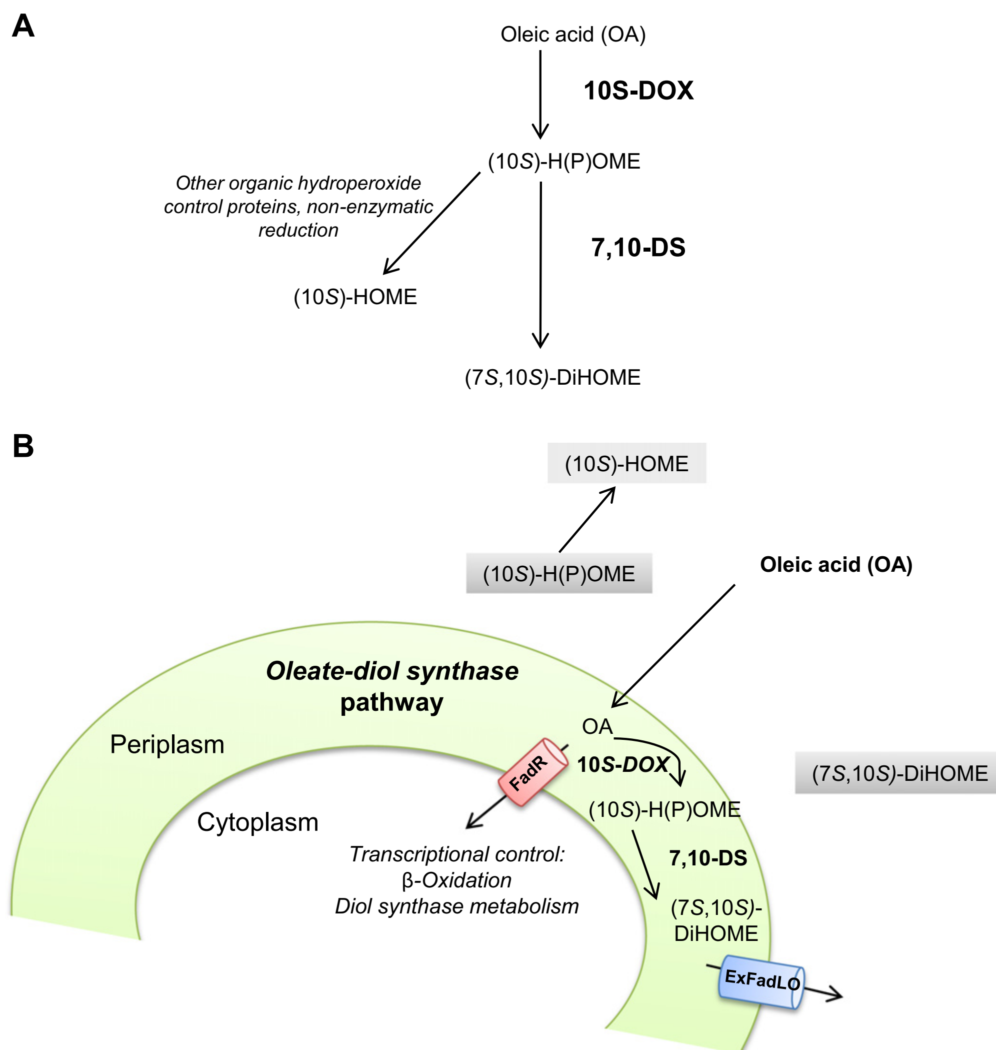


Fig. 6. A) New definition of oleate-diol synthase metabolic pathway in *P. aeruginosa* based on our current knowledge and previous results [23,24]. The genes coding for the enzymes involved in diol synthase activity have been identified and the two enzymes responsible have been functionally identified here, allowing the description of the corresponding metabolic pathway. B) In a fatty acid enriched environment, exogenous free fatty acids such as OA are available for *P. aeruginosa*. Exogenous OA overpass bacterial OM and locates in both the cytosol and periplasm. OA would induce transcription of fatty acid metabolism genes, where the diol synthase operon described here is upregulated, and 10*S*-DOX (PA2077) and (7*S*,10*S*)-DS (PA2078) enzymes are expressed. These proteins are transported to the periplasm, where they metabolize OA into 10-H(P)OME and sequentially to 7,10-DiHOME by 10*S*-DOX and (7*S*,10*S*)-DS enzymatic activities. The resulting oxygenated fatty acids are exported by ExFadLO OM-transporter, being possibly involved in environmental adaptation.

diol synthase inhibition by carbon monoxide and other specific P_{450} inhibitors was found [23]. Therefore, attempts to solve the function and evolution of the genetic elements coding for such unprecedented prokaryotic activity were done here. For this purpose, selected *P. aeruginosa* PAO1 mutants [25] were analyzed for OA bioconversion. Two genes (*PA2077* and *PA2078*), contiguously located in *P. aeruginosa* genome, were identified and shown to be responsible for this diol synthase activity. After gene amplification and cloning, recombination and complementation assays unambiguously showed that OA bioconversion is initiated by (10*S*)-dioxygenase (10*S*-DOX) PA2077 to produce hydroperoxide 10-H(P)OME, which is then used as a substrate by 7,10-hydroperoxide-diol synthase (7,10-DS) PA2078 to release the dihydroxylated compound 7,10-DiHOME (Fig. 6).

Taking into consideration that in bacteria genes involved in the same biochemical pathways are commonly located in clusters expressed under the same signals, we have demonstrated here that *PA2078*–*PA2077* genes constitute a new operon that generates a bicistronic mRNA where *PA2078* and *PA2077* are co-transcribed. In fact, the expression of the two-gene set in *trans* could completely restore the specific activity blocked in each individual mutant. The lower bioconversion shown by mutant ΔPA2078 complemented with the two genes (Table 3, D) could be due either to altered bioconversion kinetics caused by an equal gene dose of *PA2078* and *PA2077* in the plasmid-based expression system, or it could be the result of different catalytic constants for each enzyme separately, in agreement with the previous observations showing that 10-H(P)OME was transformed to the 7,10-diol less efficiently than its precursor OA [23]. In addition, 10*S*-H(P)OME is rapidly reduced to 10-HOME, thus limiting the production of 7,10-DiHOME. Therefore, we discard any possible effect of a putative internal promoter upstream *PA2077*. This hypothesis was supported by the finding of two functional RBSs, which would allow for differential translation rates of the two genes.

We also explored the transcriptional regulators that might affect oleate-diol synthase activity, and determined that general transcription factors involved in flagellar gene regulation, mucin adhesion, quorum-sensing or nitrate-response [34] are not essential for regulation of OA-induced oleate-diol synthase. These results indicate that transcription co-occurs as a result of a finely tuned regulation process that remains unknown. On the other hand, the previous results revealed different expression rates (2:1) for ORFs PA2078:PA2077 [43]. This expression discoordination would be justified by the in silico finding of a palindromic hairpin structure at the intergenic region *PA2078*–*PA2077*

(Fig. 2B), which would act as a *rho*-independent terminator producing transcriptional attenuation of the downstream gene *PA2077* (Fig. 2B).

According to phylogenetic studies (Fig. 5), the same gene order has been conserved among all known *P. aeruginosa* genomes, reflecting the probable gene disposition in a common ancestor, with an inparalog relation [45] of a duplication event leading to neofunctionalization of one of the genes [26]. Additionally, the finding that the set *PA2077*–*PA2078* is conserved only in *P. aeruginosa* and not in other *Pseudomonas* species confirms that the encoded oleate-diol synthase activity is indeed species-specific of this versatile pathogen, where gene duplications are usually related to unessential but useful functions for environmental adaptation [1]. The identification, characterization and regulation of these genes are interesting by themselves and can enable huge potential and impact in green chemistry and microbial biotechnology.

Abbreviations

OA oleic acid
LCFAs long-chain fatty acids
HFA hydroxylated fatty acids;
10-H(P)OME 10*S*-hydro(per)oxide-octadecenoic acid
10-HOME (10*S*)-hydroxy-(8*E*)-octadecenoic acid
7,10-DiHOME (7*S*,10*S*)-dihydroxy-(8*E*)-octadecenoic acid
OM outer membrane
LOX lipoxygenase
COX cyclooxygenase
CYP450 cytochrome P_{450}
DS diol synthase
AOS allene oxide synthase
PUFA polyunsaturated fatty acid
TF transcriptional factor
10*S*-DOX 10*S*-dioxygenase
7,10-DS (7*S*,10*S*)-hydroperoxide diol synthase
TLC thin-layer chromatography
HPLC high performance liquid chromatography
MS mass spectrometry
LC liquid chromatography
RP-HPLC reversed phase-HPLC
10*S*-dioxygenase activity (10*S*-DOX) responsible for the first step of the reaction
PA2078 encodes for the (7*S*,10*S*)-hydroperoxide diol synthase enzyme (7,10-DS)

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.06.010.

Competing interests

The author(s) declare that they have no competing interests.

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SUPPLEMENTAL MATERIAL (Estupiñán *et al.*, 2014)

Table S1. Plasmid constructions used in this work.

Plasmids[1]	Relevant characteristics[2]	Reference
pBBR1MCS	Cm^r, *lacPOZ' mob*$^+$, broad-host-range plasmid	(1)
pBBR-DS1	Cm^r, the tandem (*PA2078-2077)* from pGEMT-DS1 in *Sph*I/*Spe*I of pBBR1MCS1 shuttle vector (under native promoter)[3]	This study
pBBR-DS2	Cm^r, the tandem (*PA2078-2077)* from pGEMT-DS2 in *Sph*I/*Spe*I of pBBR1MCS1 shuttle vector (under native promoter)[3]	This study
pBBR-77	Cm^r, *PA2077* from pGEMT-K77B in *Kpn*I/*Bam*HI of pBBR1MCS under *lac* promoter	This study
pBBR-78	Cm^r, *PA2078* from pGEMT-K78B in *Kpn*I/*Bam*HI of pBBR1MCS under *lac* promoter	This study
pBBR-RBS78	Cm^r, native *PA2077* RBS fused to *PA2078* by amplification with megaprimer RBS77-PA2078F from pGEMT-K78B in *KpnI/Bam*HI of pBBR1MCS under *lac* promoter	This study
pMMB207	Cm^r, expression shuttle vector with IPTG-inducible P*tac* promoter	(2)
pMMB-77	Cm^r, *PA2077* from pGEMT-K77B in *KpnI/Bam*HI of pMMB207 under *lac* promoter	This study
pMMB-78	Cm^r, *PA2078* from pGEMT-K78B in *Kpn*I/*Bam*HI of pBBR1MCS under *lac* promoter	This study
pMMB-DS	Cm^r, pMMB207-*(PA2078-2077)*	This study
pGEMT-easy	Amp^r, A/T cloning vector	Invitrogen
pGEMT-K77B	Amp^r, *PA2077* gene amplified (K77-F/B77-R) including native RBS[4]	This study
pGEMT-K78B	Amp^r, *PA2078* gene amplified (K78-F/B78-R) including native RBS[4]	This study
pGEMT-KDSB	Amp^r, *PA2078-PA2077* tandem amplified (K78-F/B77-R) including RBS	This study
pGEMT-78	Amp^r, *PA2078* gene, 1875 bp length (N78-F/78-R)	This study
pGEMT-DS1	Cm^r, with a 4.1 Kbp fragment from tandem *PA2078-PA2077* including 5'UTR region (DS1-F/DS-R)	This study
pGEMT-DS2	Cm^r, harbouring a 3.8 Kbp fragment of tandem *PA2078-PA2077* excluding 5'UTR region (DS2-F/DS-R)	This study
pEX100Tlink	Cb^r, Amp^r, *sacB*+, vector for allelic exchange by homologous recombination	This study
pEX100T-78	Cb^r, Amp^r, *PA2078*from pGEMT-78 in *EcoR*Isites of pEX100Tlink	This study

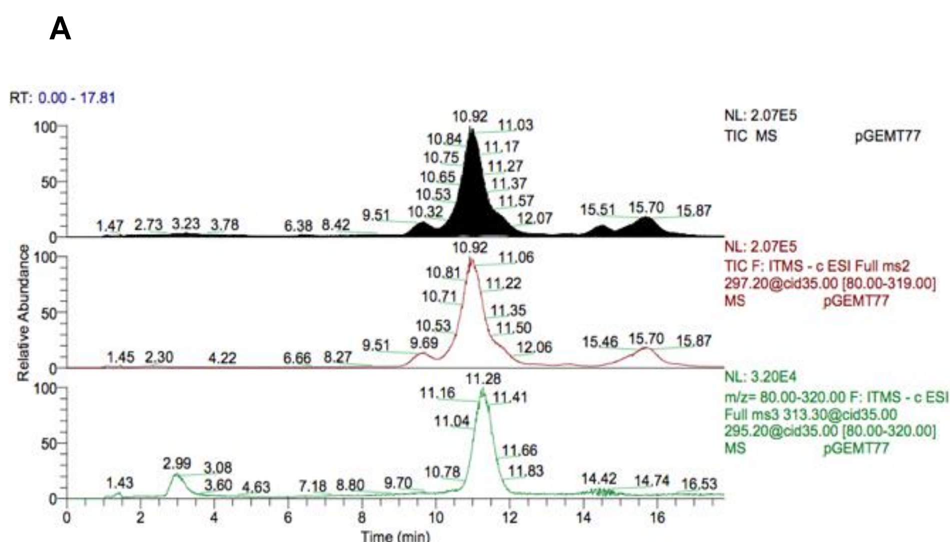
[1] Broad-host-range genetic constructions were transformed in *E. coli* DH5α and cellular extracts of recombinant strains were assayed for OA or 10-H(P)OME bioconversions. [2]Sequences amplified from *P. aeruginosa* PAO1 genomic DNA.[3] Proposed native promoter.[4] Native RBS (ribosome binding site). (1) (Kovach *et al.*, 1994). (2) (Morales *et al.*, 1991). Antibiotic resistances are indicated as follows: Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cb^rcarbenicillin resistance.

Table S2. Primers used in this study.

Primers	Sequence	Restriction sites
N78-F	CAT**ATG**CATCCGACCTTTTCGC	*Nde*I
78-R	AGGATGGCAAGGCTGAGTC	
DS1-F*	CAGGGATTGTCCGATAGAACG	
DS2-F*	TCAGGGACGAAGTGGCGGCAC	
DS-R	CGCCAGCGACACCCAGTTCG	
K77-F*	AGGTACC**CCACATGCCCAA**	*Kpn*I
B77-R*	AGGATCCGACACCCAGTTCG	*BamH*I
K78-F*	AGGTACC*AGGGGA*ACACG**ATG**C	*Kpn*I
B78-R*	AGGATCCTTGGGCATGTGGGC	*BamH*I
RBS77-PA2078F	AGGTACC**AGA*GAGAAC*CGCCCGATG**CATCCGACCTTTTCG	*Kpn*I
INT78-F*	ATCCACAAGCTCAAGG	
INT78-R*	CGTGGATGATCTCGGC	
INT77-F*	CTCGGCGGTTCCAGCG	
INT77-R*	GCGGCAGGTTGAAGTG	
RT-F*	GAAGGCAACTCCAACG	
RT-R*	TAGCCCTGCACCACCT	

* Primers with asterisk were used for amplification in both, *P. aeruginosa* PAO1 and 42A2 oleate-diol synthase DNA genomic fragment. Intergenic region is shown in bold and the theoretical RBS in *italics*.

Figure S1. **A)** LC showing the elution of 10-H(P)OME resulting from bioconversion of OA in cellular extract from recombinant *E. coli* DH5α/pGEMT-77. **B)** Identification of 10-HOME after analysis of the MS^2 spectrum (m/z 297 full scan→ 279) producing predominant signals at m/z 277, 251, 155 (upper) and identification of 10-H(P)OME through analysis of the MS^3 spectrum (m/z 313 full scan→ 295), producing signals at m/z 277, 251, 155 and 139 (bottom). Both LC/MS analyses are equal to data obtained previously produced for these compounds within *E. coli* DH5α/pMMB-77.



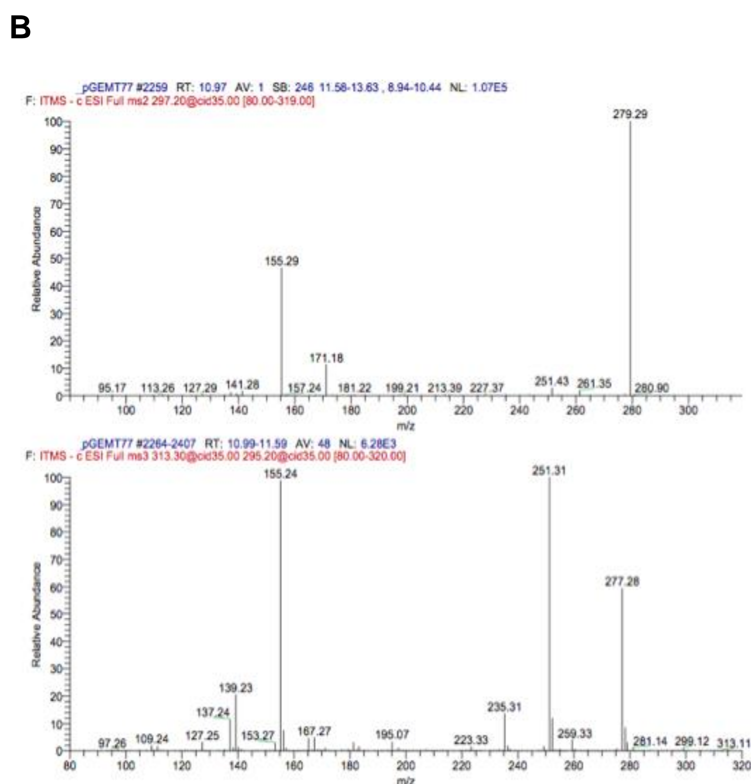
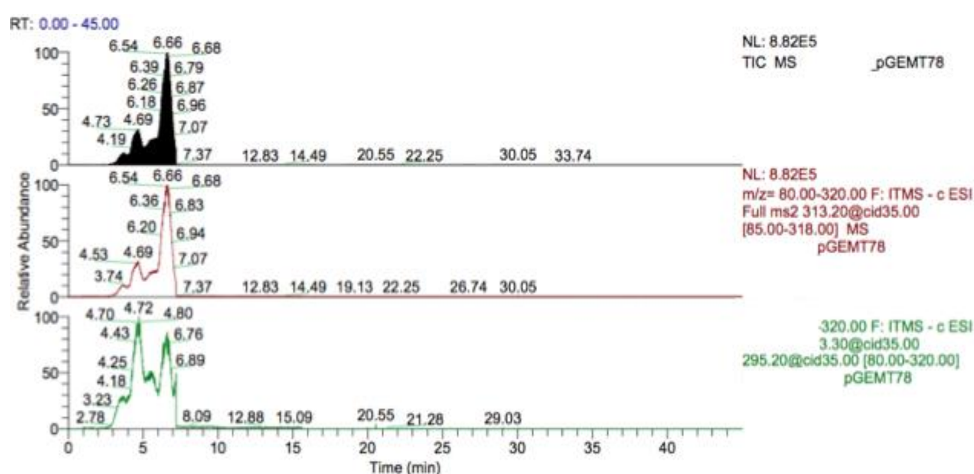
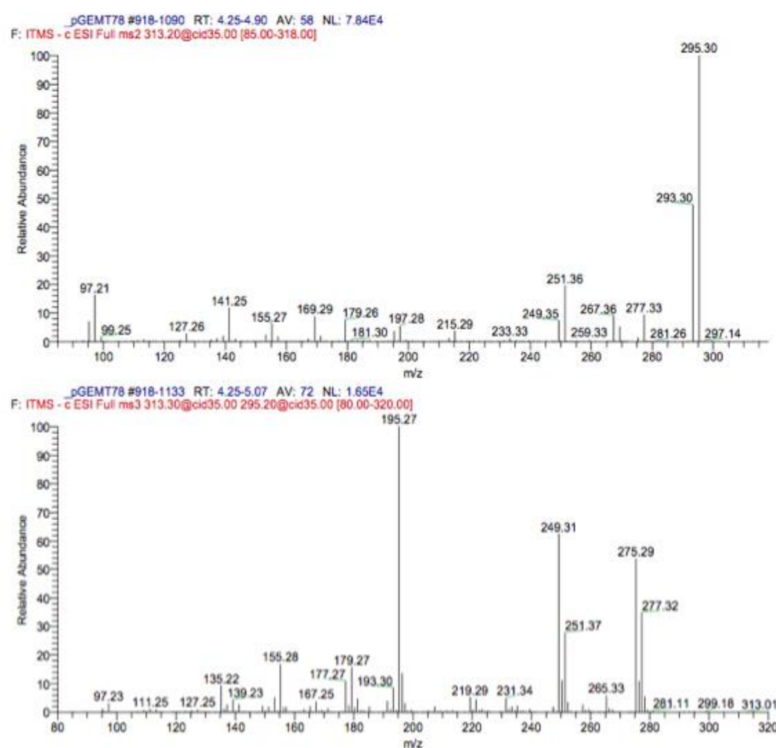


Figure S2.A) LC showing the elution of 7,10-DiHOME resulting from bioconversion of purified (10*S*)-H(P)OME in cellular extract recombinant *E. coli* DH5α/pGEMT-78. **B)** MS^2 spectrum of 7,10-DiHOME (m/z 313 full scan → 295) leaving predominant signals at m/z 251, 141 and 97 (upper) and identification through MS^3 spectrum of 7,10-DiHOME (m/z 313 full scan → 295) leaving predominant signals at m/z 251, 195 and 155 (bottom). Both LC/MS analyses are equal to data obtained previously produced for these compounds within *E. coli* DH5α/pMMB-77.

A



B



3.4. PAPER III

In silico/in vivo **insights into the functional and evolutionary pathway of *Pseudomonas aeruginosa* oleate-diol synthase. Discovery of a new bacterial di-heme cytochrome c peroxidase subfamily**

3.4.1. Resumen

Aproximaciones *in silico* e *in vivo* a los aspectos evolutivos de la oleato-diol sintasa de *Pseudomonas aeruginosa*. Descubrimiento de una nueva familia de di-hemo citocromo c peroxidasas bacterianas

Mónica Estupiñán, *Daniel Álvarez-García, Xavier Barril, Pilar Diaz, Àngels Manresa*

Como se describió anteriormente, los genes *PA2077* y *PA2078* de *P. aeruginosa* codifican para las enzimas 10*S*-DOX (10*S*-dioxigenasa) y 7,10-DS (7,10-diol sintasa) implicadas en la oxigenación de ácidos grasos de cadena larga a través de la vía oleato diol sintasa, recientemente descrita. El análisis de la secuencia de aminoácidos de ambas enzimas reveló la presencia de dos motivos de unión a hemo (CXXCH) en cada proteína. Además, el análisis filogenético mostró la relación de ambas proteínas a citocromo c peroxidasas di-hemo bacterianas (CPP), con similitud a la oxidasa del látex de *Xanthomonas* sp. 35Y, denominada RoxA. El modelo 3D por homología de PA2077 y PA2078 se obtuvo utilizando la estructura de RoxA (4b2n pdb) como molde. A partir de los modelos en 3D de los grupos funcionales obtenida, se observaron variaciones significativas en los aminoácidos en torno a los grupos hemo. Además, se detectó la presencia de repeticiones palindrómicas localizadas en las regiones codificantes de las enzimas, que pueden actuar como elementos de evolución de proteínas, siendo la primera vez que se describen en el genoma de *P. aeruginosa*. Estas observaciones y el árbol filogenético obtenido para las dos proteínas, permiten proponer una vía evolutiva para el operón diol sintasa de *P. aeruginosa*. Teniendo en cuenta el conjunto de resultados *in silico* e *in vivo* obtenidos, concluimos que las enzimas PA2077 y PA2078 constituyen los primeros miembros de una nueva subfamilia de peroxidasas bacterianas, designada como citocromo c peroxidasas di-hemo de ácidos grasos (FadCcp).

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RESEARCH ARTICLE

In Silico/In Vivo Insights into the Functional and Evolutionary Pathway of *Pseudomonas aeruginosa* Oleate-Diol Synthase. Discovery of a New Bacterial Di-Heme Cytochrome C Peroxidase Subfamily

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Abstract

As previously reported, *P. aeruginosa* genes *PA2077* and *PA2078* code for 10S-DOX (10S-Dioxygenase) and 7,10-DS (7,10-Diol Synthase) enzymes involved in long-chain fatty acid oxygenation through the recently described oleate-diol synthase pathway. Analysis of the amino acid sequence of both enzymes revealed the presence of two heme-binding motifs (CXXCH) on each protein. Phylogenetic analysis showed the relation of both proteins to bacterial di-heme cytochrome c peroxidases (Ccps), similar to *Xanthomonas sp*. 35Y rubber oxidase RoxA. Structural homology modelling of PA2077 and PA2078 was achieved using RoxA (pdb *4b2n*) as a template. From the 3D model obtained, presence of significant amino acid variations in the predicted heme-environment was found. Moreover, the presence of palindromic repeats located in enzyme-coding regions, acting as protein evolution elements, is reported here for the first time in *P. aeruginosa* genome. These observations and the constructed phylogenetic tree of the two proteins, allow the proposal of an evolutionary pathway for *P. aeruginosa* oleate-diol synthase operon. Taking together the *in silico* and *in vivo* results obtained we conclude that enzymes PA2077 and PA2078 are the first described members of a new subfamily of bacterial peroxidases, designated as Fatty acid-di-heme Cytochrome c peroxidases (FadCcp).

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Introduction

P. aeruginosa is a gram-negative rod known for its metabolic versatility, which allows its distribution in numerous environmental niches, being host in higher plants, invertebrates and vertebrates [1,2]. *P. aeruginosa* can metabolize xenobiotics and also unsaturated long-chain fatty acids (LCFAs), widely spread in the environment, to convert them into hydroxylated derivatives (HFAs) known as oxylipins [3,4].

Hydroxylated fatty acids have a wide range of biological functions, being involved in inflammation, signaling, plant pest defense, germination or fungal reproduction [5–7]. Furthermore, the biotechnological applications of HFAs have extensively been studied, constituting important emulsifying agents in food and cosmetics industries, acting as antibacterial or antifungal substances, or being used as intermediate compounds for fine chemistry industry, with an important role in the pharmaceutical area [4,8,9]. However, the bottleneck of their chemical production is the low reactivity of the fatty acid hydrophobic chain [10]. This problem is solved in nature through several enzymatic strategies including LCFA biotransformation by diol synthases (DS), lipoxygenases (LOX), heme-containing fatty acid dioxygenases (DOX), cyclooxygenases (COX), hydratases, allene oxide synthases (AOS) or cytochromes P_{450} (CYP450) [11–15]. Moreover, these oxylipin-releasing enzymes are capable to generate products with high regio- and enantioselectivities from a broad range of substrates [16].

We previously described that a diol synthase activity is responsible for the conversion of oleic acid (OA) into oxylipins in *P. aeruginosa* [3,4]. Oleic acid is initially converted into hydroperoxide 10-H(P)OME ((10*S*)-hydroxy-(8E)-octadecenoic acid) by a dioxygenase (DOX), followed by conversion of the hydroperoxide intermediate into 7,10-DiHOME ((7*S*,10*S*)-dihydroxy-(8E)-octadecenoic acid) by an oleate-diol synthase (DS). We recently identified the genes coding for these activities, which are linked in a finely tuned operon. Gene *PA2077* codes for the 10*S*-dioxygenase activity (10*S*-DOX) responsible for the first step of the reaction, whereas *PA2078* encodes the (7*S*, 10*S*)-hydroperoxide diol synthase enzyme (7,10-DS), which allows conversion of 10-H(P)OME into 7,10-DiHOME, in a metabolic pathway unique for *P. aeruginosa* [3].

Here we report the results of a detailed *in silico/in vivo* study of the nucleotide and amino acid sequences of PA2077 and PA2078, including comprehensive insights into their functional and structural features, with rational mutagenesis analysis of important residues, identification of probable evolutionary elements and phylogenetic analysis. From the results obtained we conclude that proteins PA2077 and PA2078 would be the first described and functionally characterized members of a new di-heme cytochrome c peroxidase enzyme subfamily, acting on long-chain fatty acids.

Materials and Methods

Materials

Oleic acid 99% (Sigma) was used as a substrate for bioconversion assays in LC/MS analysis. (10*S*)-hydroperoxy-8E-octadecenoic acid (10*S*-H(P)OME), provided by Dr. Martín-Arjol, was purified from *P. aeruginosa* 42A2 culture supernatants as described previously [8,17].

Bacterial strains

Bacterial strains and plasmids used in this work are listed in S1 Table. All strains were routinely grown in TSB (17 g casein peptone, 3 g soymeal peptone, 2.5 g glucose, 5 g NaCl, and 2.5 g KH_2PO_4) at 37°C on a rotary shaker operated at 200 rpm. Antibiotics were added for *P. aeruginosa* mutant recombinant strains growth when required at the following concentrations:

tetracycline 5 μg ml^{-1}; chloramphenicol 200 μg ml^{-1}, and ampicillin 100 μg ml^{-1} for *E. coli* DH5α recombinant strains.

Site-directed mutagenesis

Site-directed mutagenesis of selected amino acids in the heme binding or putative P_{450} regions of PA2077 and PA2078 (pGEMTe-77 and pGEMTe-78 variants) was carried out using a Quik-Change Site-Directed Mutagenesis Kit (Stratagene), with Pfu/Phusion High-Fidelity DNA polymerase (New England BioLabs) and the primers stated in S2 Table. Amplification was performed at 98°C for 30 s followed by 25 cycles (98°C for 10 s, 60°C for 30 s, and 72°C for 3 min) in a thermocycler PTC200, MJ Research or GeneAMP PCR system 2400 (Perkin Elmer). The amplified products were digested with *Dpn*I (Thermo Scientific) for 2 h and checked by agarose gel electrophoresis. *Dpn*I–resistant plasmid molecules were confirmed by sequencing using plasmid internal primers (S2 Table). Sequencing was performed using an ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems), available at the Serveis Científics i Tecnològics of the University of Barcelona. Plasmids carrying the desired mutations were transformed into *E. coli* DH5α, as described [18].

Bioconversion assays

500 μl of 10x concentrated crude cell extracts were incubated with 100 μM oleic acid (OA) or 0.1% (v/v) 10*S*-H(P)OME in 0.5 M Tris-HCl buffer pH 7. Reactions were incubated for 2 h at 37ºC with occasional vortexing, and terminated by acidification to pH 2 with 0.5 M HCl. Released products were extracted with ethyl acetate (1:1; v:v), routinely analyzed by TLC (hexane:diethyl ether:acetic acid; 75:15:10), and further identified by LC–MS/MS analysis.

Protein expression in *P. aeruginosa* mutant strains

In order to obtain functional enzymes for purification, *P. aeruginosa PA2078* and *PA2077* genes, cloned into pMMB207 fused to the *tac* promoter [3] were over-expressed or co-expressed in mutants ΔPA2078 and ΔPA2077. Cultures were grown to exponential phase (D. $O._{600nm}$ = 0.6), induced with 1 mM IPTG and incubated overnight at 37ºC. Bradford assay [19] was used to measure soluble protein concentration to determine the effect caused by expression of these genes on both mutant strains.

Computational analysis of ORFs PA2077 and PA2078

The nucleotide sequences of ORFs PA2077 and PA2078 and their respective orthologous genes were retrieved from *Pseudomonas aeruginosa* database (www.pseudomonas.com) [20]. The recently published nucleotide sequences of *P. aeruginosa* 42A2 oleate-diol synthase operon (GenBank #KJ372239) were also included in the analysis [3]. Nucleotide and amino acid sequences were submitted to the basic local alignment search tools BLASTn and BLASTp respectively, to identify possible homologues in the databases available at EMBL/EBI and NCBI (http://www.ncbi.nlm.nih.gov/), and to retrieve identity and similarity percentages by pairwise alignment [21].

Elements involved in enzymatic processing like post-translational modifications were predicted using PSORTb v. 2.0 for bacterial sequences (http://www.psort.org/) [22]. SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) was used for prediction of protein subcellular location [23]. Transmembrane helices prediction was achieved using TMPred Server (http://www.ch.embnet.org/software/TMPRED_form.html) [24]. ExPASy proteomics server (http://us.expasy.org/tools/protparam.html) was used to analyze the protein physico-chemical

parameters (ProtParam tool) and to predict isoelectric point and molecular mass of non-processed and mature proteins. GPMAW tool was used for detection of aromatic amino acids [25]. Nucleotide and amino acid sequence alignments were obtained using T-Coffee (http://tcoffee.crg.cat/) [26] or ClustalO (http://www.ebi.ac.uk/Tools/msa/clustalo/) [27] multiple sequence alignment software.

Search for functional similarity motifs

InterProScan 5.2 was used for domain identification [28]. HMMER (http://hmmer.janelia.org/) was used to create a Hidden Markov Model in order to visualize conserved protein motifs [29]. Amino acid sequences of both proteins were compared with protein sequences of functionally characterized enzymes bearing similar catalytic mechanisms: diol synthases, allene oxide synthases, cytochrome P450 monooxygenases, lipoxygenases, cyclooxygenases, heme-dioxygenases, *cis-trans* isomerases or catalase-peroxidases, available in the literature and in UniprotKB or Protein Data Bank (PDB) (http://www.rcsb.org/pdb/), always considering their catalytic residues [30,31].

Selected protein sequences were used to analyze the phylogenetic relationship between functionally related proteins using MEGA 6 software [32]. A phylogenetic study by maximum likelihood, employing WAG+G as the amino acid substitution model from MEGA 6 software [33] was performed to analyze specific relationships of proteins PA2077 and PA2078 with previously characterized di-heme Ccp enzymes.

3D homology model construction and optimization

PA2077 and PA2078 amino acid sequences were compared by BlastP PSI-BLAST (Position-Specific Iterated) using a BLOSUM 45 matrix with PDB database [21] in order to identify homologous proteins with available 3D structure. Template for 3D model construction was selected as that showing the highest identity score (RoxA, pdb *4b2n*) compared with target proteins in multiple amino acid sequence alignments obtained by T-Coffee [26]. For alignment correction, secondary structure prediction was performed using the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/) [34]. Alignment of conserved amino acid motifs and modeling corrections of the protein core were performed by comparison with the structure of the selected template sequence, using the visualization tool Pymol Molecular Graphics System, Version 1.5.0.4, Schrödinger, LLC (http://www.pymol.org).

A 3D homology model based on sequence alignment between proteins PA2077 and PA2078 and the template RoxA was obtained using Modeller 9.10 [35]. Both heme groups were included in the model generation but no other special restrictions were applied. The coordinate PDB files were used for structure comparison and overlapping structures were monitored using Pymol Molecular Graphics System, Version 1.5.0.4, Schrödinger, LLC.

Significant evolutionary elements

Presence of palindromic elements in both ORFs was confirmed with Emboss 6.2.0. (http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome) [36]. Further phylogenetic analysis were performed to establish a relationship pattern between the palindromic sequences found in *PA2077* and *PA2078* and orthologous nucleotide sequences, employing the JC model from MEGA 6 software [33].

Results and Discussion

Predicted parameters

P. aeruginosa ORFs PA2077 and PA2078 (UniProt Q9I238 and Q9I237) code for proteins of 634 and 624 amino acids with molecular masses of 68.6 and 67.3 kDa (66.5 and 65 kDa in mature form) and a theoretical *p*I of 5.37 and 5.38, respectively. As inferred from UniProt electronic annotation, the two proteins are described as putative heme-binding proteins with electron transport activity. In agreement with the periplasmic location of oleate-diol synthase activity [37], both proteins display type I signal export sequences that allow transport of the synthesized products to the periplasm, a fact experimentally confirmed by PhoA fusion screening [38]. According to SignalP, signal peptidase cleavage site is located at positions 21 and 23 in PA2077 and PA2078, respectively (Table 1). Despite being periplasmic proteins [4,37], presence of a transmembrane helix was predicted by TMpred for PA2077, but not for PA2078, suggesting that while PA2077 could be a periplasmic membrane-related protein, PA2078 would stay completely soluble in the periplasm. As observed before, PA2077 and PA2078 display very low homology to other proteins in the databases, except for those corresponding to their orthologues in other *P. aeruginosa* strains [3]. Moreover, PA2077 and PA2078 bear 43.4% identity (58% similarity) with respect to each other, probably pointing to a common predecessor during evolution, as previously suggested [3] (Fig 1).

Functionally significant sequence motifs in PA2077 and PA2078

Rational comparison of amino acid sequences among previously characterized oxylipin-forming enzymes allowed identification in PA2077 and PA2078 of several significant motifs or specific residues (Fig 1) related to heme/iron-binding sites or relevant in oxygenation reactions. Thus, a sequence (**Y**RQ**H**) similar to the tyrosil radical (**Y**R**WH**) involved in dioxygenation of fungal linoleate-diol synthase activity [39] could be found in PA2077 but not in PA2078. The latter displays several consensus motifs like an **EYD** sequence presumably related to iron binding, the **HD** motif found in peroxidases, and a possible P450 sequence (**E**SQ**R**), all of them located in the 22 C-terminal amino acid positions of the protein (Fig 1). PA2077 contains

Table 1. Features and attributes of PA2077 and PA20778 nucleotide and amino acid sequences.

Attribute	PA2077	PA2078
ORFs (bp)	1905	1875
Molecular mass (KDa)	68.6	67.3
Mature protein	66.5	65.0
Mature protein + 2 hemes	68.7	67.5
P_i (theoretical)	5.37	5.38
% aromatic amino acids (nº of F, W and Y amino acids)	8.2 (21/10/20)	7.6 (23/10/15)
Heme attachment[a] (N terminus/C terminus)	$CAGCH_{130}$/ $CAACH_{375}$	$CAGCH_{130}$/ $CAACH_{365}$
Axial heme ligands[a] (N terminus/C terminus)	H_{130}/H_{375} H_{611}	H_{130}/H_{365} H_{603}
MauG motif	$PYFH_{553}NGSVP$	$PYFH_{543}NGSVP$
F_{317} equivalent	F_{269}	F_{260}
W_{302} equivalent	W_{251}	S_{243}
Signal Peptide cleavage site	21	23
IR in protein-coding sequence (+ strand)	GACGTCGGCG	CCATCTGCAA
IR length	57 pb	68 bp

[a]: Numbering includes signal peptide.

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PA2078        --TEIQLEQGWNAEQRASWYDASLGSRLLPLAWAQALERPDSEERLFSEDNARRLGFPLR
PA2077        DDSGVDLDQGWNQTQKTAWLEAGQGSRMLPLAWLVALEQRASEEPLMSDALIRQYGYVPH
                : ::*:****  *:::* :*. ***:*****  ***:  *** *:*:   *: *:  :

PA2078        NWQGGELRLPRGFALDQQDDSQFSDTRLRWKARQSSSEPWVGLNCAGCHSTDISYRGSEL
PA2077        TLGGSSVKVVQGYAVDRSDDSDLTFTKLRWKALQGSREPWVGPTCSMCHTSHISYQGTQL
              .  *..::: :*:*:*:.***::: *:***** *.* ***** .*: **::.***:*::*

PA2078        TVDAGATLANVQAIFDEVLAALRRTSDDGDKFARFAGNVLGSEDSPANR-----ELLKAA
PA2077        TVYGGQTMGDLAGFQLEILGALQSTRADTAKFERFARKVLGADGLVSGYNDANKARLQAA
              ** .* *:.:: .:  *:*.**: *  *  ** *** :***::   :         *:**

PA2078        LVKRA-ALIDTLLSMSATDLQPGPGRLDATGQSLNRAAINSGARHLQANPTDAPTSFPAL
PA2077        LDATIVRLRDGSHFNLPHDPEFGPGRLDAIGSIFNSVGYELHADEQIYGAEDAPVSYPFL
              *      * *        * : ******* *. :* .. :  * .      ***.*:* *

PA2078        WHTLQMDKLQSSGFVPN-VKVLDLNGQVFDLGYLAGDIGVVQGDYGDVVSH-P--LSGLE
PA2077        WNVPQLDRVQWTGFNPNHINVVDIDNRKFDVGALARNAGEAVGVFADVKVLSPIQSALHI
              *.. *:*::* :** ** ::*:*:: : **:* ** : * . * :.**    *   :

PA2078        GYISSIRVDNLTRVEGLIHKLKAPAWPSQLFGAPDSARLAQGKRLYEENCAACHASIGRD
PA2077        GYPSSINVDNLIRIEDQLGQLKPPAWPNQLFGAPEPTRVAEGRELYRQHCSSCHTPLDRN
              ** ***.**** *:*  : :** ****.******: :*:*:*:.**.:.*::**: : *:

PA2078        DLQTPIKVRQVRLKAHGDDAPIGTDPWMACNTFTFSSPSGNYFGLFRPSLGTPSGVGIVG
PA2077        DLRTPVKTVLTHLQARGEVAPIGTDPWTACNSIAQLKTGY-VRGKPYLSFVGTGQRGFYG
              **:**:*.  .:*:*:*: ******** ***:::  . .    *    *:   .  *: *

PA2078        RTSKIADMQVPEVFQIM--------LGKKGQLADGIAE-IIHAIVTGQQTLPGSDSLQ--
PA2077        KQAYAVDVLQEVVVQALAARGLSVALGAFQTAALGIFDGQLPPLISPVPDSPDADSAEAT
              : :  .*:    *.* :        **     * ** :  :  :::     * :** :

PA2078        -AVPAGQLLLAGAAPADSQAQSLAAGEVPTDKSARKDYCLNTEHPFLGYIARPLNGIWAT
PA2077        AADAPGALLLAEN------------VAADSDKARRLEQCLAMTSDLMAYKARPLNGIWAS
               *   * ****                . :**: * : **     ::.* *********:

PA2078        APYLHNGSVPSLYDLLLPQEQRPATFYTGSHEFDPSRVGYLTAPGPDNAFLFDTHLEGNS
PA2077        PPYLHNGSVATLYDLLLPPDLRPRTFYTGSVEFDPVNVGYITDAGGANRFLFDSGKPGNA
               ******** :******* : ** ****** **** .***:*  *  * ****:   **:

PA2078        NAGHDFAR-EYDESQRLALLEYLKTL
PA2077        NGGHDYGNAQFNEQQRRALVEYMKTL
              *.***:.. :::*.** **:**:***
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Fig 1. Amino acid sequence alignment of PA2077 and PA2078 proteins, obtained by ClustalO. Significant amino acid motifs are highlighted in squares and the functionally identified amino acids are shown in **bold**. Conserved heme sequences (CXXCH) are shown in red. The predicted motif for ferrous ion union is depicted in green (EGR or EYD). The signature of oxidases containing the essential histidine like in MauG is shown in blue. Predicted tyrosyl radical (YRQH in PA2077) appears in grey. P450 motifs (EXXR) are in yellow and the distal histidines are highlighted in light green. Peroxidase signature (GXHXCLPHD) is shown in pink, with the peroxidase motif (HD) in orange. A red star indicates the position of the mutated residues (underlined).

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additional conserved amino acid motifs like three putative P450 sequences (**E**PT**R, E**LY**R** and **E**QQ**R**), two **HD** peroxidase motifs, and a signature very close to the **G**S**H**F*N***L**P**HD** peroxidase consensus sequence [40]. However, the sequence FXX**G**P**H**X**CL**G, responsible for hydroperoxide isomerase activity in *Aspergillus fumigatus* linoleate-diol synthase [41] could neither be found in PA2077 nor in PA2078. Interestingly, both proteins bear a leucine as the C-terminal residue (Fig 1), a hydrophobic amino acid close to the isoleucine shown to play an essential role in *P. aeruginosa* lipoxygenase (LOX) activity [42].

Search for conserved functional amino acid domains in discontinuous pattern mode allowed further identification of short, relevant elements shared by both proteins (Fig 1), such as the presence of two conserved cytochrome c-like domains of 19 amino acids (368–386) in

PA2077 and 21 amino acids (349–370) in PA2078 that exhibit identity to cytochrome c oxydases (cbb3-type) [43]. Also, the two heme-binding motifs (**C**XX**CH**), used for covalent attachment of heme in bacterial di-heme cytochrome c peroxidases (Ccps) were found in both proteins. Moreover, the signature 'PYL**H**N**G**S**V**', containing the essential histidine of oxydases and described for MauG (methylamine dehydrogenase) as a cytochrome c peroxidase domain [44], was also found in PA2077 and PA2078 (Fig 1, Table 1). These findings suggest that the two proteins involved in *P. aeruginosa* oleate diol synthase activity are members of the cytochrome c peroxidase (Ccp) enzyme family.

Comparison of PA2077 and PA2078 with other bacterial Ccps

Known bacterial Ccp protein family is predominantly spread among proteobacteria (Fig 2), with an average molecular mass of 35–40 KDa, whose active form purifies as a homodimer [45–48]. However, like rubber oxygenase RoxA from *Xanthomonas* sp. 35Y and its orthologous genes [49], PA2077 and PA2078 show about double molecular size compared to other bacterial Ccps, and display activity as monomers. RoxA was previously classified as a Ccp protein showing homology to PA2078 [49], which allows inclusion of protein PA2078 in the same enzyme family. Moreover, PA2078 and PA2077, share a high degree of identity between them, indicating that PA2077 can also be assigned to the Ccp protein family, even if displaying different biochemical functions [3].

To further prove this preliminary assignment, PA2077 or PA2078 and their orthologs were aligned to obtain two phylogenetic join-trees with either other known oxylipin-forming proteins (not shown) or with the 13 known members of bacterial Ccps (Fig 2). In both cases, PA2077 and PA2078 appeared localized together in the same node of the phylogenetic tree. In the first tree they appeared far related to the other oxylipin-forming enzymes analyzed, which share less than 18% identity with PA2077 and PA2078. In the second phylogenetic tree, two well-differentiated groups of bacterial Ccps appeared (Fig 2). One group includes those proteins functionally characterized as cytochrome c peroxidases, together with MauG (methylamine dehydrogenase), whereas the second protein group includes *Xanthomonas* sp. 35Y RoxA with its orthologs, and *P. aeruginosa* PA2077, PA2078 homologous proteins (Fig 2). According to these results, protein PA2077 (10*S*-DOX) constitutes, together with RoxA and MauG, the third functionally characterized bacterial oxygenase-Ccp protein. These evidences further confirm that the proteins responsible for *P. aeruginosa* oleate-diol synthase activity are quite unique, not only among bacteria but also with respect to other oxylipin-forming enzymes previously reported like those of *Aspergillus fumigatus* [41,50] or linoleate diol synthase (LDS) from the rice blast fungus *Gaeumannomyces graminis*, which consist on a single protein bearing two domains [51].

Comparative 3D modeling of PA2077 and PA2078

Taking into consideration the difficulties found in purifying PA2077 and PA2078 proteins for crystallization purposes, a comparative homology 3D model was obtained here. Based on the results from multiple sequence alignment, pdb *4b2n* from RoxA, the closest crystallographic structure related to PA2077 and PA2078 known so far, was selected as a template for modeling the 3D structure of the two proteins (S1 Fig). RoxA catalyzes the oxidative cleavage of natural rubber (poly-[*cis*-1,4-isoprene]), a complex hydrophobic biopolymer. As other Ccp proteins, RoxA bears di-heme binding domains, which act as low potential (LP; N-terminal domain; -65 mV) and high potential (HP; C-terminal domain; -130 mV to -160 mMV) hemes. Both heme domains are linked by W302, which provides an inter-heme electron bridge. RoxA active site is located in residue H195, acting as a proximal axial ligand to the heme iron. The closely

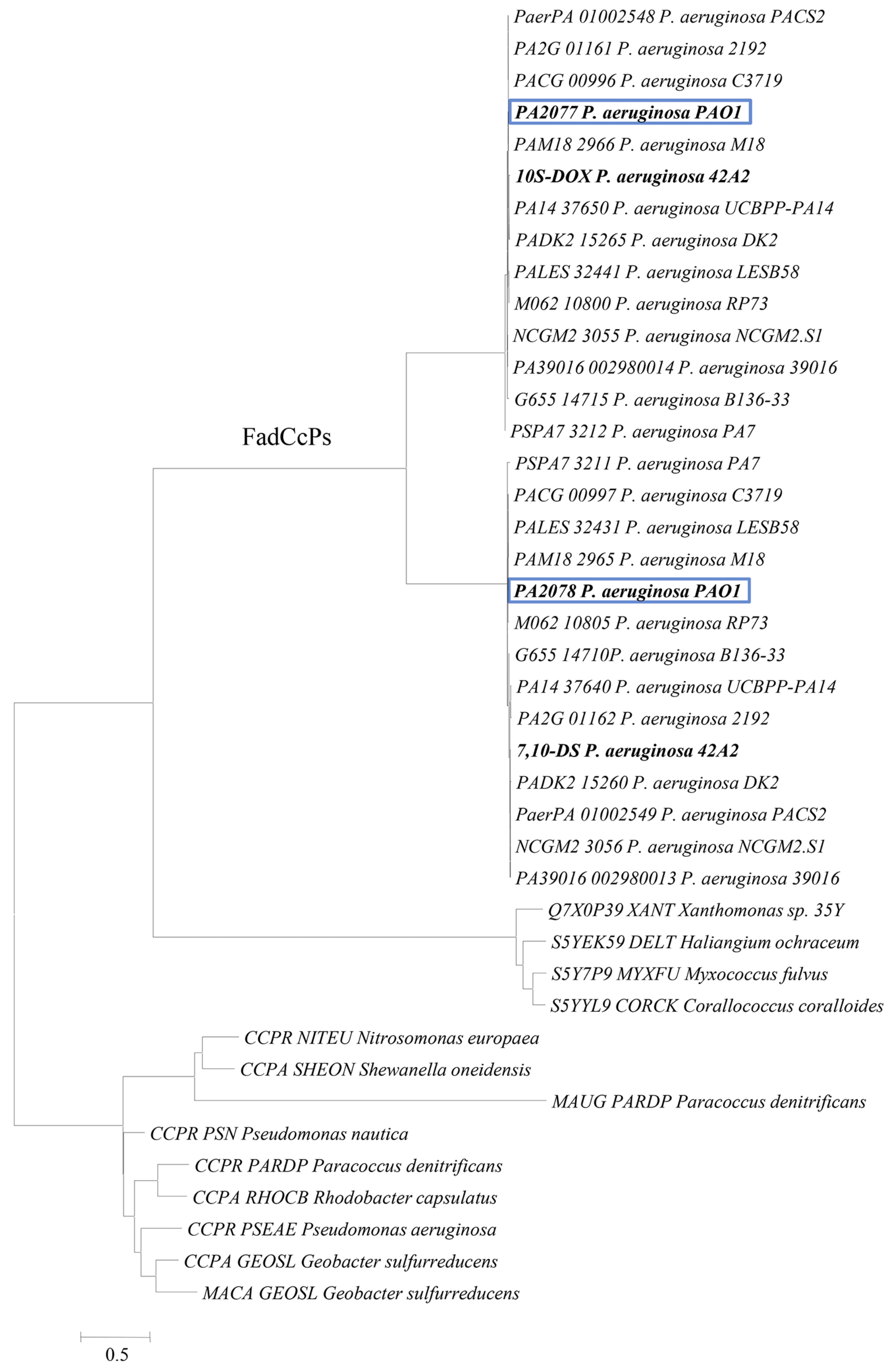

Fig 2. Phylogenetic relationship between amino acid sequences of bacterial di-heme cytochrome *c* peroxidases (Ccps). NITEU–*Nitrosomonas europaea*, SHEON–*Shewanella oneidensis*, PSN–*Pseudomonas nautica*, PARDP–*Paracoccus denitrificans*, RHOCB–*Rhodobacter capsulatus*, PSEAE–*Pseudomonas aeruginosa*, GEOSL–*Geobacter sulfurreducens*. XANT–*Xanthomonas sp*. 35Y, DELT–*Haliangium ochraceum*, MYXFU–*Myxococcus fulvus*, CORCK–*Corallococcus coralloides*. Oleate-diol synthase enzymes (PA2077 and PA2078 orthologues) constitute the newly described subfamily of FadCcps (Fatty-acid di-heme cytochrome c peroxidases).

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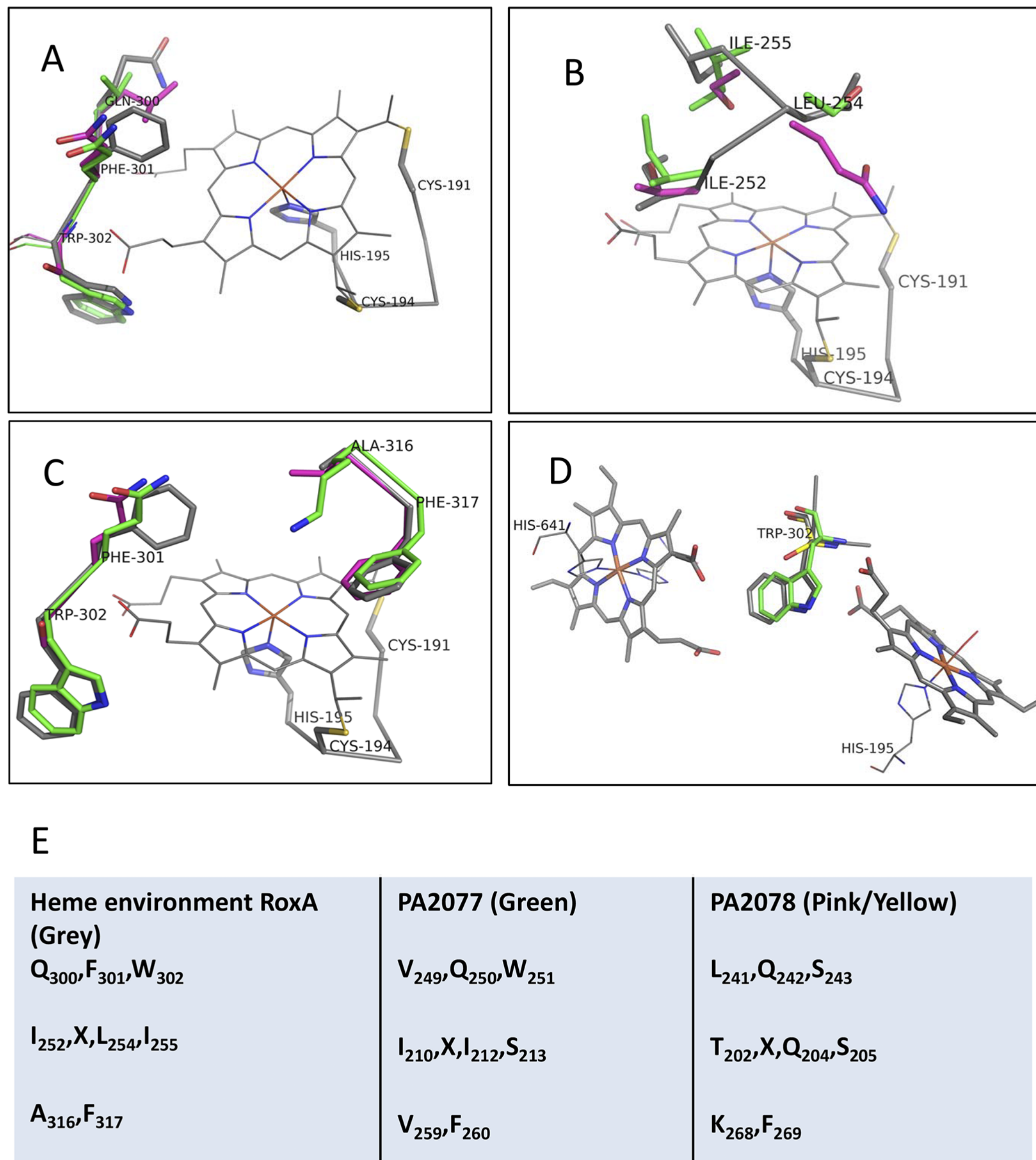
located hydrophobic residues F317, A251, I252, F301, L254, I255, and A316 constitute the heme environment, being F317 essential for activity. Furthermore, the complex flexibility of three hydrophobic amino acid loops located in the distal active site possibly create a transient channel for substrate access and accommodation [52,53]. Although RoxA, PA2077 and PA2078 have a similar molecular mass (71.5, 66.5 and 65 KDa, respectively), they share low percentage of sequence identity (coverage 20% and 32%, identity 43% and 65%, PA2077 and PA2078 respectively in front of RoxA) and, as shown in the multiple sequence alignment (S2 Fig), insertions and deletions occur at different regions of their sequences, suggesting a different spatial loop arrangement. Heme-binding sites are separated by 245 and 235 amino acids in PA2077 and PA2078 respectively, further than the 199 amino acid distance found in RoxA [52], confirming this assumption. The low similarity and complex fold of RoxA, together with ubiquitous loops, made the global process of modeling difficult to validate. Although the surface loops were poorly modelled in the 3D-models obtained, the core region holding both heme groups, well conserved according to the sequence alignment (S2 Fig), provided enough confidence to trust this modelled region for analysis. Therefore, the conserved dual-heme environment core, constituting the common trait of Ccps [52], could be modeled with enough confidence to be studied in detail, providing insights into the catalytic preferences of both proteins. To obtain a reliable model, a multiple sequence alignment including PA2077, PA2078 and RoxA (S2 Fig) was used instead of pairwise alignment, to magnify similarities around the heme cavities. Important amino acid differences could be identified in the oxygen-binding axial heme cavity (His195 in RoxA), which might alter reactivity by modifying the heme active conformation and substrate-binding pocket. As shown in Fig 3, important, non-neutral variations with respect to RoxA were identified in PA2077 and PA2078 heme environments, where hydrophobic residues are mainly substituted by polar amino acids: F301Q in both proteins, L254S in PA2077, and I255S, I252T and L254Q in PA2078. Moreover, a drastic A316K substitution is predicted for PA2077 heme-binding site. Presence of this positive charge, partially stabilized by a serine residue sitting nearby, might be important in the catalytic mechanism of this protein (Fig 3).

A general heme-binding site scheme for RoxA, PA2077 and PA2078 is depicted in Fig 4. The catalytic N-terminal heme environment of RoxA and PA2077, where the monomeric assembly of the oxygen molecule is produced, is mainly constituted by hydrophobic amino acids (excluding the lysine change in PA2077 previously described). However, a more hydrophilic environment was found in PA2078 model (Fig 4). These features can explain the polar nature of the substrate preferred by each enzyme, as RoxA transforms natural rubber, a highly hydrophobic polymer, and PA2077 substrates are mainly monounsaturated LCFAs with a polar head, whereas PA2078 acts on more polar hydroperoxide fatty acids [3,54]. Supporting the higher substrate specificity similarity of RoxA and PA2077, both proteins conserve a tryptophan located between the two heme groups (W302 in RoxA and W251 in PA2077), which can act as a linker of the redox reaction between the two hemes (Fig 3D; Table 1). On the contrary, presence of a serine instead of a tryptophan at this position in PA2078 (S243) may alter the electron transfer process between the two hemes, which would thus function as a monoheme enzyme like cytochrome CYP450, AOS or *cis-trans* isomerase (Cti) enzymes [55]. Therefore, the amino acid variations found in the heme-binding structures of the analyzed proteins may explain the catalytic differences in behavior found between these three enzymes.

Protein evolution elements found in genes *PA2077* and *PA2078*

Analysis of the nucleotide sequence of ORFs PA2077 and PA2078 revealed the presence of short inverted repeats (IR) of 19 and 22 amino acids, inserted at positions 716 and 870,



Heme environment RoxA (Grey)	PA2077 (Green)	PA2078 (Pink/Yellow)
Q_{300},F_{301},W_{302}	V_{249},Q_{250},W_{251}	L_{241},Q_{242},S_{243}
I_{252},X,L_{254},I_{255}	I_{210},X,I_{212},S_{213}	T_{202},X,Q_{204},S_{205}
A_{316},F_{317}	V_{259},F_{260}	K_{268},F_{269}

Fig 3. A, B, C, Homology models of surrounding amino acids of the low potential heme environment in PA2077 and PA2078, obtained using rubber oxidase RoxA protein (pdb *4b2n*) as a template ($C_{191}XXC_{194}H_{195}$). RoxA structure is shown in grey, PA2077 in green and PA2078 in pink. **D,** Amino acid relationships between both heme-binding motifs (H_{195}, H_{641}) in RoxA compared to those of PA2077 and PA2078. **E,** Heme environment changes observed in PA2077 (green) and PA2078 (pink/yellow) in comparison with those of RoxA (grey).

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respectively (Table 1). On the contrary, no IR insertions were found in *rox*A nucleotide sequence. *In silico* removal of the IR insertions from the original nucleotide sequence of *PA2077* and *PA2078* produced a frame-readout from amino acid 240 in PA2078, which would result in loss of the C-terminal heme-binding motif CAA**CH,** supporting the previous idea that PA2078 might not use the distal heme-binding site for activity, acting as a mono-heme enzyme. On the other hand, when the same procedure was applied to *PA2077*, the two **C**XX**CH** motifs were maintained, suggesting that they are required for activity. To prove this hypothesis, mutants of PA2077 and PA2078 at the proximal and distal heme motifs (Fig 1) were experimentally constructed and tested for activity on their corresponding substrates. Mutants at

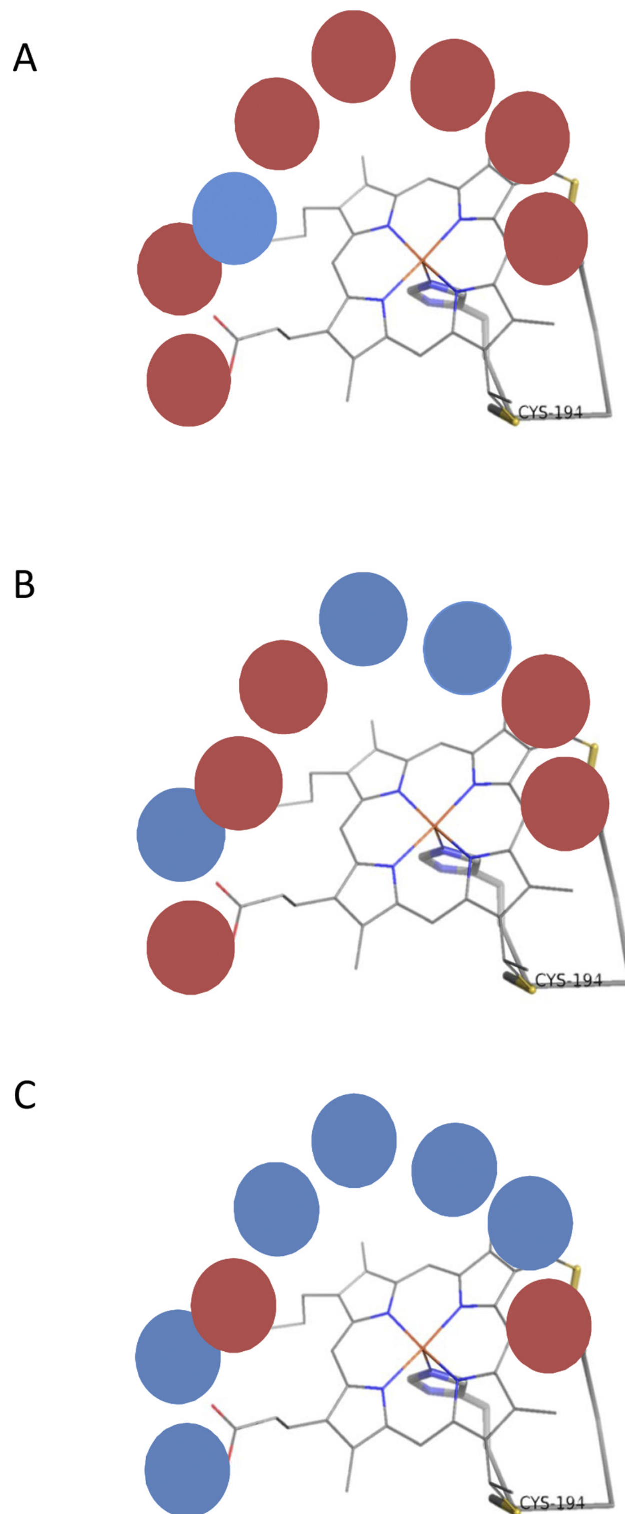

Fig 4. Heme environment hydrophobicity is shown for RoxA (A), PA2077 (B) and PA2078 (C). Polar amino acids are shown as blue circles and hydrophobic amino acids appear in red.

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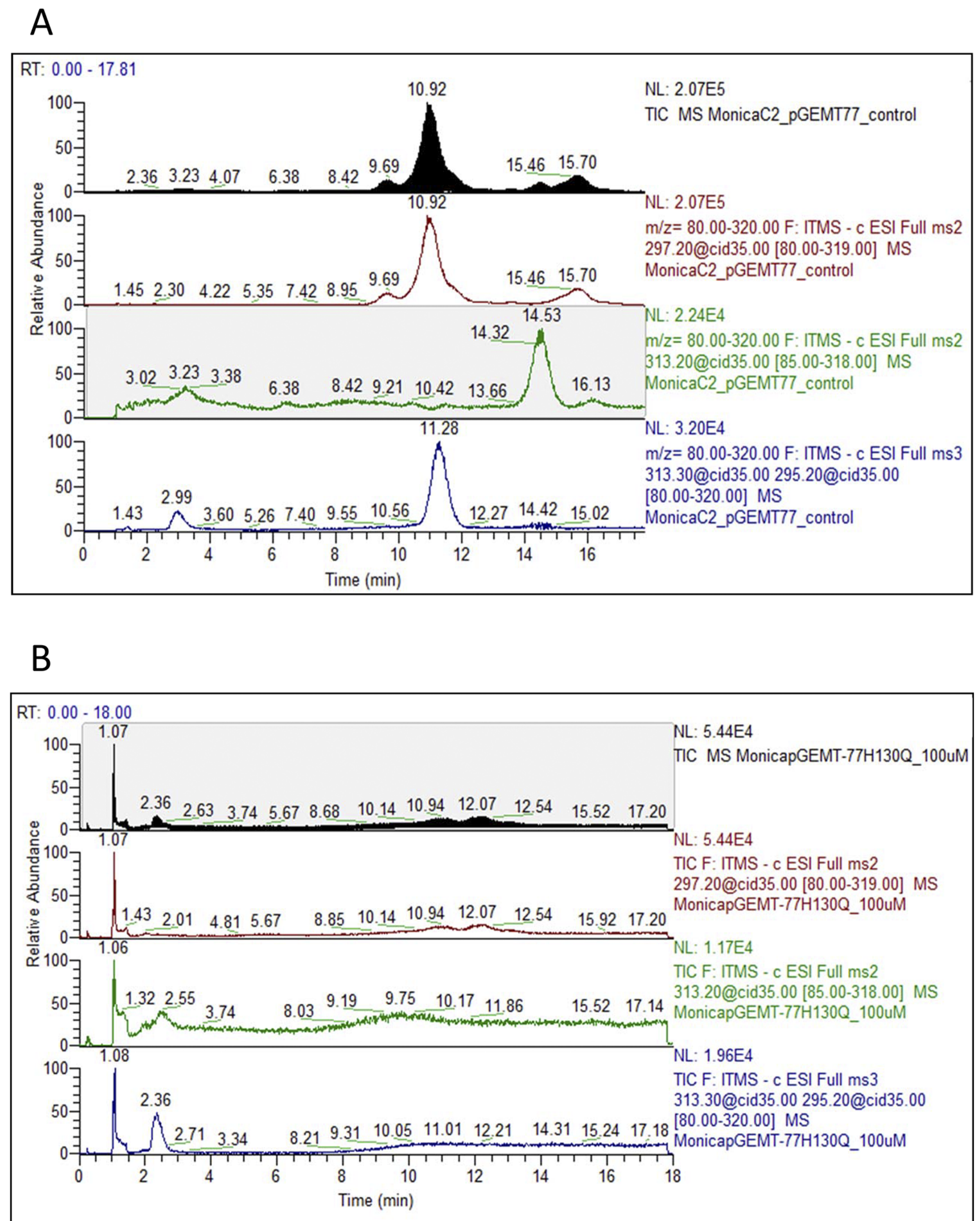

Fig 5. Products released from oleic acid by wild type PA2077 (A) and mutant PA2077 H130Q (B). Hydroperoxide 10-H(P)OME (RT = 11) could only be detected for wild type PA2077, whereas no conversion of oleic acid occurred when both mutants, PA2077 H130Q and PA2077 H375Q, were assayed.

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either position H130 or H375 (residue numbering with signal peptide) in protein PA2077 failed to convert oleic acid into 10-H(P)OME (Fig 5), definitely showing that both histidines (the two hemes) are required by PA2077 to display activity. However, mutants H130Q, H365Q and C518S constructed for PA2078 (numbering includes signal peptide) produced the same pattern as wild type PA2078 when assayed for conversion of 10-H(P)OME into 7,10-DiHOME (S3 Fig). These results indicate that neither the proximal or distal heme groups, nor the cysteine putatively related to P_{450} enzymes are involved in the catalytic activity of PA2078. Thus, despite being a di-heme enzyme, none of the heme groups of PA2078 seems to be involved in 10-H(P)

OME conversion. Further mutagenesis approaches are required to completely elucidate the catalytic environment of PA2078, which must be located elsewhere in the protein sequence, out from the heme region.

The lack of activity of the heme groups in PA2078 supports the previous hypothesis that these motifs might have been inherited from an ancestor di-heme protein and maintained during evolution independently of the present activity of the enzyme (3). In fact, the in-frame repeat insertions found in protein-coding regions like those of *PA2077* and *PA2078* have been proposed to constitute evolutionary mechanisms for enrichment of protein diversity in other proteobacteria genomes [56,57], and could be responsible for the loss of functionality of the heme groups in PA2078, thus contributing to the high metabolic versatility and adaptability shown by *P. aeruginosa* strains.

Insights into the evolutionary pathway of PA2077 and PA2078

As suggested in a previous work [3] and confirmed here after site directed mutagenesis, genes *PA2077* and *PA2078* would derive from a common phylogenetic ancestor, involving a gene duplication event followed by functional divergence into two different catalytic activities. Moreover, no functionally characterized orthologues exist in the databases or in the literature, thus constituting the first reported elements for oleic acid metabolism in *P. aeruginosa* strains [3]. Here we further analyzed the nature of this ancestor and the probable evolutionary model of such a process. From the results obtained above, we can conclude that protein PA2077 conserved the two heme-binding functional domains for dioxygenase activity even after the IR insertions, so it can be assumed that it is more closely related to the ancestral enzyme than PA2078, where the CXXCH motifs can be abolished without losing activity. Moreover, the IR sequence GACGTCGGCG is conserved in most *PA2077* homologous genes, whereas the IR sequence found in *PA2078* varies among the corresponding orthologous genes, which means that they have suffered a higher rate of mutations (Table 1). From both, the experimental and the *in silico* data obtained, we can conclude that the enzymes responsible for *P. aeruginosa* oleate diol synthase activity are inparalogs, where the activity of PA2078 would have been more recently acquired than the dioxygenase activity of PA2077, which seems to be much closer than PA2078 to the antecessor protein.

Evolutionary conservation of diol synthase operon

The evolutionary significance of *P. aeruginosa* diol synthase operon was tested by *in vivo* plasmid-based expression of gene *PA2077*, the hydroperoxide-forming enzyme, in the genetic context of mutant ΔPA2078 where the whole operon expression is blocked and there is no conversion of oleic acid at all [3]. However, this construction demonstrated to be lethal and no growth could be obtained (Fig 6A). Moreover, when gene *PA2077* was expressed in mutant ΔPA2077, where protein PA2078 is functional, a similar toxic effect was found, with an overall 66% decrease in soluble protein production (Fig 6A). These results show that overexpression of gene *PA2077* has a toxic effect due to the properties of the hydroperoxide product released by the encoded functional PA2077 (10(*S*)-DOX). On the contrary, overexpression of gene *PA2078* in either mutant under the same conditions caused no decrease of soluble protein concentration compared to the wild type strain. As described before [3], the 10(*S*)-DOX encoded by gene *PA2077* produces oxygenation of OA and could possibly act on other cellular unsaturated fatty acids, leading to accumulation of high concentrations of organic hydroperoxides, which could damage DNA, proteins and membrane phospholipids [42,58] thus causing cell lysis and a concomitant soluble protein reduction. In fact, although conversion of several unsaturated fatty acids has already been explored with the operon PA2078-PA2077 [4], the physiological range

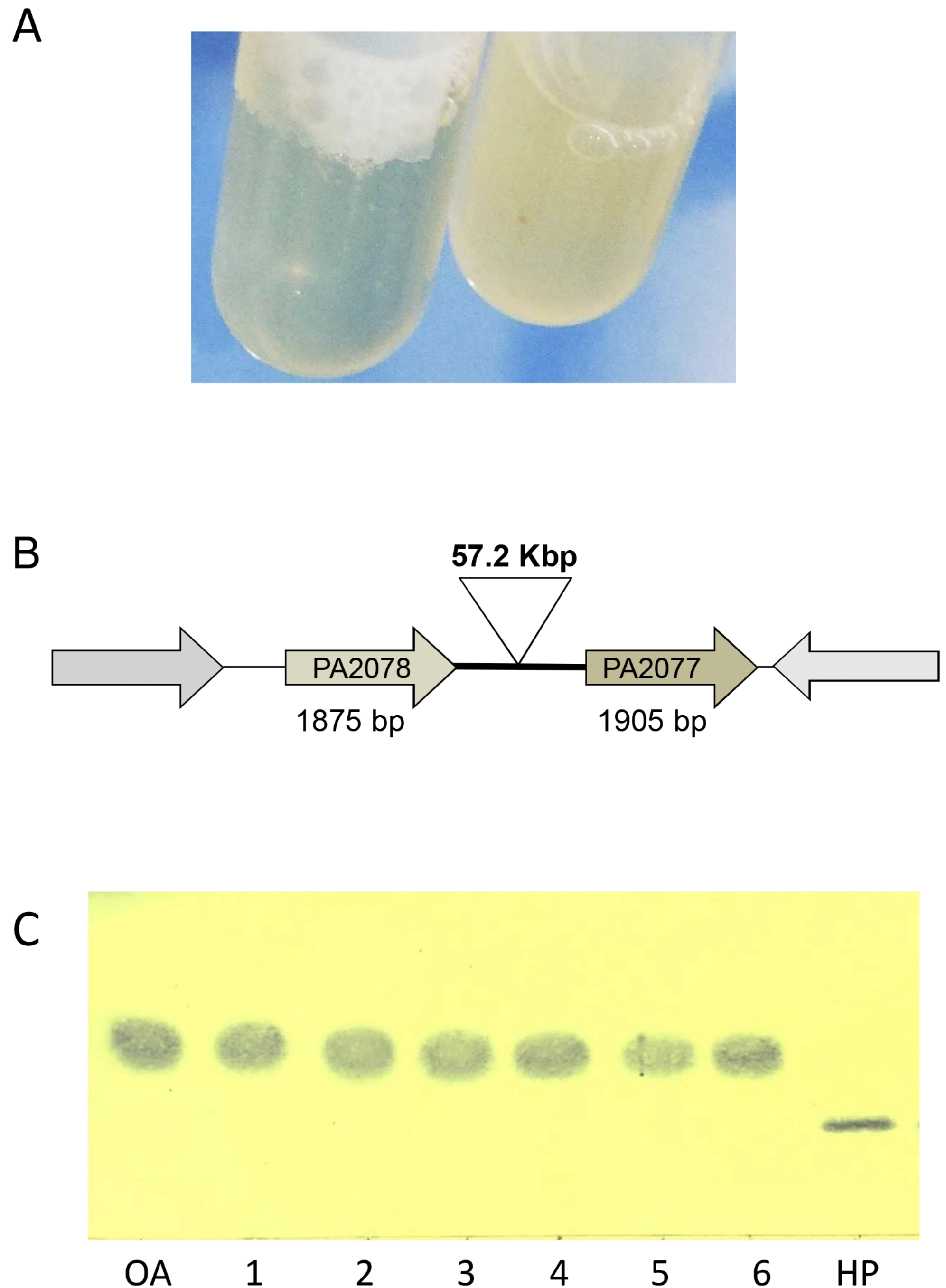

Fig 6. (A) Effect of gene *PA2077* overexpression on cell viability of mutant ΔPA2078, measured as soluble protein and culture cell density. Non IPTG-induced (right tube; 1.21 mg mL^{-1} soluble protein) and IPTG-induced (left tube; 0.8 mg mL^{-1} soluble protein) cultures of mutant ΔPA2078 carrying gene *PA2077*, shown as example of cell lysis caused by overexpression of gene *PA2077*. An overall 66% decrease in soluble protein was found in mutants overexpressing gene *PA2077*, suggesting a toxic effect of the products released by the encoded functional 10(*S*)-DOX. (B) Schematic representation of the *PA2078-PA2077* operon showing the 57 Kbp DNA insertion affecting the operon architecture. (C) Thin layer chromatography analysis showing lack of oleic acid conversion by KK strains (1,2: KK1; 3,4: KK14; 5,6: KK72) incubated for 2 [1,3,5] or 4 hours [2,4,6] with oleic acid. Oleic acid and 10-H(P)OME are shown as control markers.

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of PA2077 substrates, as for other novel dioxygenases, remains still to be elucidated. Taking into consideration these observations, the tandem disposition of genes *PA2078-PA2077* in the diol synthase operon, and its genetic regulation as a dis-coordinated operon (gene *PA2078* is expressed to a double extent than gene *PA2077*), probably allows enzymatic control of organic hydroperoxide production, thus avoiding the cellular damage caused by organic hydroperoxide accumulation. Therefore, the operon nature and disposition of oleate-diol synthase genes (transcribed *PA2078* (2X)→*PA2077*) would have evolved to acquire the differentiated functions of the two encoded enzymes, probably addressed to long chain fatty acid oxygenation (PA2077) and further detoxification/control mechanism (PA2078) to avoid the intracellular effects of hydroperoxide accumulation. This was further confirmed after expression of the complete operon in either a ΔPA2077 or a ΔPA2078 background. Normal growth rates and full activity were achieved in both mutants, indicating that when the two enzymes are co-expressed *in trans*, both maintain their respective functions, with PA2078 abolishing toxicity. These results strengthen the idea that presence of the diol synthase operon represents an evolutionary advantage for bacterial strains, constituting a non-essential metabolic pathway which, nevertheless, can provide benefits to the cell when present [3]. This fact is supported by the recently isolated *P. aeruginosa* KK-related strains which contain a non-phagic insertion of 57.2 Kb located upstream *PA2077* (Fig 6B), a phenomenon occurred later after gene duplication, which involves mercuric resistant coding-proteins [59]. These strains constitute the first *P. aeruginosa* cells reported so far in which oleate-diol synthase activity was absent when we tested them for bioconversion of oleic acid (Fig 6C). Therefore, interruption of the oleate-diol synthase operon does not affect the viability of the KK-related strains in the environment but prevents them from long-chain fatty acid oxygenation.

PA2077 and PA2078 constitute a unique, new subfamily of di-heme peroxidases

According to the previous functional, structural and phylogenetic results, we conclude that PA2077 and PA2078 are di-heme proteins that can be classified as bacterial Ccps, analogous to the well-characterized and heterogeneous group of fungal and bacterial diol synthases. They show low homology to other proteins in different databases, preventing construction of a valid complete 3D homology model. However, the 3D model structure of the heme environment allowed identification of relevant differences in residue composition that might justify the substrate specificity of each enzyme. Both proteins have different but complementary enzymatic functions, constituting a set of metabolic elements for fatty acid or fatty acid-derivatives metabolism in the cell periplasm. Nevertheless, the phylogenetic results obtained here indicate that PA2077 and PA2078 do not group in the main phylogenetic branch described so far for functionally characterized Ccps, constituting thus a new cluster of functional and structural enzymes. Therefore, we propose the inclusion of proteins PA2077 and PA2078 as the first functionally characterized members of a new subfamily of enzymes of the Ccp family, for which we suggest the designation of Fatty acid di-heme Cytochrome c peroxidases (FadCcps).

Supporting Information

S1 Fig. Expanded view of the constructed 3D-homology model structures of PA2077 (A) and PA2078 (C). The structure of RoxA (B), used as a template, is shown as a reference. Conserved di-heme core is colored in each model.
(TIF)

S2 Fig. Multiple amino acid sequence alignment of RoxA, PA2077 and PA2078 obtained by T-COFFE. The amino acids shown in the model of Fig 3 are highlighted by green boxes. (TIF)

S3 Fig. MS/MS spectrum (m/z 313→full scan) of the diol 7,10-DiHOME released from 10-H(P)OME by wild type PA2078 and mutant PA2078 H365Q. The same conversion pattern was obtained for all PA2078 mutants (H130Q, H365Q and C518S), indicating that the mutated residues are not involved in activity.
(TIF)

S1 Table. Bacterial strains used in this study.
(DOCX)

S2 Table. Primers used for directed mutagenesis and sequencing.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ME, DA-G, AM, PD. Performed the experiments: ME, DA-G. Analyzed the data: ME, DA-G, AM, PD. Contributed reagents/materials/analysis tools: PD, AM, XB. Wrote the paper: ME, AM, PD.

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SUPPLEMENTAL MATERIAL (Estupiñán *et al.,* 2015)

Figure S1. Expanded view of the constructed 3D-homology model structures of PA2077 (A) and PA2078 (C).

The structure of RoxA (B), used as a template, is shown as a reference. Conserved di-heme core is colored in each model.

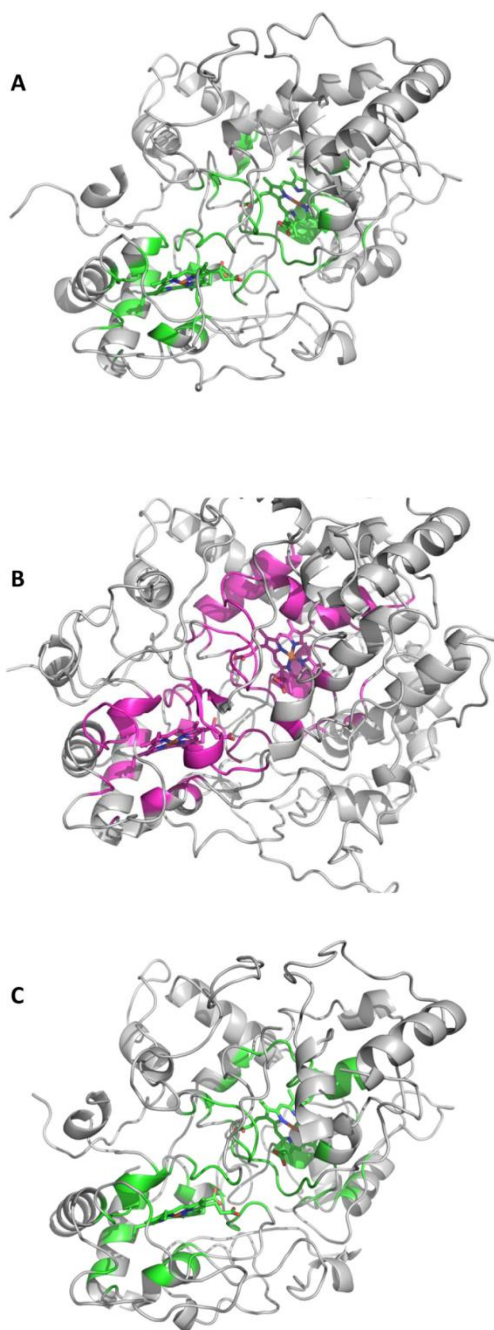

Figure S2. Multiple amino acid sequence alignment of RoxA, PA2077 and PA2078 obtained by T-COFFE.

The amino acids shown in the model of **Fig.3** are highlighted by green boxes.

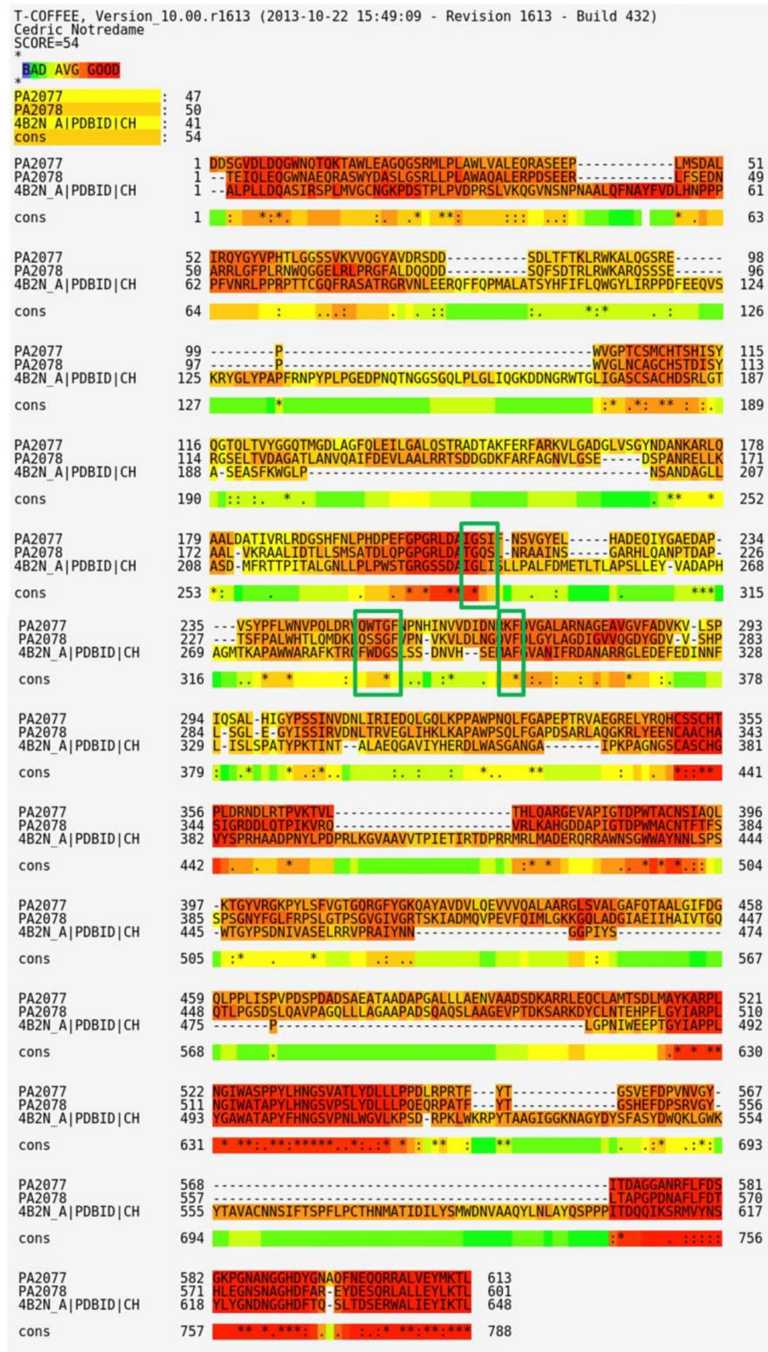

Figure S3. MS/MS spectrum (m/z 313→full scan) of the diol 7,10-DiHOME released from 10-H(P)OME by wild type PA2078 and mutant PA2078 H365Q.
The same conversion pattern was obtained for all PA2078 mutants (H130Q, H365Q and C518S), indicating that the mutated residues are not involved in activity.

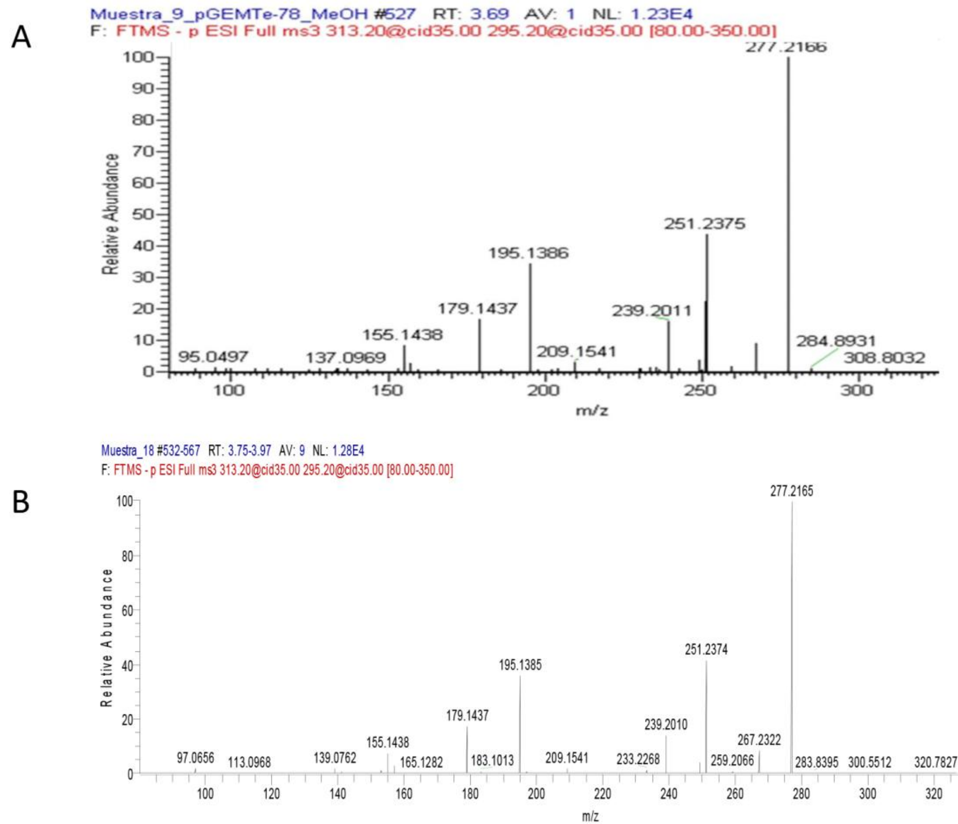

Table S1. Bacterial strains used in this study.

Strains	Relevant features	Reference
P. aeruginosa PAO1 strains		
PAO1	Wild-type, positive control strain	UWGS
ΔPA2077/77	Tet^{r}Cm^r ΔPA2077 mutant carrying pMMB-77	[3]
ΔPA2077/78	Tet^{r}Cm^r ΔPA2077 mutant carrying pMMB-78	[3]
ΔPA2078/77	Tet^{r}Cm^r ΔPA2078 mutant carrying pMMB-77	[3]
ΔPA2078/78	Tet^{r}Cm^r ΔPA2078 mutant carrying pMMB-78	[3]
ΔPA2077/77+78	Tet^{r}Cm^r ΔPA2077 mutant carrying pMMB-77+78	This work
ΔPA2078/77+78	Tet^{r}Cm^r ΔPA2078 mutant carrying pMMB-77+78	This work
P. aeruginosa KK strains	Isolated from a cystic fibrosis (CF) patient at different stages of disease	
KK1,KK14, KK72	Primo-colonization, 18 and 156 months later, respectively	[56]

Table S2. Primers used for directed mutagenesis and sequencing.

Primers	Sequence
77H130QF	GACCTGCAGCATGTGC*CA**G**ACCTCGCACATCAGCTACCAG
77H130QR	CTGGTAGCTGATGTGCGAG*GT**C**TGGCACATGCTGCAGGTC
77H375QF	GGCAACATTGCTCCAGCTGC*CA**G**ACGCCGCTGGACCGCAACG
77H375QR	CGTTGCGGTCCAGCGGC*GT**C**TGGCAGCTGGAGCAATGTTGCC
78H130QF	GGCCGGCTGC*CA**G**TCCACCGACATCAGCG
78H130QR	CGCTGATGTCGGTGGA***C**TG*GCAGCCGGCC
78H365QF	GCGCGGCCTGC*CA**G**GCGAGCATCGGCCGCG
78H365QR	CGCGGCCGATGCTCGC***C**TG*GCAGGCCGCGC
78C518SF	GTGCGCGCAAGGACTAC***A**GC*CTGAATACCGAGCATCCATTCC
78C518SR	GGAATGGATGCTCGGTATTCAG*GC**T**GT*AGTCCTTGCGCGCAC

Site-directed mutagenesis modified codons are shown in underlined italics. Nucleotide changes are in **bold.**

3.5. ADDITIONAL RESULTS

Functional and biotechnological approach to *P. aeruginosa* oleate-diol synthase activity

(In progress)

The following chapter includes relevant additional information retrieved during the development of this PhD project, which was not included in the previous publications but which can provide new insights into the functional properties of *P. aeruginosa* oleate-diol synthase activity and can contribute to the understanding of the physiological role of such an activity. Moreover, a newly developed strategy for biotechnological production of oxylipins using a GRAS *(Generally Recognized as Safe) Pseudomonas putida* KT2440 strain expressing *P. aeruginosa* 10*S*-DOX is also described.

3.5.1. Growth of oleate-diol synthase mutants in minimal medium supplemented with exogenous fatty acids

In order to better understand the role of oleate-diol synthase activity in *P. aeruginosa*, several phenotypic features of the mutants lacking this activity were studied. As an initial approach, growth of mutants ΔPA2077 and ΔPA2078 and wild type strain PAO1 was performed in order to determine if fatty acid metabolism was altered in oleate-diol synthase mutants and to evaluate if the corresponding derived oxylipins could contribute to growth by adding an exogenous energy source. Therefore, the three strains were grown in minimal medium supplemented with either oleic acid or the exported 10-HOME and 7,10-DiHOME oxylipins as the sole carbon source.

As shown in Fig. 19, the growth rates and overall cell densities between the wild-type PAO1 and mutant strains were not significantly different when they were grown with either substrate used. Therefore, oleic acid can be metabolized and used for biomass production by all strains, indicating that diol synthase activity is not a strict requirement for nutrient uptake and biomass production. However, the use of 10-HOME and 7,10-DiHOME is slightly less efficient, both being poorer substrates for growth. As fatty acid energy metabolism is not compromised in oleate-diol synthase mutant strains, including the 7,10-DS negative ΔPA2078 mutant, which does not express any of the enzymes involved, we suggest that oleate-diol synthase metabolism is probably more related to environmental adaptation or detoxyfication.

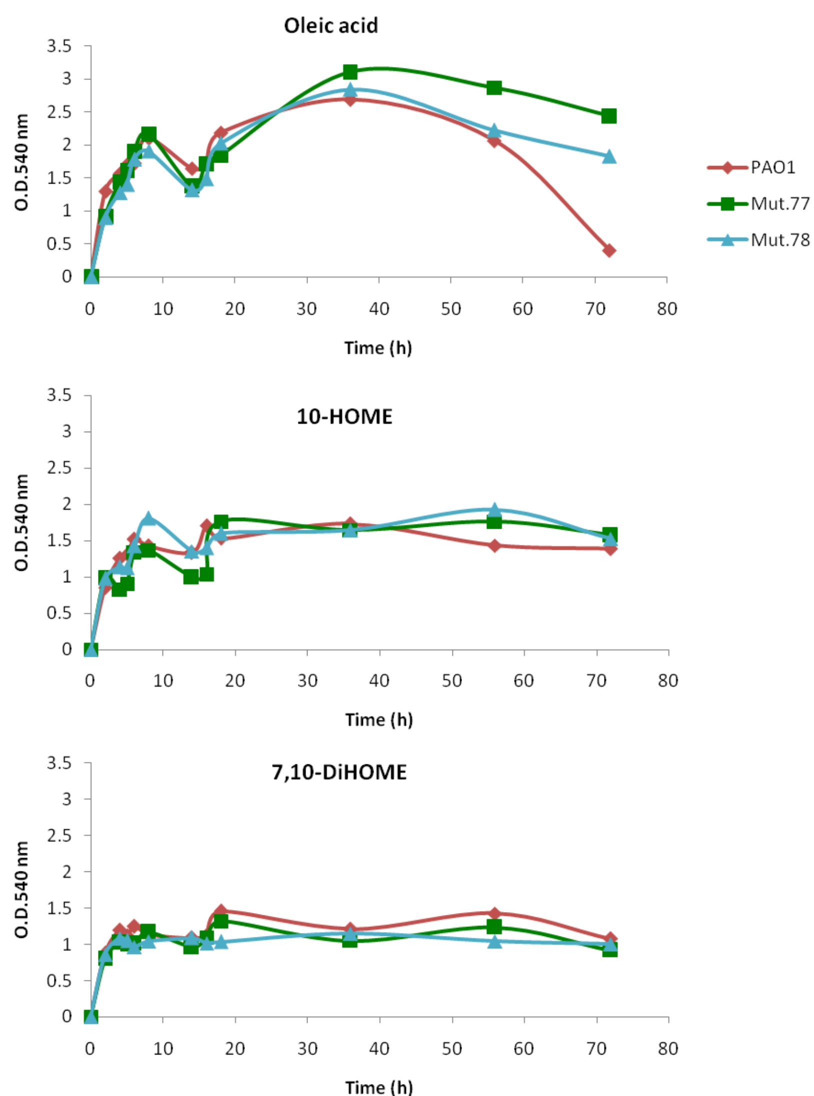

Figure 18. Growth curves of wild type PAO1 andthe mutant strains ΔPA2077 and ΔPA2078. The three strains were grown in 1 x M9 minimal medium supplemented with 0.1% different substrates as the sole carbon source, in the presence of 1% Triton X-100. From top to bottom oleic acid, 10-HOME and 7,10-DiHOME. The y-axes show optical density (O.D.) at 540 nm and the x-axes show time in hours. The method used, with some modifications, is described in Kang *et al.*, 2008 (Kang *et al.*, 2008). Briefly, *P. aeruginosa* strains were grown overnight at 37°C in TSB medium. Overnight cultures were washed twice with one volume of 1 x M9 buffer and resuspended in an equal volume of the same buffer. Resuspended cultures were then diluted 100-fold into fresh 1 x M9 + 0.1% of each substrate. Growth was then initiated and at each time point, aliquots of the culture were diluted four-fold in 4% Triton X-100 and O.D. 540 nm measurements were registered.

3.5.2. Phenotype variation in absence of substrate: Biofilm formation

Additional information concerning the role of oleate diol synthase of *P. aeruginosa* was obtained from the study of the phenotypic variations in mutants ΔPA2077 and ΔPA2078 through the study of biofilm formation. Biofilms have been related since long ago to niche colonization and are considered a persistence feature of great relevance in *P. aeruginosa* strains for environmental adaptation or pathogenesis (Høiby *et al.*, 2010). It is also known that biofilms are communities of microbes attached to surfaces that form stable communities, which can be found in medical, industrial and natural settings. In fact, life in a biofilm probably represents the predominant mode of growth for microbes in most environments. Biofilm microbes are typically surrounded by an extracellular matrix that provides structure and protection to the community. Microbes growing in a biofilm also have a characteristic architecture, generally constituted by macrocolonies (containing thousands of cells) surrounded by fluid-filled channels which are also notorious for their resistance to a range of antimicrobial agents including clinically relevant antibiotics (O'Toole, 2011).

One of the main features found in relation to biofilm formation in the absence of oleic acid (Fig. 19) was the great variability shown by the different strains, a fact that probably reflects the specific properties of each strain in terms of capacity of colonization and/or pathogenicity, as happens for the two wild type strains assayed (PAO1 and 42A2), showing different biofilm formation patterns after overnight incubation. An interesting finding was that in both mutants ΔPA2077 and ΔPA2078, there was a significant increase of biofilm formed at 4 hours growth, but this biofilm production capacity was inhibited later, as shown by the sharp decrease found after overnight incubation, an opposite phenotype behaviour compared to the rest of evaluated strains. This phenomenon was more drastic in ΔPA2077 mutant, where the hydroperoxide-converting enzyme (PA2078; 7,10-DS) is fully functional.

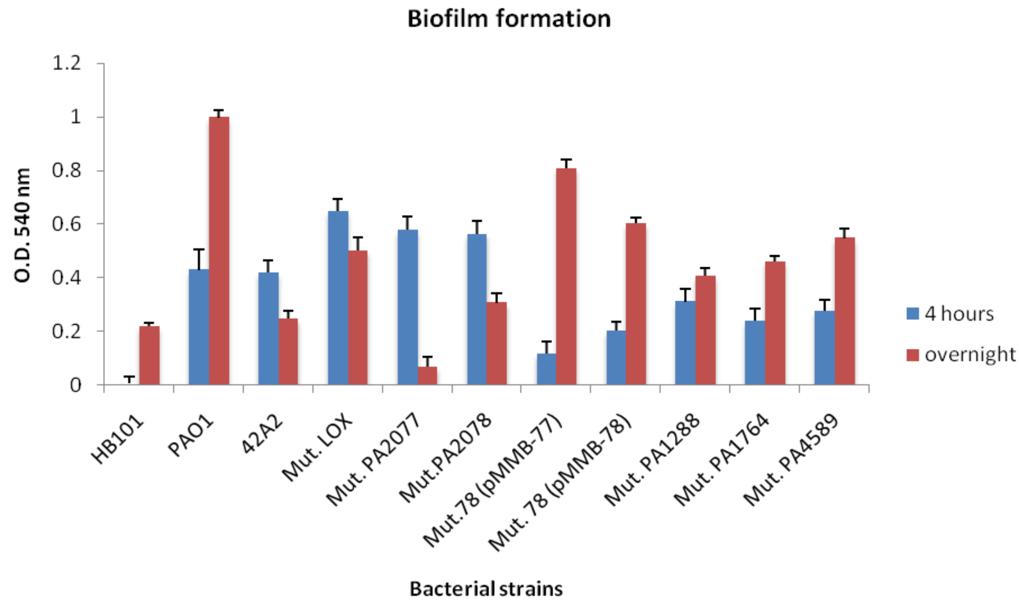

Figure 19. Quantitative microtiter plate biofilm-formation assay. Measures were performed at 4 h and overnight for the different strains assayed, incubated in M63 minimal medium supplemented with casaminoacids at 37°C, following the procedure describe by (O'Toole, 2011)**.** (1) *E. coli* HB101, used as a negative control, (2-3) PAO1 and 42A2 strains exhibiting wild-type phenotypes, (4) LOX mutant strain (ΔPA1169), (5-6) ΔPA2077 and ΔPA2078 mutant strains, (7) ΔPA2078 mutant with PA2077 (10*S*-DOX) expressed in pMMB207 plasmid, (8) ΔPA2078 mutant with PA2078 (7,10-DS) expressed in pMMB207 plasmid, (9-11) FadL-like mutant strains: ΔPA1288 (ExfadLO), ΔPA1764 and ΔPA4589.

Therefore, this results suggest that PA2078 would avoid long-term biofilm formation, probably by consumption of the hydroperoxides contained by the cell, which could be related to the construction of the biofilm matrix.Moreover, in an oleate-diol synthase-free background, the plasmid-based expression of *PA2077* and *PA2078* genes enable a relative decrease of the four hours-incubation biofilm but produce a notable increase of overnight biofilm formation (Fig. 19). The higher biofilm formation observed in mutant ΔPA2078 expressing *PA2077* in *trans* would contribute to support the idea that hydroperoxydes or their corresponding reduced compounds like monohydroxylated fatty acids, could contribute to the biofilm matrix construction. Whereas, overexpression of *PA2078* in a void diol synthase background seems to increase the four hours biofilm but avoids the overnight biofilm formation suggesting a probable regulation of PA2077. An additional interesting feature that contributes to support a relatioship between the oxylipins produced by *P. aeruginosa* and biofilm formation is the fact that all Fad-like transporter mutant strains produced a reduced

amount of biofilm at either incubation time, especially after overnight cultivation, suggesting that lack of oxylipins and other fatty-acid derived compounds in the extracellular medium can indeed affect synthesis of the biofilm matrix.

The microtiter dish assay is an important tool for the study of the early stages in biofilm formation. Because this assay uses static, batch-growth conditions, it does not allow formation of the mature biofilms typically associated with flow cell systems (O'Toole, 2011). Being biofilm formation a multifactorial-dependent trait, the exact implication of the two diol synthase enzymes in biofilm matrix synthesis in absence of oleic acid still remains to be completely elucidated but the results obtained here suggest that the diol synthase operon could contribute to regulate its production by decreasing biofilm primary adhesion. However, when the cell population increases, these enzymes could aid in biofilm formation, probably by late growth-accumulation of hydroperoxide-reduced compounds. This hypothesis could be justified after identification of the presence of changing hydroxyfatty acids in the biofilm matrix (Chao *et al.*, 2010).

3.5.3. Biotechnological production of 10-HPOME and 10-HOME in a GRAS bacterium

Pseudomonas putida is a metabolically versatile saprophytic soil bacterium that has been certified as a biosafety host for the cloning of foreign genes (Federal Register, 1982). Sequence analysis of the 6.18 Mb genome of strain KT2440 reveals diverse transport and metabolic systems. Although showing a high level of genome conservation with the pathogenic Pseudomonad *P. aeruginosa* (85% of the predicted coding regions are shared), key virulence factors including exotoxin A and type III secretion systems are absent. Therefore, these genome features points to potential new applications in agriculture, biocatalysis, bioremediation and bioplastic production (Nelson *et al.*, 2002).

The identification of oleate-diol synthase genes and their distribution in nature, through PCR specific amplification in different strains genomic DNA or by *in silico* analysis of sequenced genomes has led to the conclusion that oleate-diol synthase is unique for *P. aeruginosa* genomes *(Paper I)*. Here, we experimentally demonstrated that diol synthase activity is also absent in the cellular extracts and supernatant of *P.*

putida KT2440 wild type (Fig. 20). However (10*S*)-DOX activity could be detected in *P. putida* when gene *PA2077* was expressed in the shuttle plasmid (pBBRMCS1-77), reflecting that this newly developed estrategy can be useful for biotechnological production of such compound. Moreover, in Fig. 21, released products were identified by LC/MS, and the presence of 10-HPOME and 10-HOME were confirmed in cell extracts of recombinant *P. putida* KT2440/77 incubated with buffer TrisHCl 50 mM pH 7 supplemented with 0.1% oleic acid.

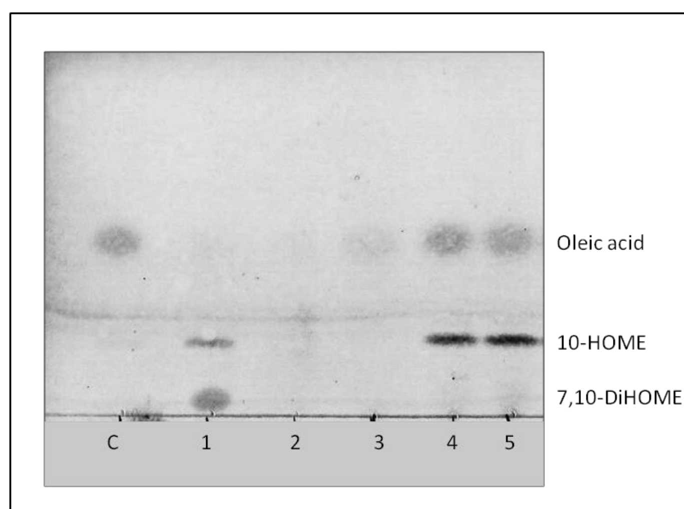

Figure 20. TLC analysis of oxylipins found in crude cell extracts of *P. putida* KT2440 and recombinant clones from *in vitro* bioconversion of 0.1% oleic acid. (C) OA control; (1) cell extract from wild type PAO1, used as positive control; (2) supernatant of *P. putida* KT2440 wild-type (3) cell extract of *P. putida* KT2440 wild-type; (4-5) bioconversions with cellular extract of *P. putida* KT2440 expressing in *trans P. aeruginosa* gene PA2077.

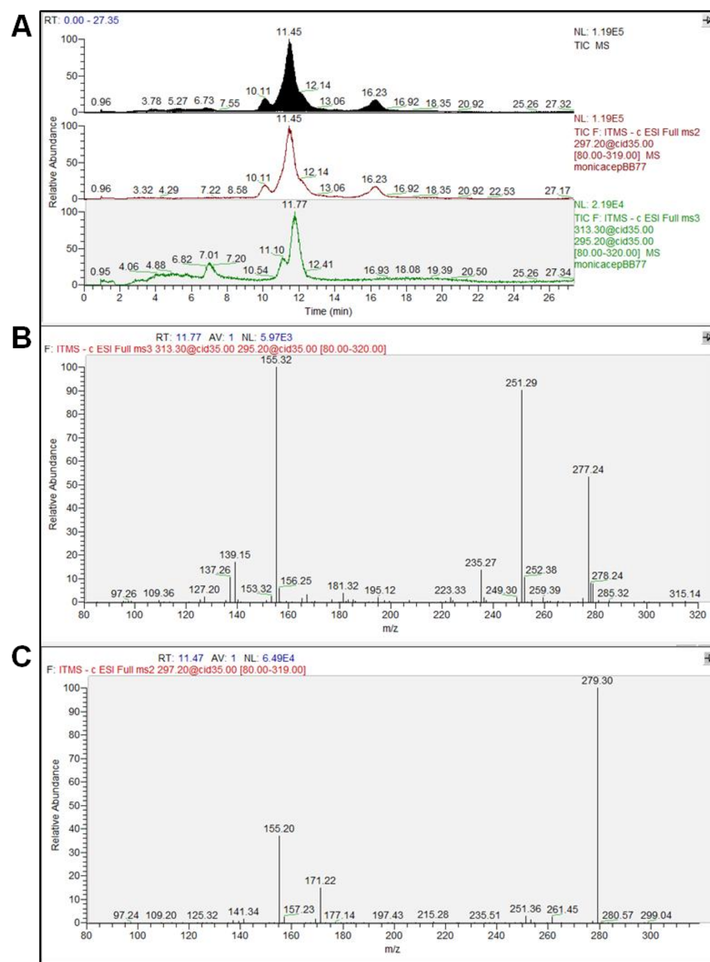

Figure 21. LC/MS analysis of oxylipins found in crude cell extracts of *P. putida* KT2440/*77* from *in vitro* bioconversion of 0.1% oleic acid. A) Total ion current chromatogram shows the most intense peak at 11.45 min retention time. This peak was selected for further analysis. **B)** MS^3 characteristic spectrum (m/z 313→295→full scan) of 10*S*-HPOME yielded signals at m/z 277, 251, 155 and 139. **C)** MS/MS spectrum (m/z 297→full scan) of 10*S*-HOME yielded a characteristic signal at m/z 155, whereas the base peak was m/z 279 (Nilsson *et al.*, 2010).

This constitutes the first biotechnological approach to reproduce oleate-diol synthase activity in microbial cell factories like the non-pathogenic *P. putida* KT2440 strain.The use of a GRAS microorganism for production of the biotechnologically interesting oxylipins naturally released only by *P. aeruginosa* opens up new possibilities for production and contributes to the widening of their industrial uses, providing a new methodology for biotechnological production of such compounds.

4. DISCUSSION

This research work was focused in the study of *P. aeruginosa* oleate-diol synthase system, and was accomplished within the group of Biotransformations of oily-wastes, located at the Department of Microbiology and Sanitary Parasitology of the Faculty of Pharmacy, in collaboration with the group of Microbial enzymes at the Department of Microbiology of the Faculty of Biology, at the University of Barcelona.

In order to obtain bioactive compounds from environmental microbial sources, one key option is the isolation of new bacterial strains from environment or microbial screening of contaminated media such as oily-waste water, where the extreme and metabolic stressfullly conditions function as a selective pressure for bacterial survival and adaptation. Therefore, several environmental strains of *Pseudomonas aeruginosa* (42A2, 47T2, or PR3 among others) were previously isolated and identified in these environmental niches (Robert, 1989; Kuo *et al.*, 1998; Hasanuzzaman *et al.*, 2004). These strains were shown to be able to use fatty acids as the sole carbon source and to generate oxygenated substrate-derivated products, identified as microbial oxylipins, which were found to accumulate in the extracellular medium (Mercadé *et al.*, 1988; Guerrero *et al.*, 1997). These natural hydroxy fatty acids are interesting by their physical and chemical features as biosurfactants, and also due to their antibacterial and antifungal properties (Mercadé *et al.*, 1988; Culleré *et al.*, 2001; Shin *et al.*, 2004; Martín-Arjol *et al.*, 2010). Moreover, they can act as monomers for the synthesis of polymeric emulsifiers, the estolides, and as building blocks for fine chemical synthesis in the pharmaceutical area (Hou, 2008; Martín-Arjol *et al.*, 2015). Nowadays, the biotechnological potential of oxylipins is constantly increasing mostly due to the limited sources of hydroxyl fatty acids and their expansive use in industry. Indeed the specificity of microbial enzyme biotransformations competes with a non-sustainable although cheaper and complex chemical synthesis (Hou, 2008; Andreou *et al.*, 2009). Thus, the basic and applied research to get knowledge of *P. aeruginosa* oxylipin synthesis is at its peak. In this context, recent research has focussed on the study of different fatty acids susceptible of being used as substrates for creating new hydroxylated compounds, the use of residual fatty acids as sources for fermentation to reduce production costs and to get green-technology process, the bioreactors processes, the optimization of the production rates, the improvement of downstream

methods for product purification, and the chemical formation of new derived products.

However, although oxylipins are well-described and characterized molecules in animals, plants and fungal microorganisms and their biological relevance and formation have been discussed over the years (Bergström *et al.*, 1968; Vick and Zimmerman, 1984; Smith, 1989; Hamberg and Gardner, 1992; Stahl and Klug, 1996; Brodhun and Feussner, 2011), microbial oxylipins are still understudied (Andreou *et al.*, 2009). Basic research has revealed a heterogeneous and vast number of oxylipin-forming enzymes, which catalyze fatty acid oxygenation, but the identification and biochemical characterization of the systems involved in bacterial oxylipin formation continues to be a blank sheet.

In 2010, more than twenty years after the first identification of an oxylipin derived form oleic acid in *P. aeruginosa* (Parra *et al.*, 1990), the oleate-diol synthase activity was biochemically characterized with description of the enzymatic mode of action. *P. aeruginosa* oleate-diol synthase displays a particular activity divided in two catalytic steps, a feature also described for other oxylipin-forming enzymes, where the substrate is oxygenated to a hydroperoxyde fatty acid and then isomerized to the diol form, as it happens for example in fungal linoleate diol synthases but with monounsaturated oleic acid as the preferred substrate (Martínez *et al.*, 2010).

Taking into consideration the development of the research field, this investigation was aimed at the comprehensive analysis of *P. aeruginosa* oleate-diol synthase system, which was achieved through functional characterization and *in silico* analysis of new genes, transporters and enzymes involved in the process in order to get further knowledge on its biological nature and to provide the required tools for microbial biotechnology. Due to the availability of *P. aeruginosa* PAO1 genome, this strain was selected as a blueprint for most assays performed in order to understand the physiology of oxygenated fatty acid production.

Nowadays, genome projects are generating an unprecedented amount of information regarding the identification and structure of genes. The vast number of uncharacterized genes and proteins found in different organisms, has led to an explosion of molecular biological information (Kandpal *et al.*, 2009). The *Pseudomonas* genome database (http://www.pseudomonas.com) has played an important role in

maintaining updated information via community-based sequence annotation and more recently, providing valuable tools for comparative genomics (Stover *et al.*, 2000; Winsor *et al.*, 2011).

However, after 20 years of microbial genome sequencing and analysis, and 15 years of *Pseudomonas* genome sequencing, we have barely scratched the surface of the complex microbial world. As an example, half of the genes in the *P. aeruginosa* genome have either no homology to any previously reported sequence or are homologs to previously reported genes of unknown function (Wiehlmann *et al.*, 2007). Between the original manual PAO1 genome annotation in 2000 and a subsequent reannotation (Stover *et al.*, 2000; Weinel *et al.*, 2003), up to 75% of the 5570 ORFs have been assigned probable functions but only approximately 7% of the genes have been experimentally assigned a function in *P. aeruginosa* (Levesque, 2006).

The era of –omics has been complemented by a new post-omics approach, which is mostly based in functional genomics studies by the use of highly effective methods like transposon-based strategies for functional complementation (Suen *et al.*, 2007). Moreover, expression data as transcriptome, proteome and phenoarray or metabolome analysis should be sought as a matter of priority to verify transcriptional activity and to identify the signals and environments that trigger expression (Nouwens *et al.*, 2003; Silby *et al.*, 2011; Lecoutere *et al.*, 2012). Therefore, a selective search of available –omics data and bioinformatics sources constitute nowadays strong tools for getting closer to gene role-assignment.

In this project, our first attempt to identify oleate-diol synthase activity-related genes was to accomplish a reverse approach, starting up from a known activity to find the gene or genes involved in the process (Fig. 22). For doing so, we took advantadge of previously designed and published partial purification results obtained from oleic acid-induced periplasmic extracts of *P. aeruginosa* 42A2 (Martínez *et al.*, 2010).

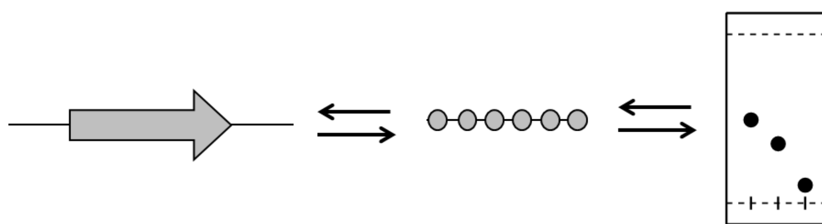

Figure 22. Flux diagram of reverse/direct approach for oleate-diol synthase-coding genes identification. From right to left: Gene→Protein→Activity, shown in a TLC plate.

According to Martínez and coworkers, *P. aeruginosa* oleate-diol synthase activity displayed the following features: the two activities involved in the oleate-diol synthase system co-eluted after gel-filtration and ionic exchange chromatography purification, showing complete bioconversion of oleic acid even when using small, 10 µL aliquots of the purified extracts. The molecular mass was estimated to be 50 kDa, with optimum pH and temperature at 7.0 and 37°C, respectively. However SDS-PAGE analysis showed several protein bands at the final stage of purification. MALDI-TOFF analysis of the most significant bands revealed no homology to any previously described oxygenase, indicating that the enzyme or enzymes involved in diol synthase activity were not the major purified proteins, although full bioconversion was found. The finding of activity even when a minimal protein concentration was present in the partially purified samples, indicate that very low amount of enzyme is required for activity, being the diol synthase activity a non-majority protein in the extract (Martínez *et al.*, 2010). Taking into consideration these features, a further selective-periplasmic subproteome was obtained by 2D electrophoresis and subsequent proteomic analysis of defined spots was achieved. Again, no oxygenases could be identified (data not shown), confirming the hypothesis that the enzymes or enzymes involved in oleate-diol synthase activity are minoritary among periplasmic proteins.

As mentioned before, transposon-based signature-tagged mutagenesis and genetic footprinting strategies have pinpointed essential genes and individual proteins. Also, protein complexes can be dissected by transposon-mediated scanning linker mutagenesis (Hayes, 2003; Silby *et al.*, 2011). The complementation of the mutated genes constitutes a test of function, which determines if two putative alleles, being in the same cell and acting independently, can supply all functions necessary for restoration of mutant to wild-type phenotype. This kind of strategy could be

considered as a direct approach as in this case, gene expression is altered as revealed by lack of activity.

However, obtaining a saturated transposon-mutant library of the whole genome is complex and must be coupled to a rapid and effective screening method for analyzing the mutated phenotypes. In addition, the procedure to identiy mutated genes and to confirm transposon location is time-consuming and the results involve genes that could be directly or indirectly linked to the enzymatic activity under study like transcriptional regulators, chaperones, transport proteins or unknown enzymes andgene products. Accordingly, the complementation and recombinant expression of each gene for the detection of new enzymes may be a challenge.

In 2003 and 2005 Jacobs and Lewenza and coworkers constructed saturated transposon mutant libraries in PAO1 strain (Jacobs *et al.*, 2003; Held *et al.*, 2012). The first library was sequence-verified by Held *et al.*, in 2012, and mutants of both PAO1 libraries are completely available to the research community (http://pseudomutant.pseudomonas.com/), creating a huge potential base for increasing the number of functional genomics analysis. Such mutant library was used in the present project for functional characterization and annotation of three previously undescribed genes from *P. aeruginosa* PAO1 genome and the study of possible transcriptional regulators putatively related with theoleate-diol synthase system through several approximations.

After the demonstration that PAO1 oleate-diol synthase activity was located in the periplasm, associated to the bacterial cell, like in *P. aeruginosa* 42A2, whereas the released products 10-HOME and 7,10-DiHOME were accumulated in the extracellular medium, we found by genome homology and by comparative genomics approach, three putative FadL-like proteins (ORFs PA1288, PA1764 and PA4589) in *P. aeruginosa* PAO1 that might be involved in fatty acid and oxylipin transport. Therefore, we tested if the FadL-like proteins were involved in the transport of these oxygenated LCFAs across the OM from the periplasm to the extracellular medium, confirming that *PA1288* gene, named as ExFadLO, was indeed involved in the exportation of oleate-derived oxylipins, being the first outer membrane transporter for exportation of fatty acids described in bacteria that belongs to the FadL-family of proteins *(Paper I).*

Additionally, in view of the complexity of the selective oleic acid-induced periplasmic subproteome (Imperi *et al.*, 2009) and due to the existence of extensive sequencing projects and the availability of powerful bioinformatics tools, we decided to follow a reverse strategy, from gene to function (Fig. 22), in order to catch the gene or genes involved in oleate-diol synthase activity in *P. aeruginosa* genome. For such purpose, a rational genome-based approach was folowed, taking into consideration those enzymes with similarity and analogous activity to lipoxygenases, diol synthases, cyclooxygenases, dioxygenases and P450, either functionally or *in silico* annotated *(Paper II)*. This search, in combination with a blind-approximation strategy by means of full screening of all possible diol synthase candidate genes in the oleate-induced transcriptome and periplasmic subproteome, using the data available from other parallel research projects (Nouwens *et al.*, 2003; Kang *et al.*, 2008; Imperi *et al.*, 2009; Kang *et al.*, 2009; Martínez *et al.*, 2010), allowed identification of several candidates that were further experimentally tested for oleic acid bioconversion. Among the set of selected candidates, a unique gene, *PA4447* accomplished all proposed criteria: i) genes being over expressed during OA induction, ii) genes coding for enzymes that could be involved in oxygenation like copper/heme-containing proteins and other oxidoreductases, iii) genes with probable periplasmic location and iv) proteins with a predicted size of 30-70 KDa. However, *PA4447* mutant strain exhibited the wild type phenotype. On the contrary, the phenotype of the transposon-mutated selected candidates was anomalous in two of them, the mutants at ORFs PA2077 and PA2078. These mutant strains, with the transposon inserted in genes coding for ‘hypothetical proteins’, did not display accumulation nor formation of oleate-derived oxylipins. Subsequent analyses were performed to unveil if the two identified oleate-diol synthase-related genes involved a single enzyme and its transcriptional regulator or two independent enzymes. These assays were mainly performed by mutant complementation tests. Under the conditions used, a *cis*-dominant mutation was observed as the insertion of the transposon in ORF PA2078, affecting expression of ORF PA2077, tandemly located in the chromosome, regardless of the nature of the *trans* copy. Moreover, we described that both genes constitute an operon, a fact that justifies the *PA2078* polarity affecting expression of *PA2077* and correlates well with the finding of defined features at the intergenic region *(Paper II)*.

In addition to the mutant complementation results showing that *P. aeruginosa* oleate-diol synthase activity is the result of the combined and sequential action of two different enzymes (PA2077 and PA2078), coded by finely tuned operon, the individual recombinant expression of each gene in *E. coli (Paper I)* and *P. putida* KT2440 *(Adittional results)* has demonstrated the same oxylipins pattern, confirming the results obtained in complementation tests in which PA2077 is involved in the initial 10*S*-DOX activity and PA2078 corresponds to the 7,10-DS activity, with no requirement for heterocomplex formation. Both strains were able to export oleate-derived oxylipins to the extracellular medium even if the diol synthase is not present (data not shown). This may be due to the existance of *exFadLO* homologous genes *(Paper I)*. These data and the fact that *exFadLO* is transcribed and expressed without any known inductor (Nouwens *et al.*, 2002; Bauman and Kuehn, 2006) suggest that although it directs the export of oleate-derived oxylipins, it may also display a role in the transport of other hydrophobic molecules.

As previous studies of *P. aeruginosa* oleate-diol synthase had been performed using strain 42A2, which might exhibit a different genotype (Fernández *et al.*, 2005), we sequenced the oleate-diol synthase operon in this strain and showed an overall 98% sequence identity to *P. aeruginosa* PAO1, with an identical *PA2078-PA2077* intergenic region (#GenBank KJ372239), being a completely homologous systems *(Paper II).*

In order to find out the evolutionary genomic model for oleate-diol synthase genes, we evaluated the phylogenetic relationship between homologous genes available in the genome databases. This transcriptional operon unit is present only in *P. aeruginosa*, where all genomes sequenced so far display the same genes with identical architechture. These results indicate that this unique operon could be the result of a gene duplication event inherited from a common ancestor, maintaining their adjacent location. However, as biochemical activity has been demonstrated to be different for each gene, we have proposed that a neofunctionalization of one of the genes should also have occurred *(Paper II).*

Gene duplication has been described as a form of adaptation to stress environment, leading to either dosage benefits or neofunctionalization of the duplicated copies (Serres *et al.*, 2009; Bratlie *et al.*, 2010). In a recently proven theory of innovation-amplification-divergence, an evolutionary successful model has been proposedin

which the diversification of the gene function appears after duplication and stabilizes it providing gains. The subsequent specialization of the paralogous (duplicated genes) under continuous selection would allow for an efficiency increase that outweighs adaptation costs directly linked to gene duplication itself (Näsvall *et al.*, 2012). In the oleate-diol synthase context, the subspecialization of the paralogous genes could indeed lead to their conservation, as observed for all *P. aeruginosa* strains. A possible explanation for the evolutionary conservation of *P. aeruginosa* diol synthase operon was hypothethized by the observations of *in vivo* overexpression of gene *PA2077*, the hydroperoxyde-forming enzyme, in the genetic context of mutant ΔPA2078, where the whole operon expression is blocked, which resulted to be lethal *(Paper III)*. These results suggest that overexpression of gene *PA2077* has a toxic effect due to the properties of the products released by the encoded functional PA2077 (10*S*-DOX) that could possibly act on other cellular unsaturated fatty acids, leading to accumulation of high concentrations of organic hydroperoxides, which could damage DNA, proteins and membrane phospholipids, causing cell lysis (Imlay, 2008; Rhea M Miller *et al.*, 2008; Garreta *et al.*, 2013). In fact, the physiological range of PA2077 substrates, as for other novel dioxygenases, remains still to be elucidated and no other characterized bifunctional diol synthase activity has been overexpressed in the original strain so far. Moreover, the genome disposition of the diol synthase operon has been observed previously in an ancient relative of a cyclooxygenase in cyanobacteria, which is a linoleate 10*S*-dioxygenase that works in tandem with a catalase-related protein with specific 10*S*-hydroperoxide lyase activity (Brash *et al.*, 2014).

The observed cytotoxicity of 10*S*-DOX could also explain the existence of ExFadLO for specific efflux of oleate-diol synthase oxylipins, acting as a detoxifying element. However, the fact that *exFadLO* is not over-expressed in the oleate-induced transcriptome, being also found under non-induced culture conditions (Bauman and Kuehn, 2006; Kang *et al.*, 2009; Lecoutere *et al.*, 2012) or in other strains without oleate-diol synthase genes, suggest that oxylipin exportation is not the unique function of this outer membrane transporter.

The finding that the set *PA2077-PA2078* is conserved only in *P. aeruginosa* and not in other *Pseudomonas* species by *in silico* and *in vivo* analysis evidences the paralog relationship of the duplication event and confirms that the encoded oleate-diol

synthase activity is indeed unique among bacteria. This explains why oleate-diol synthase activity has only been described and characterized in *P. aeruginosa (Paper II)*.

Recently, three *P. aeruginosa* isolates named KK, were sequentially collected from the sputum samples of a cystic fibrosis (CF) patient reported at the CF clinic of Hannover (Germany). The KK1 strain corresponds to the primo-colonization stage, the second KK14 strain was isolated 18 months later, and the third strain KK72 was recovered 156 months after the KK1 strain during antibiotic treatment. The genome of the three strains was compared to that of PAO1, and regions of genome plasticity were identified. Surprisingly, one of these regions, the KK12_region is conformed by a non-fagic insertion of 57.2 kb involving mercuric resistant coding-proteins (*merE*, *merD*, *merA*, *merP*, *merR* and *merT* homologs, corresponding to an RND multi-drug efflux pump and a regulator of EAL (Lucchetti-Miganeh *et al.*, 2014). Interestingly, the insertion is located upstream gene *PA2077*, at the intergenic region of the diol synthase operon. Although both *PA2078* and *PA2077* genes are present in the genome of these strains in a wild type, non-mutated way, oleate-diol synthase activity was absent in all these strains when they were evaluated for *in vivo* bioconversion using oleic acid as substrate *(Paper III)*. These results confirm the following previous conclusions: *i*) oleate-diol synthase activity requires that *PA2078* and *PA2077* genes are tandemly positioned in *P. aeruginosa* genome, constituting a specific type of operon*; ii*) oleic acid is not a substrate for PA2078 enzyme, as shown by the fact that no oleate-diol synthase activity was detectedin KK-strains bearing a genomic background where the 5'-untranslated region upstream *PA2078* remains intact; *iii*) absence of an internal promoter located at the *PA2078-PA2077* intergenic region, as shown by lack of oleic acid bioconversion to 10-HPOME; *iv*) the proposed gene duplication event preceeded the 57.2 Kb insertion of mercury-resistance genes in KK strains. Therefore, *P. aeruginosa* KK-related strains constitute the first *P. aeruginosa* strains reported so far without oleate-diol synthase activity and indeed reinforce our previous conclusions.

Although *P. aeruginosa* genome mining has constituted a useful tool for identification and functional characterization of oleate-diol synthase genes, the nature of the coded enzymes was still unsolved. Therefore, *in silico* and *in vivo* site-directed based mutagenesis analysis were conducted to classify 10*S*-DOX and 7,10-DS enzymes among

other known oxygenases, as we observed that they are unprecedented enzymes among bacteria, showing no relation with other oxylipin-forming enzymes described so far. These features prevented construction of a valid homology 3D model and the visualization of a predictable 3D structure for fatty acid docking predictions. Therefore, the possible catalytic regions of the two enzymes were identified after in *silico* prediction. Protein sequence analysis and *in vivo* mutagenesis studies confirmed that both proteins have di-heme groups, being related to other bacterial di-heme cytochrome c peroxydases (CCPs), although constituting a new subfamily named FadCCPs due to their functional relation to fatty acids. No obvious phylogenetic relationship between fungal and *P. aeruginosa* diol synthases was found. Thus, the oleate-diol synthase activity studied here constitutes the first case described where two separated enzymes (corresponding to DOX and DS activity) are responsible for the bioconversion of oleic acid instead of the bifunctional enzymes (DOX-CYP) described before for other fungal diol synthases or monomeric enzymes like PaLOX *(Paper III)*.

As for other fatty acid-oxygenases, 10-DOX and 7,10-DS display two heme groups, constituting essential regions for catalysis. Both hemes are required by 10*S*-DOX to perform oleic acid bioconversion, whereas surprisingly mutation of these groups in PA2078 does not affect 7,10-DS activity, indicating that the catalytic center is located somewhere else in the sequence of this enzyme. The loose of these functional groups in 7,10-DS could be explained by accumulation of mutations, as we observed the insertion of numerous and variable palindromic insertion sequences in protein-coding regions, leading to enzyme activity diversification (Claverie and Ogata, 2003; Ogata *et al.*, 2005; Suyama *et al.*, 2005). To our knowledge, these evolutive insertion elements are described for the fist time here in *P. aeruginosa* genome. Diversification could preceede neofunctionalization, being PA2077 probably closer to the original antecesor gene as it maintains the functionality of the di-heme groups. Moreover, oxygenase activity of a di-heme protein has been reported once in a distant relative of Pseudomonads, *Xanthomonas* sp. 35Y strain, which expresses a Rubber Oxygenase A enzyme (RoxA), involved in the degradation of this highly hydrophobic polymer (Jendrossek and Reinhardt, 2003). It was not until ten years after the gene product identification, that crystallization and functional mutagenesis studies of RoxA were achieved, indicating a complex catalytic mechanism of these type of enzymes (Seidel *et*

al., 2013), among which *P. aeruginosa* 7,10-DS activity remains still far from being biochemically characterized. Therefore, heterologous-expression systems should be developed and optimized for overexpression, purification and crystallization of the new oleate-diol synthase proteins described here, to allow description of their catalytic requirements and chemical features, using new substrates in order to fully understand the whole process of LCFA bioconversion into oxylipins.

Unexpectedly, attempts to reproduce expression in *trans* of *PA2078–PA2077* operon under the native promoter in each mutant strain, simulating the original genome conditions, failed *(Paper II)*. This suggests that specific regulation elements may be involved in diol synthase activity expression control. Therefore, in order to assign a functional role to diol synthase activity in the global biological cell processes of *P. aeruginosa*, we selected possible transcriptional regulators that could affect expression of the operon, and analyze oleic acid bioconversion in their respective mutants. Specific transcription regulators were selected by bioinformatic prediction of several transcriptional factor-DNA binding sites described through the position weight matrices-based method for *P. aeruginosa* PAO1 (Mu *et al.*, 2005). These data, displayed in combination with the folded DNA structures and hybridization prediction (Zuker, 2003), revealed the presence of specific binding sites exposed to several transcription factors in the region upstream *PA2078*.

Among the predicted and selected transcriptional regulators, NarL and FleQ regulators (Jyot *et al.*, 2002; Schreiber *et al.*, 2007) not only were *in silico* predicted here but also have been shown to increase the number of controlled genes when OA or phosphatidiletanolamide (PE) are present (Fig. 23), as shown in a comparative study of the differential global regulatory networks of OA-induced versus non-induced and OA-induced versus PE-chemotaxis induced transcriptome in *P. aeruginosa* PAO1 (Klein *et al.*, 2008; Miller *et al.*, 2008; Kang *et al.*, 2009).

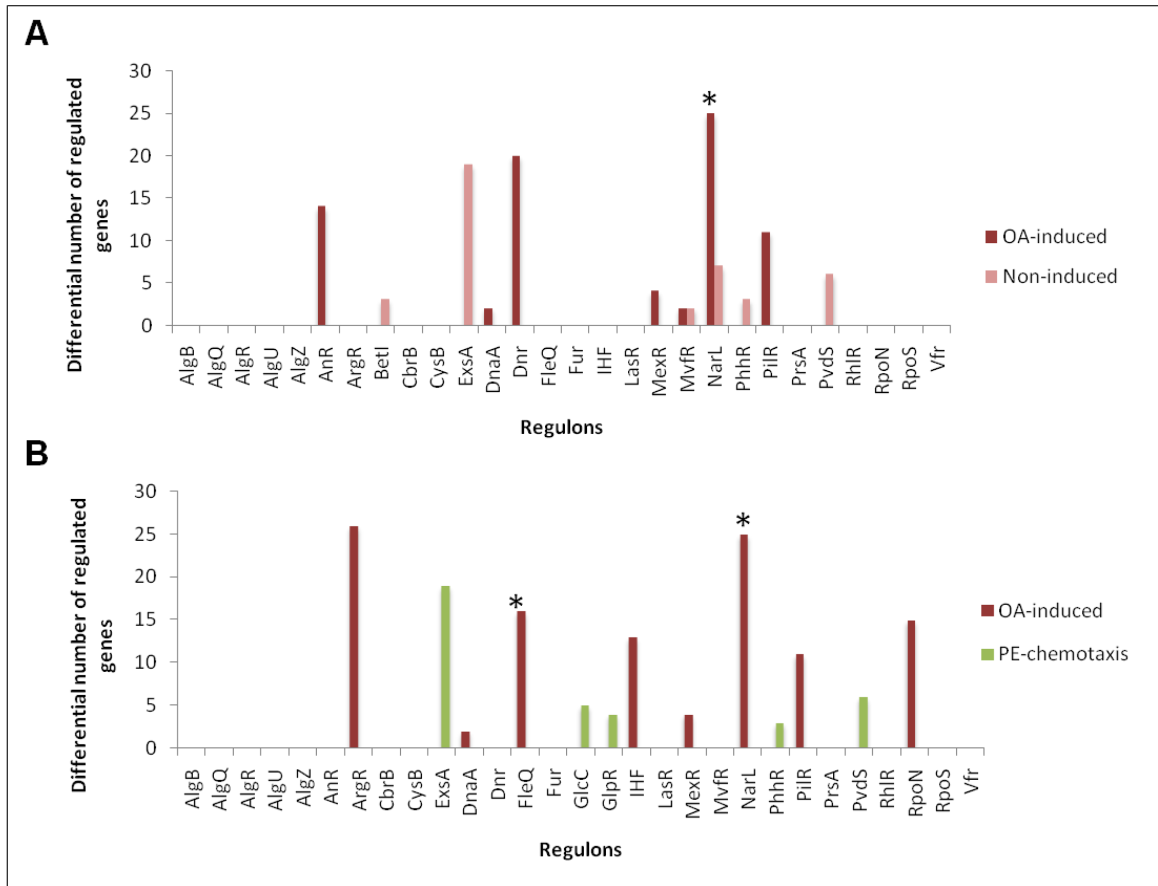

Figure 23. **Differential number of regulated genes in the regulatory networks inof *P. aeruginosa* PAO1. A)** Oleic acid-induced versus non-induced transcriptome. **B)** Oleic acid (OA)-induced versus phosphatidiletanolamide(PE)-chemotaxis induced transcriptome. Zero values indicate no variation rates in the number of regulated genes. Asterisks point to those regulons whose variation is higher and show predicted binding-sites in the *PA2079-PA2078* intergenic region (Miller *et al.*, 2008; Kang *et al.*, 2009). Number of regulated genes has been obtained by Prodonet software (http://www.prodoric.de/prodonet/) (Klein *et al.*, 2008).

Moreover, taking into consideration the genomic environment of ORFs PA2077 and PA2078, a homologous gene of LysR transcriptional regulator PA2076 was predicted, being conserved in all *PA2078-PA2077* orthologs found up to date.

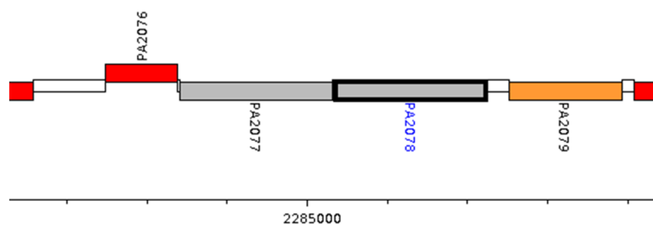

Figure 24. Genomic environment of oleate-diol synthase operon. The exact genomic location of ORF PA2076 is at position 2282480-2283382 (+ strand) of *P. aeruginosa* PAO1 chromosome (www.pseudomonas.com).

This gene was also selected as a putative transcriptional regulator element due to its homology to LysR-type transcriptional regulators, involved in controlling a diverse set of genes, including those related to virulence, metabolism, quorum sensing and motility in *P. aeruginosa* (Maddocks and Oyston, 2008; Reen *et al.*, 2013). Unfortunately, the results suggested that the global transcriptional regulators explored so far involved in flagellar gene regulation, mucin adhesion, quorum-sensing or nitrate-response (Galán-Vásquez *et al.*, 2011) are not essential for regulation of oleic acid-induced oleate-diol synthase, whose actual transcription elements remain still unknown *(Paper II).*

Besides the previous considerations, the diol synthase operon must be finely regulated because a transcriptonal discoordination in expression of the two genes was observed in proteomic analysis, where gene *PA2078* was found to be expressed to a double extent than gene *PA2077* (Miller *et al.*, 2008; Kang *et al.*, 2008), a fact that probably allows for an accurate enzymatic control of organic hydroperoxide production and accumulation. Therefore, the operon nature and disposition of oleate-diol synthase genes within the genome would have been maintained during evolution to keep the differentiated functions of the two encoded enzymes, probably addressed to LCFA oxygenation (PA2077, 10-DOX) and further detoxification/control mechanism (PA2078, 7,10-DS) to avoid the intracellular effects of hydroperoxide accumulation.

Nevertheless, although the occurrence of oxylipins and enzymes involved in their biosynthesis has been studied for nearly three decades, knowledge about microbial oxylipins is still scarce as compared with the situation in plants and mammals (Brodhun and Feussner, 2011; Pohl and Kock, 2014). The physiological function of fungal oxylipins has been mostly studied in the genus *Aspergillus* where the producing enzymes are named as psi-factors, involved in the regulation of the sexual and asexual sporulation rates (Tsitsigiannis *et al.*, 2005; Horowitz B *et al.*, 2008) and host-pathogen communication, promoting fungal infection of plants (Tsitsigiannis and Keller, 2007; Christensen and Kolomiets, 2011). Recently, a mutation in *lds1* (linoleate-diol synthase 1) in the fungal maize pathogen *Fusarium verticillioides* has demonstrated that LDS1-derived oxylipins are involved in controlling growth, sporulation and mycotoxin (fumonisin) production, as well as in shaping of the oxylipin fungal profile during host-

pathogen interaction (Scala *et al.*, 2014). However, the physiological relevance of bacterial oxylipins is undescribed so far.
We have observed that the interruption of the operon oleate-diol synthase does not affect the viability of the KK-related strains as it happens for PA2078 mutant strains in the environment under non-selective pressure. Accordingly, these genes seem to be non-essential for bacterial survival although profitable for environmental adaptation. Moreover, as the oleate-diol synthase deficient *P. aeruginosa* KK-related strains are in fact human pathogens, we conclude that oleate-diol synthase activity is probably not essential for bacterial pathogenesis, at least for cystic fibrosis infections.
We must also keep in mind that oleic acid and its derived-oxylipins can be metabolized for bacterial biomass production in both, wild type and mutant strains, as no significant differences were observed in growth of ΔPA2078 and ΔPA2077 mutants compared to the wild-type PAO1 strain, when they were cultured in minimal medium with oleic acid and oxylipins as a sole carbon source *(Additional results).* Bioreactor studies of the yield in the production of OA-derived oxylipins in *P. aeruginosa* 42A2 have confirmed that the detection of the 10-HPOME, 10-HOME and 7,10-DiHOME correspond to the stationary growth phase, being thus defined as secondary metabolites as 10-HOME and 7,10-DiHOME remains accumulated in the extracellular medium (Gross and Loper, 2009; Torrego-Solana *et al.*, 2012; Martín-Arjol *et al.*, 2014).
Moreover, oleate-diol synthase products can act as biosurfactants, being 7,10-DiHOME more efficient than 10-HOME (Parra *et al.*, 1990). As previously reported, non oleic acid emulsifiers are needed in bioreactor productions of these oxylipins as themselves act as such compounds (Kuo *et al.*, 2003; Martín-Arjol *et al.*, 2014). This suggests that oleate-diol synthase products, especially the final product 7,10-DiHOME, could indeed improve the solubilization of the substrate in the medium thus allowing a higher proportion an upper rate of available oleic acid even though used at high concentrations (Mercadé *et al.*, 1988).
Emulsifiers or biotensioactive compounds are produced by a wide variety of microorganisms and may exhibit different biological functions like enhancing the growth of bacteria on hydrophobic water-insoluble substrates by increasing nutrient bioavailability, presumably by widening their surface area, desorbing them from surfaces and increasing their apparent solubility (Shreve *et al.*, 1995), or by increasing

the bioavailability of hydrophobic substrates by rising their apparent solubility or desorbing them from surfaces (Rosenberg and Ron, 1999). In addition, they are involved also in regulating the attachment–detachment of microorganisms to and from surfaces (Ron and Rosenberg, 2001). This could represent the perfect strategy to improve the solubility of hydrophobic substrates in the culture medium, increasing both, biomass and derivate-products formation.

Based on the unique described biological properties of the products released by oleate diol synthase activity, diverse strategies for environmental adaptation of *P. aeruginosa* could be attributed to oleate-diol synthase metabolism. To start with, several oxygenated fatty acids, derived from oleic, linoleic, ricinoleic and eicosanoic fatty acids have been previously described as antibacterial and antifungal compounds (Shin *et al.*, 2007; Hou, 2008; Bajpai *et al.*, 2009). Moreover, 7,10-DiHOME specifically displays antimicrobial activity against plant pathogenic bacteria and food-borne pathogenic bacteria (Sohn *et al.*, 2012; Sohn *et al.*, 2013). These activities may provide an evolutive advantage against other microorganisms competing for the same environmental niche.

Interestingly, when oleic acid is absent, diol synthase operon genes are up and down-regulated with diverse substrates and culture conditions (Kim *et al.*, 2008; Lee *et al.*, 2009). Here, we have explored the biofilm formation in oleate-diol synthase mutant strains and determined a possible relationship with this phenotype, opening new implications of diol synthase enzymes in the environmental behavior of *P. aeruginosa* living-system *(Additional results).*

A more detailed study of the differentially regulated genes to different functional categories in oleate-induced and non-induced transcriptome of *P. aeruginosa* PAO1 is illustrated in Fig. 25 (data extracted from *Annexes*). Genes that exhibited highly variable gene expression (4-fold increased expression or greater) have been related to their functional category, constituting 12.5% and 64% of the total identified genes in oleate-induced and non-induced transcriptome, respectively (Kang *et al.*, 2008).

The functional class of transport of SmallTrans, small molecules, showed the highest fraction of highly variably expressed genes in oleate-induced and non-induced conditions. However, energy metabolism (EnergyMb), fatty acid and phospholipid metabolism (FAPOL) and biosynthesis of cofactors, prosthetic groups and carriers (Cofactors) showed substantially higher variability in the oleate-induced culture condition (Fig. 25), which may reflect the effect of a phenotypic response to oleic acid. In contrast, carbon compound catabolism (CCatab), central intermediary metabolism (CIM), membrane proteins (Membrane) and transcriptional regulators (TRs) were more variable under non-induced conditions (Winsor *et al.*, 2011). Moreover, if we analyze the functional categories of genes shared in oleate-induced versus phosphatidiletanolamide-chemotaxis induced transcriptomes, adaptation and protection (AdpProt), fatty acid and phospholipid metabolism (FAPOL) and putative enzymes are the more representatives, followed by energy metabolism (EnergyMb). This could justify the existence of the oleate-diol synthase pathway as a part of the environmental adaptation of *P. aeruginosa* to free fatty acids enriched media.

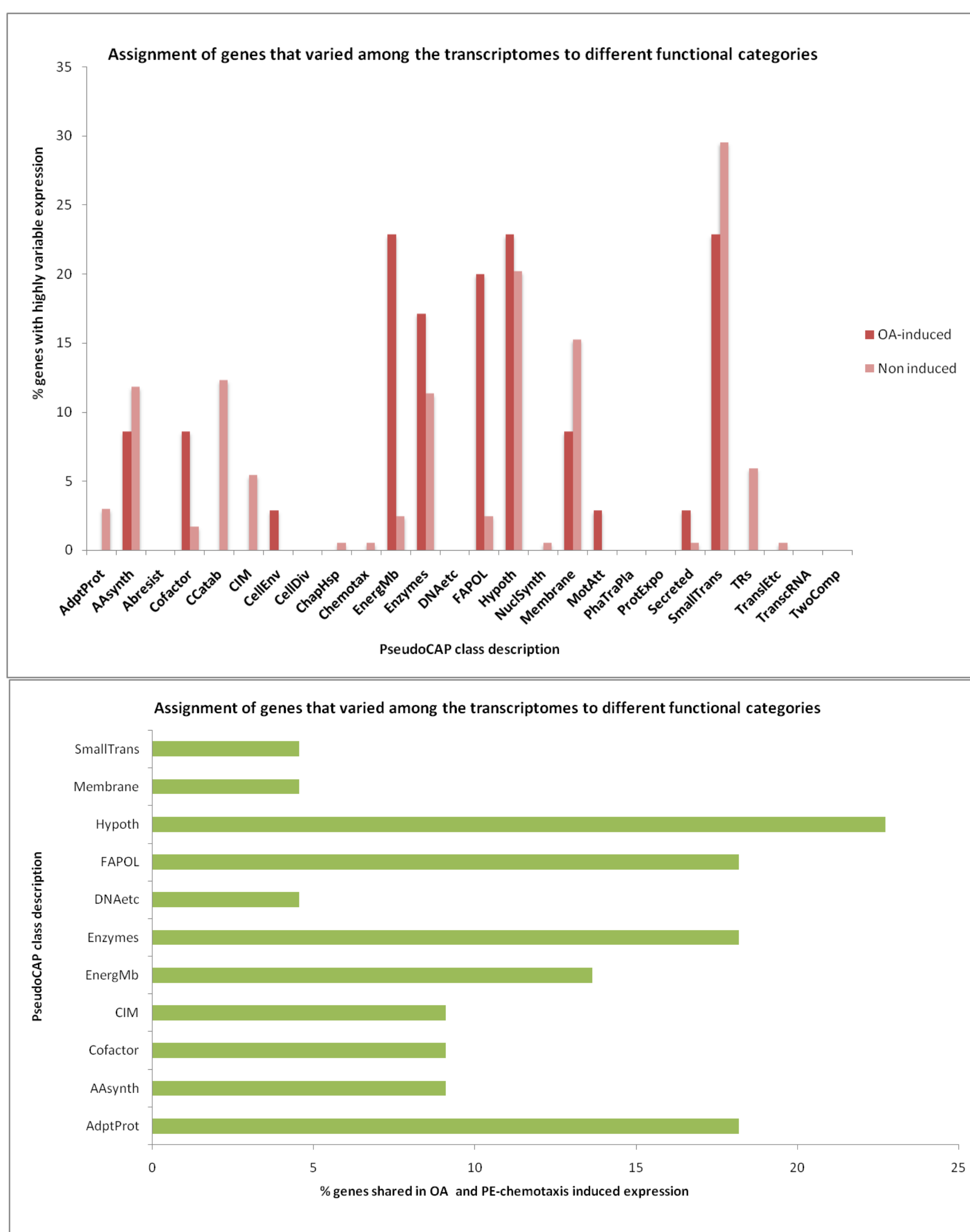

Figure 25. Assignment of genes that varied among the transcriptomes to different functional categories. Top) Bars indicate the percentage of genes that exhibited highly variable gene expression (4-fold increase or greater) within each functional category. **Bottom)** Bars indicate the percentage of genes shared in OA-induced and PE-chemotaxis induced expression within each functional category. Functional categories were assigned according to the annotation for strain PAO1 available at the *Pseudomonas* genome database (Winsor *et al.*, 2011). The PseudoCAP functional classes are abbreviated as follows: AAsynth, amino acid biosynthesis and metabolism; ABresist, antibiotic resistance and susceptibility; AdptProt, adaptation and protection; CCatab, carbon compound catabolism; CellDiv, cell division; CellEnv, Cell wall/lipopolysaccharide (LPS)/capsule; ChapHsp, chaperones and heat shock proteins; Chemotax, chemotaxis; CIM, central intermediary metabolism; Cofactor, biosynthesis of cofactors, prosthetic groups, and carriers; DNAetc, DNA replication, recombination, modification, and repair; EnergMb, energy metabolism; Enzymes, putative enzymes; FAPOL, fatty acid and phospholipid metabolism; Hypoth, hypothetical, unclassified, and unknown; membrane, membrane proteins; MotAtt, motility and attachment; ncRNA, noncoding RNA (rRNA and tRNA were excluded in this study); NuclSynth, nucleotide biosynthesis and metabolism; PhaTraPla, related to phage, transposon, or plasmid; ProtExpo, protein secretion/export apparatus; Secreted, secreted factors (toxins, enzymes, alginate); SmallTrans, transport of small molecules; TranscRNA, transcription, RNA processing and degradation; TranslEtc, translation, posttranslational modification, degradation; TRs, transcriptional regulators; TwoComp, two-component regulatory systems.

Indeed, if we focuss in the oleate-induced metabolome of *P. aeruginosa* (Kang *et al.*, 2009) and construct a plot to visualize the overexpression gene patterns that were obtained under this experimental condition by grouping the genes whose expression changes were from 2 to 4-fold, from 4 to 16-fold and more than 16-fold, it can be observed that the number of genes decreases in a reverse proportion to the expression-fold changes (Fig.26).

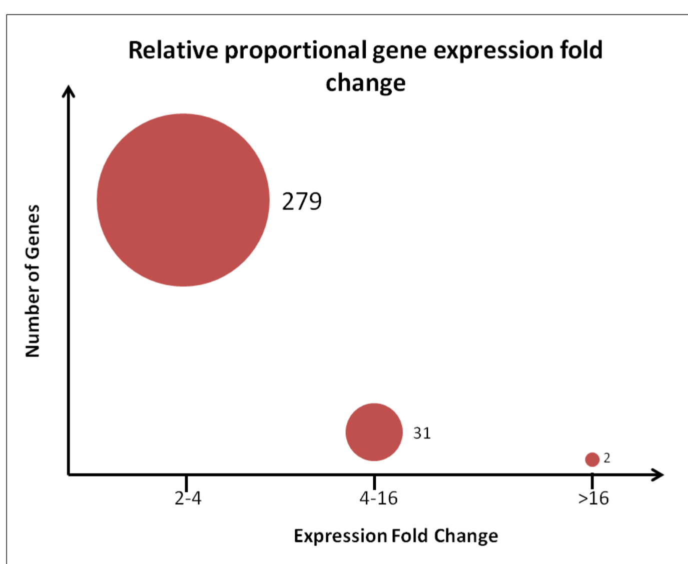

Figure 26. Relative proportional gene expressions fold change. Diameters of the circles correspond to the number of genes. (Raw data obtained from (Kang *et al.*, 2009).

P. aeruginosa fad-like genes are overexpressed in the oleic acid induced metabolome, being located at the upper-limit of the second group of genes. With an expression fold change of 15.9 and 15.6 are *fadE* homologous genes, probably acyl-CoA dehydrogenases participating in the first step of the β–oxidation pathway (Kang *et al.*, 2010; Zarzycki-Siek *et al.*, 2013). Interestingly, only two genes out of 312 are overexpressed more than 16 fold (146.2 and 73.0 fold change, respectively), corresponding to ORFs PA2078 and PA2077. These results confirms that when an excess of oleic acid is present in the environment, two main mechanisms for LCFAs metabolism are activated: i) the central energy metabolism via intracellular β–oxidation, and ii) periplasmic production of oxylipins via the oleate-diol synthase pathway, belonging to the secondary metabolism. The fact that the latter pathway is overexpressed 9 and 5 times more than the β–oxidation cycle might be due to an excess of carbon source in the assay performed.

The oleate-diol synthase pathway of *P. aeruginosa* has been re-defined based on the results obtained in this research project together with previously described data and the interpretation of other research works obtained up to date *(Papers I, II)*. A simplified diagram of the newly proposed pathway is shown in Fig.27.

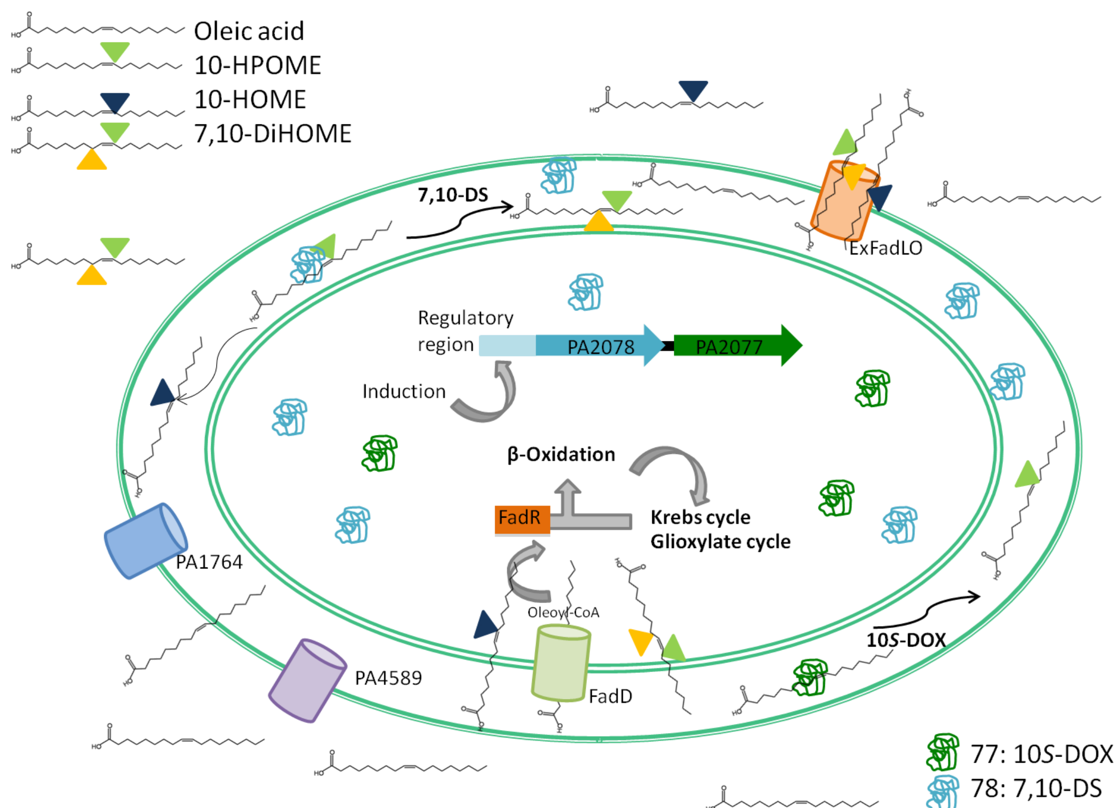

Figure 27. Diagram providing a representation of the proposed metabolic pathways relevant to this study.

This scheme includes also data obtained from microarrays analysis revealing that when triacylglycerides or phospholipids like phosphatidiletanolamide are present in the medium, a notable assortment of genes encoding lipid-degrading enzymes like extracellular phospholipase plcB and lipases LipA, LipC or EstA, LCFAs transport systems, and enzymes associated with oxidation, as well as glyoxylate shunt enzymes, showed significantly increased expression (Miller *et al.*, 2008). Explanation to the increased production of fatty acids-related elements can be due to the fact that free extracellular LCFAs move through the outer membrane into the periplasm, where they are either transported to the cytoplasmic acyl-coenzyme A (Acyl-CoA) by means of

FadD or a Fad-like transporter. There, *P. aeruginosa* converts them to long-chain acetyl-CoAs via oxidation to acetyl-CoA, which is then processed by isocitrate lyase and malate synthasethrough the glyoxylate shunt and ultimately through the tricarboxylic acid cycle to generate energy (Miller *et al.*, 2008).

The process of LCFA transport is partially sensitive to osmotic imbalance, suggesting that either a protein or the integrity of the chemical milieu within the periplasmic space is required for this process. Osmotic imbalance not only disrupts the protein constituents of the periplasmic space but likewise the chemical makeup, including pH. Under normal respiratory conditions, the periplasmic space is slightly acidic, which is predicted to increase the number of protonated (uncharged) fatty acids. The disruption of the periplasmic space upon osmotic shock will increase pH, which is likely to reduce the number of protonated fatty acids and depress the rate of fatty acid transport (Azizan *et al.*, 1999).

Therefore, we suggest that when oleic acid is predominant in the extracellular medium and metabolic energy is supplied, non-activated − via Acyl-CoA enzyme − LCFAs, which could participate in the disruption of periplasmic space chemical conditions by osmotic shock, are metabolized by the diol synthase pathway releasing oxylipins. Then, the oxylipins, 10-HPOME, 10-HOME and 7,10-DiHOME, accumulate in the periplasm, where 10-HPOME is non-enzymatically reduced to 10-HOME. Both, 10-HOME and 7,10-DiHOME are exported to the extracellular medium through ExFadLO transporter. In the extracellular medium, both oxylipins could be polymerized into estolides by extracellular lipases and might serve as emulsifiers for a diverse class of purposes (Peláez *et al.*, 2003; Martín-Arjol *et al.*, 2013).

The comprehensive analysis of oleate-diol synthase activity has significantly increased its biotechnological possibilities. Even though we have set up the conditions and established only one substrate for activity assays in order to reduce the screening strategies and product identification, the actual range of substrates of both enzymes still needs further exploration. Therefore, both the use of waste material for production of biotechnologically interesting compounds, and the description of new products as other LCFAs, hydroperoxy-fatty acids and even rubber, could be an attractive research field. Furthermore, we have achieved expression *in trans* of 10*S*-DOX and 7,10-DS in *E. coli* and 10S-DOX in a GRAS *P. putida* KT2440 strain as a first step

to industrial application. New strategies have been set up for improving HFAs productivity in *E. coli* based in ExFadLO studies like the deletion of fadD, the inner fatty acid transporter and the overexpression of fadL and CYP153A, a periplasmic enzyme from *Marinobacter aquaeolei* (Bae et al., 2014). So it is clear that basic research of microbial enzymes must be in parallel to applied biocatalysis, providing tools to exploit white biotechnological approximations.

5. CONCLUSIONS

CONCLUSIONS

1. *P. aeruginosa* oleate-diol synthase activity was found to take place in the periplasmic space.
2. ExFadLO, located in the outer membrane, is required to export 10-HOME and 7,10-DiHOME from the periplasm to the extracellular medium.
3. Two new genes of *P. aeruginosa* genome have been annotated and their participation in the oleate-diol synthase pathway determined: *PA2077* is oleate 10*S*-DOX, responsible for the first step of oleate oxygenation and 10-HPOME intermediate production, and *PA2078,* the 7,10-DS, involved in 7,10-DiHOME formation. Oleate-diol synthase genes constitute a bicistronic, finely regulated operon.
4. Individual recombinant expression of 10*S*-DOX and 7,10-DS in *E. coli* confirmed their activity and demonstrated that no hetero-complex formation is required for oleate-diol synthase activity.
5. Diol synthase-coding genes could have been originated by a gene duplication event, followed by neofunctionalization of one of them, most probably *PA2078*.
6. Oleate-diol synthase is conserved in all *P. aeruginosa* species, being unique among bacteria.
7. Bioinformatic analysis of diol synthase enzymes 10*S*-DOX and 7,10-DS showed that both proteins constitute a unique, new subfamily of bacterial diheme cytochrome c peroxidases, named as FadCCPs.
8. 10*S*-DOX exhibits two functional heme-binding groups, required for oleate dioxygenation activity. On the contrary, no requirement for the heme groups was found for 7,10-DS activity.
9. Presence of inserted palindromic sequences inside diol synthase-coding genes was demonstrated in *P. aeruginosa* for the first time and described as evolutionary elements.
10. Recombinant *P. putida* KT2440 GRAS strain expressing functional 10*S*-DOX has been obtained for biotechnological production of oxylipins.

6. ABBREVIATIONS

ABBREVIATIONS

10-H(P)OME	(10*S*)-hydro(pero-)xy-8(*E*)-octadecenoic acid
10*R*-DOX	10*R*-dioxygenase
10*R*-HPODE	(10*R*)-hydro(pero-)xyoctadecadienoic acid
10S-DOX	(10*S*)-dioxygenase
10-HOME	(10*S*)-hydroxy-(8*E*)-octadecenoic acid
13S-HPODE	(13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid
15-HETE	15-hydroxy-5,8,11,13-(*E*)-eicosatetraenoicacid
5,8-DiHODE	(5*S*,8*R*)-dihydroxyoctadeca-(9*Z*,12*Z*)-dienoic acid
5,8-LDS	5,8-Linoleate diol synthase
7,10,12-TriHOME	7, 10, 12-trihydroxy-8(*E*)-octadecenoic acid
7,10-DiHOME	(7*S*, 10*S*)-dihydroxy-(8*E*)-octadecenoic acid
7,10-DS	Diol synthase
7,8-DiHODE	(7S, 8S)- dihydroxylinoleic acid
7,8-LDS	7,8-Linoleate diol synthase
8,11-DiHODE	(8*R*,11*S*)-dihydroxy-(9*Z*,12*Z*)-octadecadienoic acids
8*R*-HODE	8*R*-hydroxylinoleic
8R-HPODE	8*R*-hydro(pero-)xylinoleic
9,10,13-THOD	9,10,13-trihydroxy-11(*E*)-octadecenoic acid
9,12,13-THOD	9,12,13-trihydroxy-10(*E*)-octadecenoic acid
9S-HPODE	(9*S*)-hydro(pero)-xy-(10*E*,12*Z*)-octadecadienoic acid
AdpProt	Adaptation and protection
CCatab	Compound catabolism
CCP	Cytochrome c peroxydases
CF	Cystic fibrosis
CIM	Central intermediary metabolism
COX	Cyclooxygenases
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DOX	Dioxygenase
DS	Diol synthases
EnergyMb	Energy metabolism
FAPOL	Fatty acid and phospholipid metabolism
GRAS	Generally recognized as safe
HFA	Hydroxylated fatty acid
LCFA	Long-chain fatty acid
LDAO	Lauryldimethylamine-oxide
LDS	Linoleate diol synthases
LOX	Lipoxygenases
LPS	Lipopolysaccharide
MS	Mass spectrometry
m/z	Mass-to-charge ratio
ncRNA	Non-coding RNA
O.D.	Optical density
OA	Oleic acid
PC	Phosphatidylcholine

PE	Phosphatyldiletanolamine
PGHS	Prostaglandin H synthases
PHA	Polyhydroxyalkanoate
PHB	Poly (3-hydroxybutyrate)
PHV	Poly (3-hydroxyvalerate)
ppo	psi producingoxygenases
psi	Precocious sexual inducers
ROS	Reactive oxygen species
TR	Transcriptional regulator

7. RESUMEN *(SUMMARY)*

RESUMEN *(SUMMARY)*

INTRODUCCIÓN

El interés por el género *Pseudomonas*

Una de las características más notables del género *Pseudomonas* es su versatilidad fisiológica y metabólica, que le permite la colonización de diversos nichos ambientales tales como los hábitats terrestres y acuáticos, así como plantas, insectos y tejidos animales (Palleroni, 1992; Silby *et al.*, 2011). Aunque la patogénesis humana constituye el foco de atención del género *Pseudomonas*, estas bacterias presentan características relevantes para su aplicación en la agricultura, la biorremediación y la biotecnología debido a su capacidad de tolerar, degradar o precipitar compuestos tóxicos. En la actualidad, la industria química necesita ser reemplazada por procesos industriales más sostenibles como la denominada "biotecnología blanca" cuyo objetivo es utilizar células o enzimas de levaduras, mohos, bacterias y plantas para sintetizar productos de interés industrial, disminuyendo el gasto energético y la generación de residuos durante su producción (Frazzetto, 2003). La asombrosa diversidad catabólica de *Pseudomonas*, que incrementa su capacidad de adaptarse a fluctuantes condiciones ambientales, proporciona una fuente natural de recursos para transformar un amplio rango de sustratos en compuestos de alto valor añadido a partir de materias primas de bajo coste (Nikel *et al.*, 2014). Por lo tanto, presenta interés como factorías celulares microbianas para la producción o la biotransformación de compuestos residuales, difíciles o imposibles de obtener por síntesis química, debido a la complejidad de sus estructuras y a sus requisitos regio- o estereoquímicos (de Boer and Schmidt-Dannert, 2003).

Pseudomonas aeruginosa

P. aeruginosa es una bacteria de vida libre que se encuentran comúnmente en el suelo y el agua. Presenta una alta capacidad de adaptición y por tanto, de colonizar y persistir bajo una amplia gama de condiciones ambientales incluyendo la infección en diferentes huéspedes como seres humanos, plantas, nemátodos e insectos (Hardalo and Edberg, 1997; Tan *et al.*, 1999; Rahme *et al.*, 2000; Miyata *et al.*, 2003; Apidianakis and Rahme, 2009; Clatworthy *et al.*, 2009). Puede crecer a temperaturas de hasta 42°C, y la mayoría de los fenotipos caracterizados producen pigmentos como la

pioverdina y la piocianina (Meyer *et al.*, 2002; Dietrich *et al.*, 2006). La morfología colonial puede verse alterada debido a la producción de biopelículas, que protege a las bacterias de agentes antimicrobianos y condiciones ambientales adversas. Debido a su patogenicidad, *P. aeruginosa* está presente en infecciones crónicas del tracto respiratorio humano, además de asociarse frecuentemente a la mortalidad en pacientes immunocompromometidos, víctimas de quemaduras y afectados de fibrosis quística (Davies, 2002; Driscoll *et al.*, 2007; Goldberg *et al.*, 2008). La cepa de referencia común es *P. aeruginosa* PAO1, una mutante espontánea de la cepa original de PAO (anteriormente llamado "*P. aeruginosa* cepa 1") que había sido aislada en 1954 de una herida en Melbourne, Australia (Holloway, 1955; Holloway, 1975). La cepa *P. aeruginosa* PAO1 se ha convertido en la referencia para la genética de *Pseudomonas* y el análisis funcional de su fisiología y metabolismo.

Genómica funcional de*Pseudomonas aeruginosa*

El repertorio genético de *Pseudomonas aeruginosa* refleja el estilo de vida ubicuo de esta especie bacteriana. Su genoma es rico en G + C (65-67%) y está representado por un único cromosoma circular y un número variable de plásmidos. El cromosoma consiste en un núcleo conservado y una parte accesoria variable que permite la variación del tamaño del genoma entre 5,2 y 7 Mbp (Schmidt *et al.*, 1996; Lee *et al.*, 2006; Winsor *et al.*, 2009). Los genomas centrales de cepas de *P. aeruginosa* son en su mayoría co-lineales, exhiben una baja tasa de polimorfismo de secuencia y contienen pocos *loci* de alta diversidad de secuencias (Klockgether *et al.*, 2011). Por otra parte, el genoma accesorio consiste en elementos extracromosómicos tales como plásmidos, elementos y de bloques de ADN insertados en varios *loci*, que constituyen islotes genómicos específicos de la cepa o clon e islas genómicas de elementos de ADN. El gran tamaño y la complejidad genética del genoma de *P. aeruginosa* permite la ocurrencia de adaptaciones evolutivas para tener éxito en diversos nichos ecológicos. Esto incluye un gran número de genes que codifican proteínas de membrana externa, sistemas de transporte, y las enzimas que participan en la absorción de nutrientes y el metabolismo, así como una de las mayores proporciones de genes reguladores (8,4%) entre los genomas bacterianos. Además, *P. aeruginosa* es capaz de personalizar su genoma para adaptarse a las necesidades para la supervivencia en el medio, mediante

la adquisición específica de bloques de genes de diferentes fuentes, incluyendo otras especies o géneros (Mathee *et al.*, 2008). No obstante, de los 5.570 marcos de lectura abierta (ORF) presentes en el genoma de *P. aeruginosa* PAO1, sólo un 6,7% de genes tienen una función experimentalmente demostrada(Levesque, 2006). La genómica funcional tiene como objetivo la asignación de funciones a nuevos genes, aún no anotados y una de sus herramientas básicas es el uso de mutagénesis mediante transposones, utilizada en este trabajo (Jacobs *et al.*, 2003; Held, Ramage, Jacobs, Gallagher, and Manoil, 2012).

Oxilipinas y aplicaciones de los ácidos grasos hidroxilados

Los ácidos grasos insaturados de cadena larga son moléculas indispensables para cualquier célula viva. Incorporados en los fosfolípidos, constituyen la mayor parte de la membrana plasmática. Además de cumplir funciones estructurales, también sirven como fuente de carbono o energía y como bloques para la construcción de moléculas complejas como metabolitos secundarios. Las oxilipinas son una clase de ácidos grasos hidroxilados que pueden formarse enzimáticamente o mediante autooxidación (Porter *et al.*, 1995; Yin and Porter, 2005; Schneider, 2009). Estas oxilipinas pueden actuar en la inflamación, la señalización, en mecanismos de defensa de plantas, la germinación o la reproducción de hongos entre otros (Funk, 2001; Feussner and Wasternack, 2002; Noverr *et al.*, 2003; Andreou *et al.*, 2009; Mosblech *et al.*, 2009; Brodhun and Feussner, 2011). Además, los ácidos grasos hidroxilados presentan interés industrial debido a su gran reactividad, que permite usarlos como compuestos intermediarios para la síntesis química y farmacéutica, su capacidad emulsionante y sus propiedades antibacterianas y antifúngicas (Peláez *et al.*, 2003; Metzger and Bornscheuer, 2006; Hou, 2008; Bajpai *et al.*, 2009; Martín-Arjol *et al.*, 2010). Sin embargo, la síntesis química de estos ácidos grasos hidroxilados presenta muchos inconvenientes. Por el contrario, las reacciones enzimáticas pueden suponer una buena alternativa para su producción industrial debido a su alta especificidad, la menor demanda de energía y la disminución de subproductos (Burton, 2003; Wackett *et al.*, 2004; Cao *et al.*, 2013; Adrio and Demain, 2014).

Producción de oxilipinas en *Pseudomonas aeruginosa*

Pseudomonas aeruginosa presenta una excepcional versatilidad metabólica y es capaz de biodegradar hidrocarburos, incluyendo compuestos aromáticos y xenobióticos (Ridgway *et al.*, 1990; Zhang *et al.*, 2012). Una cepa bacteriana de *Pseudomonas aeruginosa*, denominada 42A2 (NCIB 40045), fue aislada por el Departamento de Microbiología y Parasitología Sanitarias (Facultad de Farmacia de la Universidad de Barcelona) a partir de una muestra de agua contaminada con residuos oleosos (Robert, 1989). En presencia de ácido oleico esta cepa es capaz de acumular en el sobrenadante del cultivo los ácidos (10*S*)-hidroperoxi-8*(E)*-octadecenoico (10-HPOME), que es reducido espontáneamente a (10*S*)-hidroxi-8*(E)*-octadecenoico (10-HOME), y (7*S*,10*S*)-hidroxi-8*(E)*-octadecenoico (7,10-DiHOME) (Mercadé *et al.*, 1988; Guerrero *et al.*, 1997a; Bastida *et al.*, 1999). Posteriormente, la acumulación de estas oxilipinas ha sido descrita en otras cepas de *P. aeruginosa* como la cepa PR3 (Hou *et al.*, 1993). El desarrollo de técnicas analíticas han permitido identificar numerosas oxilipinas, producto de la bioconversión de otros ácidos grasos como los ácidos ricinoleico, palmitoleico, linoleico, ecosanoico, araquidónico, etc. en *P. aeruginosa* (Kim *et al.*, 2000; Kuo *et al.*, 2001; Vance *et al.*, 2004; Bae *et al.*, 2007; Bae *et al.*, 2010; Martínez *et al.*, 2010; Back *et al.*, 2011; Seo *et al.*, 2014).

Enzimas formadoras de oxilipinas

La síntesis de oxilipinas es catalizada por oxigenasas. Estas enzimas se dividen en dioxigenasas y monoxigenasas. Las dioxigenasas incluyen las lipoxigenasas (LOX), las dioxigenasas de ácidos grasos que contienen hemo (DOX) y las diol sintasas (DS) entre otras, mientras que las monooxigenasas pertenecen a la familia de las citocromo P450.
Las lipooxigenasas son metalo-oxigenasas, que no contienen grupos hemo y que catalizan la dioxigenación estereoselectiva de ácidos grasos poliinsaturados con uno o más dobles enlaces en posición 1Z,4Z-pentadieno a hidroperóxidos de ácidos grasos (Oliw, 2002). Estas enzimas se distribuyen ampliamente en el reino eucariota y se han estudiado durante años en animales (Kühn and Thiele, 1999), plantas (Liavonchanka and Feussner, 2006), o de organismos fúngicos (Oliw, 2002; Wennman *et al.*, 2014), y por mucho tiempo, las LOXs parecían estar restringidas a estos reinos. Sin embargo, la reciente cristalización del gen de la lipoxigenasa bacteriana (*PA1169*) de *P. aeruginosa*

ha supuesto la reclasificación de estas enzimas en una nueva subfamilia de lipoxigenasas, presentando un posible rol en la interacción con fosfolípidos (Hansen *et al.*, 2013; Garreta *et al.*, 2013).

Las dioxigenasas que contiene hemo como grupo funcional son abundantes en la naturaleza y se pueden dividir en varios grupos de enzimas como las sintasas de prostaglandina H (PGHS) de vertebrados, las linoleato diol sintasas (LDS) y 10R-dioxigenasas de hongos filamentosos, y la α-DOX de plantas (Sanz *et al.*, 1998; Hörnsten *et al.*, 1999; Garscha and Oliw, 2009). Las dioxigenasas que presentan grupos hemo se producen en eucariotas, a menudo asociadas a las citocromo P450, que transforman los productos peroxidados (Brash *et al.*, 2014). Por otra parte, otros ejemplos de enzimas que contienen grupos hemo son las P450, las peroxidasas y las catalasas (Hersleth *et al.*, 2006).

Otra clase de oxigenasas de ácidos grasos recientemente descrita en bacterias son las diol sintasas. En 1995, se caracterizó la enzima linoleato 7,8-diol sintasa (DS) en el hongo *Gaeumannomyces graminis* (Su *et al.*, 1995). La actividad diol sintasa comprende la dioxigenación secuencial de ácidos grasos insaturados a hidroperóxidos y su isomerización al respectivo diol. Las enzimas LDS son proteínas bifuncionales, resultantes de la fusión de un dominio amino terminal DOX y un dominio carboxilo terminal de tipo P450 que cataliza la isomerización del hidroperóxido (Brodhun *et al.*, 2009). Desde entonces, se han identificado y caracterizado numerosas LDS en hongos y una única oleato diol sintasa bacteriana en *P. aeruginosa* 42A2 (Martínez *et al.*, 2010).

Por otra parte, las citocromo P450 (CYP450) son enzimas que contienen hemo y presentan activida monooxigenasa. Las CYP catalizan la incorporación de un átomo de oxígeno a un sustrato mediante la transferencia de un electrón desde el cofactor NADPH. Estas enzimas están presentes en toda clase de organismos y catalizan diversas reacciones, tanto en la degradación como en la biosíntesis de compuestos orgánicos (Meunier *et al.*, 2004; Guengerich, 2008).

La oleato diol sintasa de *P. aeruginosa*

En *P. aeruginosa* 42A2, la formación de oxilipinas derivadas del ácido oleico ha sido caracterizada bioquímicamente y se describe como una actividad diol sintasa, que posibilita la dioxigenación del ácido oleico mediante una actividad 10*S*-DOX, liberando

un intermediario hidroperóxido, el 10-HPOME, que es reducido de forma no enzimática a 10-HOME. Posteriormente el intermediario hidroperóxido es isomerizado mediante la actividad hidroperóxido isomerasa a 7,10-DiHOME, productos que son acumulados en el sobrenadante del cultivo. El ácido oleico es el sustrato preferido pero no el único catalizado por la diol sintasa de *P. aeruginosa* (Martínez *et al.*, 2010). Aunque la oxidación enzimática de ácidos octadecenoicos hacia hidroperóxidos y dioles se ha descrito anteriormente en hongos (Champe and El-Zayat, 1989; Brodowsky and Oliw, 1993; Garscha *et al.*, 2007; Oliw *et al.*, 2011), la oleato diol sintasa de *P. aeruginosa* es la primera descrita en bacterias.

La naturaleza de la actividad oleato diol sintasa de *P. aeruginosa* se ha discutido a fondo durante estos años. Martínez y colaboradores observaron que las actividades 10*S*-dioxigenasa y diol sintasa co-eluyen en las fracciones obtenidas mediante cromatografía de intercambio iónico y cromatografía de exclusión molecular,con un tamaño molecular aparente de ~ 50 kDa, lo que sugiere que ambas actividades podrían encontrarse vinculadas a una sola enzima bifuncional, asociada al extracto celular. La observación de que el intermediario 10*S*-HPOME se transforma en el producto 7,10-diol menos eficientemente que su precursor, el ácido oleico, también sugirieron que ambas actividades estaban acopladas funcionalmente. Sin embargo, los ácidos grasos monoinsaturados son sustratos pobres para las enzimas LOX descritas previamente como responsables de la síntesis de oxilipinas en bacterias (Clapp *et al.*, 2006). Además, el genoma de *P. aeruginosa* PAO1 no contiene homólogos evidentes de dioxigenasas de ácidos grasos (como cicloxigenasas, linoleato diol sintasas, dioxigenasas o α-DOX). Por otra parte, el genoma de *P. aeruginosa* presenta sólo dos genes homólogos a citocromo P450 (correspondientes a los ORFs PA2475 y PA3331), pero el uso de inhibidores específicos de P450 no afectaron a la actividad catalítica (Martínez *et al.*, 2010).

Transporte de ácidos grasos de cadena larga a través de la membrana externa de bacterias Gram-negativas

La membrana externa de las bacterias Gram-negativas constituye una barrera para la libre difusión de compuestos hidrofílicos e hidrofóbicos (Nikaido, 2003). Presenta porinas que permiten la entrada de nutrientes de pequeño tamaño (< 600 Da), pero

excluyen moléculas de character hidrofóbico. La proteína FadL fue la primera proteína caracterizada implicada en la difusión de compuestos hidrofóbicos a través de la membrana externa, siendo la única proteína conocida que participa en el transporte de LCFAs en *E. coli* (Nunn and Simons, 1978; van den Berg, 2010). Esta proteína, denominada EcFadL, fue cristalizada en 2004 y su estructura reveló la presencia de 14 láminas beta antiparalelas con una apertura lateral (van den Berg *et al.*, 2004). Basada en la estructura de la proteína FadL se propuso un mecanismo mediante el cual los LCFAs atraviesan la membrana externa mediante difusión a través del orificio lateral, liberándose el ácido graso en el espacio periplásmico (van den Berg, 2005). Posteriormente, se cristalizó el producto del gen *PA4589* o PaFadL de *P. aeruginosa*, que aún presentando una baja identidad de secuencia con respecto a EcFadL, conserva la apertura lateral característica (Hearn *et al.*, 2009). La difusión lateral se ha propuesto como una característica general de las proteínas de la familia FadL (Hearn *et al.*, 2009; Touw *et al.*, 2010). Además del gen *PA4589*, *P. aeruginosa* presenta dos genes con cierta homología a proteínas de la familia FadL, *PA1288* y *PA1764*. Ninguno de estos productos ha sido caracterizado funcionalmente pero un triple mutante de estos tres genes conservó la capacidad de desplazarse hacia los LCFAs presentes en el medio. Esto sugiere que *P. aeruginosa*, a diferencia de *E.coli*, dispone de una ruta alternativa para la incorporación de LCFAs.

OBJETIVOS

El enorme potencial que presenta *P. aeruginosa* 42A2 para la producción de oxilipinas, su explotación biotecnológica y la posibilidad desentrañar los efectos biológicos de los ácidos grasos hidroxilados, ha motivado el estudio exhaustivo de la actividad oleato diol sintasa.

El presente proyecto de investigación se ha centrado en el estudio de la síntesis de oxilipinas en *P. aeruginosa*, ya que abarca no sólo los intereses biológicos sino también biotecnológicos. Como se menciona en la introducción, cuando se inició este trabajo, el transporte de membrana externa de las oxilipinas y la naturaleza de la enzima/s implicadas en la bioconversión del ácido oleico por *P. aeruginosa* todavía permanecían sin descubrir.

Para resolver estas cuestiones, se definieron los siguientes objetivos específicos:

1. El estudio del sistema de transporte de oxilipinas a través de la membrana externa de *P. aeruginosa (Paper I)*
2. Identificación y caracterización funcional de la enzima/s que participan en la actividad oleato diol sintasa en *P. aeruginosa (Paper II)*
3. El análisis filogenético para llevar a cabo la clasificación de las enzimas responsables de la actividad oleato diol sintasa *(Paper III)*
4. El estudio de la vía evolutiva de los elementos que intervienen en la actividad oleato diol sintasa *(Paper III)*
5. Una aproximación funcional y biotecnológica de la actividad oleato diol sintasa de *P. aeruginosa (Resultados adicionales)*

RESULTADOS Y DISCUSIÓN

Caracterización funcional de ExFadLO, una proteína de membrana externa necesaria para la exportación de ácidos grasos de cadena larga en *Pseudomonas aeruginosa*

Las proteínas bacterianas de la familia FadL se han asociado frecuentemente a la asimilación de sustratos hidrofóbicos exógenos. Sin embargo, su ubicación en la membrana externa y su participación en la captación de sustratos se han deducido principalmente por su similaridad de secuencia con la proteína FadL de *Escherichia coli*, los primeros transportadores de ácidos grasos de cadena larga de membrana externa caracterizados en bacterias. Aquí se presenta la caracterización funcional de una proteína de membrana externa de *Pseudomonas aeruginosa* PAO1 (ORF PA1288) que muestra similitud con los miembros de la familia FadL, y es el motivo por el que proponemos el nombre de ExFadLO. Se demuestra experimentalmente que se requiere esta proteína para exportar los ácidos grasos de cadena larga 10-HOME y 7,10-DiHOME, derivados de la actividad oleato diol sintasa, desde el compartimento periplasmático al medio extracelular. La acumulación de los derivados oxigenados 10-HOME y 7,10-DiHOME en el medio extracelular de *P. aeruginosa* resultó inhibida por una mutación generada por la inserción de un transposón en el gen ExFadLO (*exFadLO*$^{-}$ mutante). Sin embargo, la actividad oleato diol sintasa se encuentra intacta en el periplasma de este mutante, lo que indica que ExFadLO participa exclusivamente

en la exportación a través de la membrana externa de estos ácidos grasos de cadena larga oxigenados. La capacidad de ExFadLO¯ mutante para exportar 10-HOME y 7,10-DiHOME se recuperó después de su complementación mediante la expresión de la proteína ExFadLO en un plásmido, restituyendo el fenotipo salvaje. Además, un ensayo de *western blot* con una variante de ExFadLO fusionada al epítopo V5 confirmó la ubicación de ExFadLO en la membrana externa bacteriana en las condiciones experimentales ensayadas. Estos resultados proporcionan la primera evidencia de que proteínas de la familia FadL, involucradas en la asimilación de sustratos hidrófobos desde el entorno extracelular, también funcionan como elementos de secreción de metabolitos de relevancia biológica.

Desvelando los genes responsables de la exclusiva actividad oleato-diol sintasa de *Pseudomonas aeruginosa*

P. aeruginosa muestra la capacidad de realizar la bioconversión de ácido oleico en una clase de ácidos grasos hidroxilados conocidos como oxilipinas. La actividad diol sintasa es responsable de tal bioconversión, que tiene lugar a través de la dioxigenación de ácido oleico para liberar un intermediario hidroperóxido 10-H(P)OME (ácido (10*S*)-hidroxi-8*(E)*-octadecenoico), seguido por la conversión del hidroperóxido en 7,10-DiHOME (ácido (7*S*,10*S*)-dihidroxi-(8*E*)-octadecenoico), los cuales se acumulan en el sobrenadante del cultivo. Se seleccionaron varios mutantes de *P. aeruginosa* PAO1, analizando su capacidad para la producción de 10-H(P)OME y 7,10-DiHOME y sorprendentemente dos de ellos (ORFs PA2077 y PA2078), fueron incapaces de liberar ácidos grasos hidroxilados, eligiéndose para posteriores análisis. La participación de los ORFs PA2077 y PA2078 en la actividad oleato diol sintasa se confirmó, y su respectivo papel en la conversión de ácido oleico se determinó mediante la complementación de las mutaciones. La restauración de la actividad reveló que el gen *PA2077* codificaba para la actividad 10*S*-dioxigenasa (10*S*-DOX) responsable de la primera etapa de la reacción, mientras que el gen *PA2078* codificaba para la actividad (7*S*,10*S*) -hydroperoxido diol sintasa (7,10-DS), que permite la conversión de 10-H(P)OME en 7,10-DiHOME. La expresión heteróloga de ambas enzimas de manera independiente mostró que no se requiere la formación de hetero-complejos para que tenga lugar la actividad enzimática. Análisis bioinformáticos y de RT-PCR revelaron que ambos genes

constituyen un nuevo operón oleato-diol sintasa finamente regulado, originado mediante un evento de duplicación de genes seguido por neo-funcionalización para la adaptación del medio ambiente, sin precedentes en procariotas.

Aproximaciones *in silico* e *in vivo* a los aspectos evolutivos de la oleato-diol sintasa de *Pseudomonas aeruginosa*. Descubrimiento de una nueva familia de di-hemo citocromo c peroxidasas bacterianas

Como se describió anteriormente, los genes *PA2077* y *PA2078* de *P. aeruginosa* codifican para las enzimas 10*S*-DOX (10*S*-dioxigenasa) y 7,10-DS (7,10-diol sintasa) implicadas en la oxigenación de ácidos grasos de cadena larga a través de la vía oleato diol sintasa, recientemente descrita. El análisis de la secuencia de aminoácidos de ambas enzimas reveló la presencia de dos motivos de unión a hemo (CXXCH) en cada proteína. Además, el análisis filogenético mostró la relación de ambas proteínas a citocromo c peroxidasas di-hemo bacterianas (CPP), con similitud a la oxidasa del látex de *Xanthomonas* sp. 35Y, denominada RoxA. El modelo 3D por homología de PA2077 y PA2078 se obtuvo utilizando la estructura de RoxA (4b2n pdb) como molde. A partir de los modelos en 3D de los grupos funcionales obtenida, se observaron variaciones significativas en los aminoácidos en torno a los grupos hemo. Además, se detectó la presencia de repeticiones palindrómicas localizadas en las regiones codificantes de las enzimas, que pueden actuar como elementos de evolución de proteínas, siendo la primera vez que se describen en el genoma de *P. aeruginosa*. Estas observaciones y el árbol filogenético obtenido para las dos proteínas, permiten proponer una vía evolutiva para el operón diol sintasa de *P. aeruginosa*. Teniendo en cuenta el conjunto de resultados *in silico* e *in vivo* obtenidos, concluimos que las enzimas PA2077 y PA2078 constituyen los primeros miembros de una nueva subfamilia de peroxidasas bacterianas, designada como citocromo c peroxidasas di-hemo de ácidos grasos (FadCcp).

CONCLUSIONES

1. La actividad oleato diol sintasa se localiza en el espacio periplásmico de *P. aeruginosa.*

2. ExFadLO, localizada en la membrana externa, se requiere para exportar 10-HOME y 7,10-DiHOME desde el periplasma al medio de extracellular.

3. Se han caracterizado funcionalmente dos nuevos genes del genoma de *P. aeruginosa* y su participación en la ruta metabólica oleato diol sintasa ha sido determinada experimentalmente: PA2077 como la oleato 10*S*-DOX, responsable de la primera etapa de oxigenación del ácido oleico y la producción del intermediario 10-HPOME y PA2078 como la enzima 7,10-DS, que participan en la formación de 7,10-DiHOME. Los genes de la diol sintasa constituyen un operón bicistrónico finamente regulado.

4. La expresión recombinante individual de las proteínas 10*S*-DOX y 7,10-DS en *E. coli* confirmó su actividad y demostró que no se requiere la formación de hetero-complejos para llevar a cabo la actividad oleato diol sintasa.

5. Los genes que codifican para la actividad enzimática diol sintasa podrían haberse originado por un evento de duplicación, seguido de la neo-functionalización de uno de ellos, lo más probable del *PA2078*.

6. La actividad oleato diol sintasa se conserva en todas las especies de *P. aeruginosa* y es única entre bacterias.

7. El análisis bioinformático de las enzimas de la diol sintasa 10*S*-DOX y 7,10-DS, muestran que ambas proteínas constituyen una nueva subfamilia de citocromo c peroxidasas bacterianas di-hemos, nombradas como FadCCPs.

8. La enzima 10*S*-DOX exhibe dos grupos de unión a hemo funcionales que se requieren para la dioxigenación del oleato. Por el contrario, se encontró que los grupos hemo no se requieren para la actividad 7,10-DS.

9. La presencia de regiones palindrómicas dentro de las regiones que codifican para la diol sintasa fueron identificadas en *P. aeruginosa* por primera vez y se describieron como elementos evolutivos.

10. Se ha obtenido una cepa recombinante de *P. putida* KT2440 GRAS que expresa la enzima 10*S*-DOX de manera funcional para la producción biotecnológica de oxilipinas.

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9. ANNEXES

9.1. ADDITIONAL INFORMATION

Available data from transcriptomic studies were manually matched with information of the Pseudomonas Community Annotation Project (PseudoCAP) functional class, Gene Ontology prediction and Category from Pseudomonas Genome Database (www.pseudomonas.com). Oleate-diol synthase genes are coloured in grey.

Table S1. Genes expressed 4 folds or greater in LB-oleate induced transcriptome versus LB (Kang *et al.*, 2008).

Table S2. Genes expressed 4 folds or greater in LB versus LB-oleate induced transcriptome (Kang *et al.*, 2008).

Table S3. Genes expressed 4 folds or greater in PE-chemotaxis induced transcriptome (Miller *et al.*, 2008).

Table S4. Shared genes in oleate-induced and PE-chemotaxis induced transcriptome (Miller *et al.*, 2008; Kang *et al.*, 2008).

Genes expressed 4 folds or greater in LB-oleate induced transcriptome vs LB				
Accession Number		PseudoCAP/functional class	COG Prediction	Category
PA0169		Hypothetical, unclassified, unknown/Motility & Attachment	c-di-GMP synthetase (diguanylate cyclase, GGDEF domain)	Signal transduction mechanism
PA0195		Transport of small molecules/Energy metabolism	NAD/NADP transhydrogenase alpha subunit	Energy production and conversion
PA0196		Hypothetical, unclassified, unknown	NAD/NADP transhydrogenase alpha subunit	Energy production and conversion
PA0383		Hypothetical, unclassified, unknown	-	-
PA0506		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0508		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0517		Biosynthesis of cofactors, prosthetic groups and carriers/Energy metabolism	Cytochrome c, mono- and diheme variants	Energy production and conversion
PA0518		Biosynthesis of cofactors, prosthetic groups and carriers/Energy metabolism	Cytochrome c551/c552	Energy production and conversion
PA0519		/Energy metabolism	Cytochrome c, mono- and diheme variants	Energy production and conversion
PA0524		/Energy metabolism	Nitric oxide reductase large subunit	Inorganic ion transport and metabolism
PA0977		Hypothetical, unclassified, unknown	-	-
PA0997		Biosynthesis of cofactors, prosthetic groups and carriers	3-oxoacyl-	Lipid transport and metabolism
PA1748		Fatty acid and phospholipid metabolism	Enoyl-CoA hydratase/carnithine racemase	Lipid transport and metabolism
PA2077	1	Putative enzymes	Oxylipins biosynthesis	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA2078	1	Putative enzymes	Oxylipins biosynthesis	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA2458		Hypothetical, unclassified, unknown	Rhs family protein	Cell wall/membrane/envelope biogenesis
PA2634		Putative enzymes	Isocitrate lyase	Energy production and conversion
PA2814		Hypothetical, unclassified, unknown	Transglutaminase-like enzymes, putative cysteine proteases	Amino acid transport and metabolism
PA3012		Hypothetical, unclassified, unknown	Uncharacterized protein conserved in bacteria	Function unknown
PA3013		Amino acid biosynthesis and metabolism/Fatty acid and phospholipid metabolism	Acetyl-CoA acetyltransferase	Lipid transport and metabolism
PA3014		Amino acid biosynthesis and metabolism/Fatty acid and phospholipid metabolism	3-hydroxyacyl-CoA dehydrogenase	Lipid transport and metabolism
PA3092		Fatty acid and phospholipid metabolism	NADH:flavin oxidoreductases, Old Yellow Enzyme family	Energy production and conversion
PA3136		Transport of small molecules	Multidrug resistance efflux pump	Defense mechanisms
PA3137		Transport of small molecules/membrane proteins	Arabinose efflux permease	Carbohydrate transport and metabolism
PA3392		Energy metabolism	Nitrous oxide reductase	Energy production and conversion
PA3608		Transport of small molecules/membrane proteins	ABC-type spermidine/putrescine transport system, permease component I	Amino acid transport and metabolism
PA3610		Transport of small molecules	Spermidine/putrescine-binding periplasmic protein	Amino acid transport and metabolism
PA4167		Putative enzymes	Aldo/keto reductases, related to diketogulonate reductase	General function prediction only
PA4514		Transport of small molecules	Outer membrane receptor for monomeric catechols	Inorganic ion transport and metabolism
PA4625	1	Hypothetical, unclassified, unknown	-	-
	2	Cell wall / LPS / capsule	-	-
	3	Secreted Factors (toxins, enzymes, alginate)	-	-
PA4710		Transport of small molecules	Outer membrane receptor proteins, mostly Fe transport	Inorganic ion transport and metabolism
PA4770		Transport of small molecules	L-lactate permease	Energy production and conversion
PA4471		Energy metabolism	Isopentenyl diphosphate isomerase (BS_ypgA, MTH48 and related proteins)	Energy production and conversion
PA4472		Energy metabolism	FAD/FMN-containing dehydrogenases	Energy production and conversion
PA5170	1	Membrane proteins	Amino acid transporters	Amino acid transport and metabolism
	2	Amino acid biosynthesis and metabolism	-	-
	3	Transport of small molecules	-	-

Genes expressed 4 folds or greater in LB vs LB-oleate induced transcriptome (Kang, 2008)				
Accession Number		PseudoCAP/functional class	COG Prediction	Category
PA0031		Adaptation, Protection	Arylsulfatase A and related enzymes	Inorganic ion transport and metabolism
PA0049		Hypothetical, unclassified, unknown	-	
PA0129	1	Transport of small molecules	Gamma-aminobutyrate permease and related permeases	Amino acid transport and metabolism
	2	Carbon compound catabolism	-	
PA0130	1	Carbon compound catabolism	NAD-dependent aldehyde dehydrogenases	Energy production and conversion
	2	putative enzymes	-	
PA0131	1	Hypothetical, unclassified, unknown	Uncharacterized conserved protein, contains double-stranded beta-helix domain	Function unknown
	2	Carbon compound catabolism	-	
PA0132	1	Amino acid biosynthesis and metabolism	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Coenzyme transport and metabolism
	2	Carbon compound catabolism	-	
PA0186		Transport of small molecules	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	Inorganic ion transport and metabolism
PA0191		Transcriptional regulators	Transcriptional regulator	Transcription
PA0197		Transport of small molecules	Periplasmic protein TonB, links inner and outer membranes	Cell wall/membrane/envelope biogenesis
PA0198		Transport of small molecules	Biopolymer transport proteins	Intracellular trafficking, secretion, and vesicular transport
PA0201		Hypothetical, unclassified, unknown	Predicted esterase of the alpha/beta hydrolase fold	General function prediction only
PA0280		Transport of small molecules	ABC-type sulfate/molybdate transport systems, ATPase component	Inorganic ion transport and metabolism
PA0281	1	Membrane proteins	ABC-type sulfate transport system, permease component	Inorganic ion transport and metabolism
	2	Transport of small molecules	-	-
PA0282	1	Membrane proteins	ABC-type sulfate transport system, permease component	Posttranslational modification, protein turnover, chaperones
	2	Transport of small molecules	-	-
PA0283		Transport of small molecules	ABC-type sulfate transport system, periplasmic component	Inorganic ion transport and metabolism
PA0284		Hypothetical, unclassified, unknown	Uncharacterized small protein	Function unknown
PA0364		putative enzymes	Choline dehydrogenase and related flavoproteins	Amino acid transport and metabolism
PA0365		Membrane proteins	-	-
PA0366		putative enzymes	NAD-dependent aldehyde dehydrogenases	Energy production and conversion
PA0493		putative enzymes	Biotin carboxyl carrier protein	Lipid transport and metabolism
PA0494		putative enzymes	Acetyl/propionyl-CoA carboxylase, alpha subunit	Lipid transport and metabolism
PA0495		Hypothetical, unclassified, unknown	Allophanate hydrolase subunit 1	Amino acid transport and metabolism
PA0496		Conserved hypothetical protein	Allophanate hydrolase subunit 2	Amino acid transport and metabolism
PA0742		Hypothetical, unclassified, unknown	-	-
PA0744		putative enzymes	Enoyl-CoA hydratase/carnithine racemase	Lipid transport and metabolism
PA0746		putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0789	1	Membrane proteins	Gamma-aminobutyrate permease and related permeases	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA0794		Energy metabolism	Aconitase A	Energy production and conversion
PA0795	1	Central intermediary metabolism	Citrate synthase	Energy production and conversion
	2	Carbon compound catabolism	-	-
PA0796	1	Fatty acid and phospholipid metabolism	PEP phosphonomutase and related enzymes	Carbohydrate transport and metabolism
	2	Central intermediary metabolism	-	-
	3	Carbon compound catabolism	-	-
PA0797		Transcriptional regulators	Transcriptional regulators	Transcription
PA0830		Hypothetical, unclassified, unknown	Predicted metal-dependent hydrolase	General function prediction only
PA0865		Amino acid biosynthesis and metabolism	4-hydroxyphenylpyruvate dioxygenase and related hemolysins	Amino acid transport and metabolism/General function prediction only
PA0866		Transport of small molecules	Gamma-aminobutyrate permease and related permeases	Amino acid transport and metabolism
PA0867	1	Hypothetical, unclassified, unknown	Predicted periplasmic protein	General function prediction only
	2	Adaptation, Protection	-	-
PA0870		Amino acid biosynthesis and metabolism	Aspartate/tyrosine/aromatic aminotransferase	Amino acid transport and metabolism
PA0872		Amino acid biosynthesis and metabolism	Phenylalanine-4-hydroxylase	Amino acid transport and metabolism
PA0887	1	Carbon compound catabolism	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	Lipid transport and metabolism
	2	Central intermediary metabolism	-	-
PA1071		Transport of small molecules	ABC-type branched-chain amino acid transport systems, ATPase component	Amino acid transport and metabolism
PA1074		Transport of small molecules	ABC-type branched-chain amino acid transport systems, periplasmic component	Amino acid transport and metabolism
PA1337	1	Amino acid biosynthesis and metabolism	L-asparaginase/archaeal Glu-tRNAGln amidotransferase subunit D	Amino acid transport and metabolism
	2	-	Translation, ribosomal structure and biogenesis	-
PA1338	1	Amino acid biosynthesis and metabolism	Gamma-glutamyltransferase	Amino acid transport and metabolism
	2	Adaptation, Protection	-	-
	3	Central intermediary metabolism	-	-
PA1409	1	Carbon compound catabolism	Deacetylases, including yeast histone deacetylase and acetoin utilization protein	Chromatin structure and dynamics
	2	-	Secondary metabolites biosynthesis, transport and catabolism	-
PA1410		Transport of small molecules	Spermidine/putrescine-binding periplasmic protein	Amino acid transport and metabolism

PA1537		putative enzymes	Short-chain dehydrogenases of various substrate specificities	General function prediction only
PA1542		Hypothetical, unclassified, unknown	Predicted metal-dependent hydrolase	General function prediction only
PA1632		Transport of small molecules	-	-
PA1634		Transport of small molecules	High-affinity K+ transport system, ATPase chain B	Inorganic ion transport and metabolism
PA1760		Transcriptional regulators	ATP-dependent transcriptional regulator	Transcription
PA1818		Amino acid biosynthesis and metabolism	Arginine/lysine/ornithine decarboxylases	Amino acid transport and metabolism
PA1819	1	Membrane proteins	Amino acid transporters	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA1837		Hypothetical, unclassified, unknown	Uncharacterized protein conserved in bacteria	Function unknown
PA1838		Central intermediary metabolism	Sulfite reductase, beta subunit (hemoprotein)	Inorganic ion transport and metabolism
PA1978	1	Transcriptional regulators	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	Signal transduction mechanisms
	2	-	Transcription	-
PA1984		putative enzymes	NAD-dependent aldehyde dehydrogenases	Energy production and conversion
PA1985		Biosynthesis of cofactors, prosthetic groups and carriers	-	-
PA1986		Biosynthesis of cofactors, prosthetic groups and carriers	Metal-dependent hydrolases of the beta-lactamase superfamily I	General function prediction only
PA1987		Biosynthesis of cofactors, prosthetic groups and carriers	Pyrroloquinoline quinone (Coenzyme PQQ) biosynthesis protein C	Coenzyme transport and metabolism
PA1988		Biosynthesis of cofactors, prosthetic groups and carriers	-	-
PA1990		putative enzymes	Dipeptidyl aminopeptidases/acylaminoacyl-peptidases	Amino acid transport and metabolism
PA1999	1	Amino acid biosynthesis and metabolism	Acyl CoA:acetate/3-ketoacid CoA transferase, alpha subunit	Lipid transport and metabolism
	2	Carbon compound catabolism	-	-
PA2000	1	Amino acid biosynthesis and metabolism	Acyl CoA:acetate/3-ketoacid CoA transferase, beta subunit	Lipid transport and metabolism
	2	Carbon compound catabolism	-	-
PA2001	1	Central intermediary metabolism	Acetyl-CoA acetyltransferase	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA2002	1	Hypothetical, unclassified, unknown	Short chain fatty acids transporter	Lipid transport and metabolism
	2	Membrane proteins	-	-
PA2003	1	Carbon compound catabolism	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	Lipid transport and metabolism
	2	-	Secondary metabolites biosynthesis, transport and catabolism	-
	3	-	General function prediction only	-
PA2004	1	Membrane proteins	H+/gluconate symporter and related permeases	Carbohydrate transport and metabolism
	2	-	Amino acid transport and metabolism	-
PA2006	1	Membrane proteins	Sugar phosphate permease	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-
PA2007		Carbon compound catabolism	Glutathione S-transferase	Posttranslational modification, protein turnover, chaperones
PA2008		Carbon compound catabolism	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)	Secondary metabolites biosynthesis, transport and catabolism
PA2009		Carbon compound catabolism	Homogentisate 1,2-dioxygenase	Secondary metabolites biosynthesis, transport and catabolism
PA2012		Carbon compound catabolism	Acetyl/propionyl-CoA carboxylase, alpha subunit	Lipid transport and metabolism
PA2013		Carbon compound catabolism	Enoyl-CoA hydratase/carnithine racemase	Lipid transport and metabolism
PA2014		Carbon compound catabolism	Acetyl-CoA carboxylase, carboxyltransferase component (subunits alpha and beta)	Lipid transport and metabolism
PA2015		Carbon compound catabolism	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA2016		Transcriptional regulators	Predicted transcriptional regulators	Transcription
PA2041	1	Transport of small molecules	Amino acid transporters	Amino acid transport and metabolism
	2	Carbon compound catabolism	-	-
PA2062		putative enzymes	Cysteine sulfinate desulfinase/cysteine desulfurase and related enzymes	Amino acid transport and metabolism
PA2073		Transport of small molecules	Purine-cytosine permease and related proteins	Nucleotide transport and metabolism
PA2109		Hypothetical, unclassified, unknown	Predicted outer membrane protein	Function unknown
PA2110		Hypothetical, unclassified, unknown	Allophanate hydrolase subunit 2	Amino acid transport and metabolism
PA2111		Hypothetical, unclassified, unknown	Allophanate hydrolase subunit 1	Amino acid transport and metabolism
PA2112		Hypothetical, unclassified, unknown	Uncharacterized proteins, homologs of lactam utilization protein B	General function prediction only
PA2113	1	Transport of small molecules	-	-
	2	Membrane proteins	-	-
PA2114	1	Membrane proteins	Arabinose efflux permease	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-
PA2116		Hypothetical, unclassified, unknown	Uncharacterized conserved protein	Function unknown
PA2123		Transcriptional regulators	Transcriptional regulator	Transcription
PA2202	1	Membrane proteins	ABC-type amino acid transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA2203	1	Membrane proteins	ABC-type amino acid transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA2204	1	Transport of small molecules	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	Amino acid transport and metabolism
	2	-	Signal transduction mechanisms	-
PA2247		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, alpha subunit	Energy production and conversion

PA2248		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, beta subunit	Energy production and conversion
PA2249		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide acyltransferase (E2) component, and related enzymes	Energy production and conversion
PA2250	1	Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	Energy production and conversion
	2	Energy metabolism	-	-
PA2310		putative enzymes	Probable taurine catabolism dioxygenase	Secondary metabolites biosynthesis, transport and catabolism
PA2312		Transcriptional regulators	Predicted transcriptional regulators	Transcription
PA2322	1	Transport of small molecules	H+/gluconate symporter and related permeases	Carbohydrate transport and metabolism
	2	-	Amino acid transport and me	-
PA2330		Hypothetical, unclassified, unknown	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA2331		Membrane proteins	Uncharacterized conserved protein	Function unknown
PA2334		Transcriptional regulators	Transcriptional regulator	Transcription
PA2359	1	Transcriptional regulators	Transcriptional regulators containing an AAA-type ATPase domain and a DNA-binding domain	Transcription
	2	-	-	Signal transduction mechanisms
PA2533	1	Membrane proteins	Na+/alanine symporter	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA2552		putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA2553		putative enzymes	Acetyl-CoA acetyltransferase	Lipid transport and metabolism
PA2554	1	putative enzymes	ehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	Lipid transport and metabolism
	2	-	Secondary metabolites biosynthesis, transport and catabolism	-
	3	-	General function prediction only	-
PA2555		putative enzymes	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	Lipid transport and metabolism
PA2557	1	Fatty acid and phospholipid metabolism	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	Lipid transport and metabolism
	2	-	Secondary metabolites biosynthesis, transport and catabolism	-
PA2594		Hypothetical, unclassified, unknown	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	Inorganic ion transport and metabolism
PA2598		Hypothetical, unclassified, unknown	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases	Energy production and conversion
PA2599		Hypothetical, unclassified, unknown	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	Inorganic ion transport and metabolism
PA2600		Hypothetical, unclassified, unknown	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases	Energy production and conversion
PA2862	1	Carbon compound catabolism	Predicted acetyltransferases and hydrolases with the alpha/beta hydrolase fold	General function prediction only
	2	Secreted Factors (toxins, enzymes, alginate)	-	-
PA3038		Transport of small molecules	-	-
PA3068		Amino acid biosynthesis and metabolism	NAD-specific glutamate dehydrogenase	Amino acid transport and metabolism
PA3186		Transport of small molecules	Carbohydrate-selective porin	Cell wall/membrane/envelope biogenesis
PA3187		Transport of small molecules	ABC-type sugar transport systems, ATPase components	Carbohydrate transport and metabolism
PA3188		Transport of small molecules	ABC-type sugar transport system, permease component	Carbohydrate transport and metabolism
PA3189		Transport of small molecules	ABC-type sugar transport systems, permease components	Carbohydrate transport and metabolism
PA3190		Transport of small molecules	ABC-type sugar transport system, periplasmic component	Carbohydrate transport and metabolism
PA3195	1	Energy metabolism	Glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase	Carbohydrate transport and metabolism
	2	Carbon compound catabolism	-	-
PA3232		putative enzymes	DNA polymerase III, epsilon subunit and related 3'-5' exonucleases	Replication, recombination and repair
PA3233		Hypothetical, unclassified, unknown	Predicted signal-transduction protein containing cAMP-binding and CBS domains	Signal transduction mechanisms
PA3234	1	Membrane proteins	Predicted symporter	General function prediction only
	2	Transport of small molecules	-	-
PA3235		Membrane proteins	Predicted membrane protein	Function unknown
PA3356	1	Hypothetical, unclassified, unknown	Glutamine synthetase	Amino acid transport and metabolism
	2	Carbon compound catabolism	-	-
PA3365		Chaperones & heat shock proteins	ATP-binding subunits of Clp protease and DnaK/DnaJ chaperones	Posttranslational modification, protein turnover, chaperones
PA3442		Transport of small molecules	ABC-type nitrate/sulfonate/bicarbonate transport system, ATPase component	Inorganic ion transport and metabolism
PA3443	1	Membrane proteins	ABC-type nitrate/sulfonate/bicarbonate transport system, permease component	Inorganic ion transport and metabolism
	2	Transport of small molecules	-	-
PA3444		putative enzymes	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases	Energy production and conversion
PA3445		Hypothetical, unclassified, unknown	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	Inorganic ion transport and metabolism
PA3446		Hypothetical, unclassified, unknown	Predicted flavoprotein	General function prediction only
PA3450		Adaptation, Protection	Peroxiredoxin	Posttranslational modification, protein turnover, chaperones
PA3510		Hypothetical, unclassified, unknown	Uncharacterized conserved protein, contains double-stranded beta-helix domain	Function unknown
PA3513		Hypothetical, unclassified, unknown	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	Inorganic ion transport and metabolism
PA3779		Hypothetical, unclassified, unknown	TRAP-type C4-dicarboxylate transport system, periplasmic component	Carbohydrate transport and metabolism
PA3780		Membrane proteins	TRAP-type C4-dicarboxylate transport system, small permease component	Carbohydrate transport and metabolism
PA3781	1	Membrane proteins	TRAP-type C4-dicarboxylate transport system, large permease component	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-
PA3922		Hypothetical, unclassified, unknown	-	-
PA3923		Hypothetical, unclassified, unknown	-	-

PA3931		Hypothetical, unclassified, unknown	ABC-type metal ion transport system, periplasmic component/surface antigen	Inorganic ion transport and metabolism
PA3932	1	Transcriptional regulators	Transcriptional regulators containing an AAA-type ATPase domain and a DNA-binding domain	Transcription
	2	-	Signal transduction mechanisms	-
PA3935		Carbon compound catabolism	Probable taurine catabolism dioxygenase	Secondary metabolites biosynthesis, transport and catabolism
PA3936	1	Membrane proteins	ABC-type nitrate/sulfonate/bicarbonate transport system, permease component	Inorganic ion transport and metabolism
	2	Transport of small molecules	-	-
PA3637		Transport of small molecules	ABC-type taurine transport system, ATPase component	Inorganic ion transport and metabolism
PA3938		Transport of small molecules	ABC-type taurine transport system, periplasmic component	Inorganic ion transport and metabolism
PA4021	1	Transcriptional regulators	Transcriptional activator of acetoin/glycerol metabolism	Secondary metabolites biosynthesis, transport and catabolism
	2	-	-	Transcription
PA4023		Transport of small molecules	Amino acid transporters	Amino acid transport and metabolism
PA4024		Central intermediary metabolism	Ethanolamine ammonia-lyase, large subunit	Amino acid transport and metabolism
PA4025		Carbon compound catabolism	Ethanolamine ammonia-lyase, small subunit	Amino acid transport and metabolism
PA4191		putative enzymes	Isopenicillin N synthase and related dioxygenases	General function prediction only
PA4192		Transport of small molecules	ABC-type polar amino acid transport system, ATPase component	Amino acid transport and metabolism
PA4193	1	Membrane proteins	ABC-type amino acid transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA4194	1	Membrane proteins	ABC-type amino acid transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA4195	1	Transport of small molecules	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	Amino acid transport and metabolism
	2	-	Signal transduction mechanisms	-
PA4198	1	putative enzymes	Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II	Lipid transport and metabolism
	2	-	Secondary metabolites biosynthesis, transport and catabolism	-
PA4290	1	Adaptation, Protection	Methyl-accepting chemotaxis protein	Cell motility
	2	Chemotaxis	-	Signal transduction mechanisms
PA4442	1	Central intermediary metabolism	GTPases - Sulfate adenylate transferase subunit 1	Inorganic ion transport and metabolism
	2	Amino acid biosynthesis and metabolism	-	-
PA4443	1	Central intermediary metabolism	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase)/FAD synthetase and related enzymes	Amino acid transport and metabolism
	2	Amino acid biosynthesis and metabolism	-	Coenzyme transport and metabolism
PA4496		Transport of small molecules	ABC-type dipeptide transport system, periplasmic component	Amino acid transport and metabolism
PA4497		Transport of small molecules	ABC-type dipeptide transport system, periplasmic component	Amino acid transport and metabolism
PA4498		Translation, post-translational modification, degradation	Xaa-Pro aminopeptidase	Amino acid transport and metabolism
PA4500		Transport of small molecules	ABC-type dipeptide transport system, periplasmic component	Amino acid transport and metabolism
PA4501	1	Transport of small molecules	-	-
	2	Membrane proteins	-	-
PA4502		Transport of small molecules	ABC-type dipeptide transport system, periplasmic component	Amino acid transport and metabolism
PA4503	1	Membrane proteins	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	Amino acid transport and metabolism
	2	Transport of small molecules	-	Inorganic ion transport and metabolism
PA4504	1	Membrane proteins	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	Amino acid transport and metabolism
	2	Transport of small molecules	-	Inorganic ion transport and metabolism
PA4505	1	Transport of small molecules	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	Amino acid transport and metabolism
	2	-	-	Inorganic ion transport and metabolism
PA4506		Transport of small molecules	ABC-type oligopeptide transport system, ATPase component	Amino acid transport and metabolism
PA4605		Hypothetical, unclassified, unknown	Uncharacterized small protein	Function unknown
PA4606		Adaptation, Protection	Carbon starvation protein, predicted membrane protein	Signal transduction mechanisms
PA4733	1	Carbon compound catabolism	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	Lipid transport and metabolism
	2	Central intermediary metabolism	-	-
PA4888		Fatty acid and phospholipid metabolism	Fatty acid desaturase	Lipid transport and metabolism
PA4979		putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA5024		Hypothetical, unclassified, unknown	Predicted permeases	General function prediction only
PA5102		Membrane proteins	Fatty acid desaturase	Lipid transport and metabolism
PA5103		Hypothetical, unclassified, unknown	ABC-type proline/glycine betaine transport systems, periplasmic components	Amino acid transport and metabolism
PA5152		Transport of small molecules	ABC-type histidine transport system, ATPase component	Amino acid transport and metabolism
PA5153	1	Transport of small molecules	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	Amino acid transport and metabolism
	2	-	-	Signal transduction mechanisms
PA5154	1	Membrane proteins	ABC-type arginine transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA5155	1	Membrane proteins	ABC-type arginine/histidine transport system, permease component	Amino acid transport and metabolism
	2	Transport of small molecules	-	-
PA5168	1	Membrane proteins	TRAP-type C4-dicarboxylate transport system, small permease component	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-

PA5169	1	Membrane proteins	TRAP-type C4-dicarboxylate transport system, large permease component	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-
PA5174	1	Fatty acid and phospholipid metabolism	3-oxoacyl-(acyl-carrier-protein) synthase	Lipid transport and metabolism
	2	-	-	Secondary metabolites biosynthesis, transport and catabolism
PA5212		Hypothetical, unclassified, unknown	-	-
PA5302		Amino acid biosynthesis and metabolism	Alanine racemase	Cell wall/membrane/envelope biogenesis
PA5303		Hypothetical, unclassified, unknown	Putative translation initiation inhibitor, yjgF family	Translation, ribosomal structure and biogenesis
PA5304	1	Energy metabolism	Glycine/D-amino acid oxidases (deaminating)	Amino acid transport and metabolism
	2	Amino acid biosynthesis and metabolism	-	-
PA5375	1	Membrane proteins	Choline-glycine betaine transporter	Cell wall/membrane/envelope biogenesis
	2	Transport of small molecules	-	-
PA5380		Transcriptional regulators	Transcriptional regulator containing an amidase domain and an AraC-type DNA-binding HTH domain	Transcription
PA5396		Hypothetical, unclassified, unknown	Zn-dependent dipeptidase, microsomal dipeptidase homolog	Amino acid transport and metabolism
PA5397		Hypothetical, unclassified, unknown	-	-
PA5398		Amino acid biosynthesis and metabolism	NADH:flavin oxidoreductases, Old Yellow Enzyme family	Energy production and conversion
PA5400		Energy metabolism	Electron transfer flavoprotein, alpha subunit	Energy production and conversion
PA5410	1	Amino acid biosynthesis and metabolism	Phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit	Inorganic ion transport and metabolism
	2	-	-	General function prediction only
PA5411		Amino acid biosynthesis and metabolism	Flavodoxin reductases (ferredoxin-NADPH reductases) family 1	Energy production and conversion
PA5416		Amino acid biosynthesis and metabolism	Glycine/D-amino acid oxidases (deaminating)	Amino acid transport and metabolism
PA5417		Amino acid biosynthesis and metabolism	Sarcosine oxidase delta subunit	Amino acid transport and metabolism
PA5419		Amino acid biosynthesis and metabolism	Sarcosine oxidase gamma subunit	Amino acid transport and metabolism
PA5420		Nucleotide biosynthesis and metabolism	Formyltetrahydrofolate hydrolase	Nucleotide transport and metabolism
PA5421	1	Central intermediary metabolism	Threonine dehydrogenase and related Zn-dependent dehydrogenases	Amino acid transport and metabolism
	2	-	-	General function prediction only
PA5445		putative enzymes	Acetyl-CoA hydrolase	Energy production and conversion
PA5507		Hypothetical, unclassified, unknown	Amidases related to nicotinamidase	Secondary metabolites biosynthesis, transport and catabolism
PA5508	1	Putative enzymes	Glutamine synthetase	Amino acid transport and metabolism
	2	Carbon compound catabolism	-	-
PA5543		Hypothetical, unclassified, unknown	Uncharacterized conserved protein	Function unknown

Genes expressed 4 folds or greater in PE chemotaxis induced transcriptome (Miller, 2008)				
Accession Number		PseudoCAP/functional class	COG Prediction	Category
PA0083	1	Hypothetical, unclassified, unknown	Predicted component of the type VI protein secretion system	Function unknown
	2	Protein secretion/export apparatus	-	-
PA0084	1	Hypothetical, unclassified, unknown	Predicted component of the type VI protein secretion system	Function unknown
	2	Protein secretion/export apparatus	-	-
PA0182	1	Putative enzymes	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	Lipid transport and metabolism
	2	-	-	Secondary metabolites biosynthesis, transport and catabolism
	3	-	-	General function prediction only
PA0208		Carbon compound catabolism	Acyl CoA:acetate/3-ketoacid CoA transferase	Lipid transport and metabolism
PA0209		Putative enzymes	Triphosphoribosyl-dephospho-CoA synthetase	Coenzyme transport and metabolism
PA0210		Carbon compound catabolism	Citrate lyase, gamma subunit	Energy production and conversion
PA0211		Carbon compound catabolism	Acetyl-CoA carboxylase beta subunit	Lipid transport and metabolism
PA0212		Carbon compound catabolism	Acetyl-CoA carboxylase, carboxyltransferase component (subunits alpha and beta)	Lipid transport and metabolism
PA0213		Carbon compound catabolism	-	-
PA0214		Carbon compound catabolism	(acyl-carrier-protein) S-malonyltransferase	Lipid transport and metabolism
PA0215	1	Membrane proteins	-	-
	2	Transport of small molecules	-	-
PA0216	1	Membrane proteins	Predicted membrane protein	Function unknown
	2	Transport of small molecules	-	-
PA0278		Hypothetical, unclassified, unknown	Predicted permeases	General function prediction only
PA0506		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0508		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0518	1	Biosynthesis of cofactors, prosthetic groups and carriers	Cytochrome c551/c552	Energy production and conversion
	2	Energy metabolism	-	-
PA0519		Energy metabolism	Cytochrome c, mono- and diheme variants	Energy production and conversion
PA0565		Hypothetical, unclassified, unknown	Uncharacterized homolog of gamma-carboxymuconolactone decarboxylase subunit	Function unknown
PA0752		Membrane proteins	Uncharacterized protein conserved in bacteria	Function unknown
PA0865	1	Amino acid biosynthesis and metabolism	4-hydroxyphenylpyruvate dioxygenase and related hemolysins	Amino acid transport and metabolism
	2	-	-	General function prediction only
PA0866		Transport of small molecules	Gamma-aminobutyrate permease and related permeases	Amino acid transport and metabolism
PA1137	1	Putative enzymes	NADPH:quinone reductase and related Zn-dependent oxidoreductases	Energy production and conversion
	2		-	General function prediction only
PA1288	1	Membrane proteins	Long-chain fatty acid transport protein	Lipid transport and metabolism
	2	Transport of small molecules	-	-
PA1412		Hypothetical, unclassified, unknown	Arabinose efflux permease	Carbohydrate transport and metabolism
PA1949	1	Carbon compound catabolism	Transcriptional regulators	Transcription
	2	Transcriptional regulators	-	-
PA1974		Hypothetical, unclassified, unknown	-	-
PA1975		Hypothetical, unclassified, unknown	Uncharacterized conserved protein	Function unknown
PA1976		Two-component regulatory systems	Signal transduction histidine kinase	Signal transduction mechanisms
PA1977		Membrane proteins	Threonine/homoserine efflux transporter	General function prediction only
PA1978	1	Transcriptional regulators	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	Signal transduction mechanisms
	2	-	-	Transcription
PA1979		Two-component regulatory systems	Signal transduction histidine kinase	Signal transduction mechanisms
PA1980	1	Transcriptional regulators	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain	Signal transduction mechanisms
	2	Two-component regulatory systems	-	Transcription
PA1981		Hypothetical, unclassified, unknown	Pentapeptide repeats containing protein	Function unknown
PA1982		Carbon compound catabolism	Glucose dehydrogenase	Carbohydrate transport and metabolism
PA1983	1	Energy metabolism	Cytochrome c, mono- and diheme variants	Energy production and conversion
	2	Carbon compound catabolism	-	-
PA1984		Putative enzymes	NAD-dependent aldehyde dehydrogenases	Energy production and conversion
PA1985		Biosynthesis of cofactors, prosthetic groups and carriers	-	-
PA1986		Biosynthesis of cofactors, prosthetic groups and carriers	Metal-dependent hydrolases of the beta-lactamase superfamily I	General function prediction only
PA1987		Biosynthesis of cofactors, prosthetic groups and carriers	Pyrroloquinoline quinone (Coenzyme PQQ) biosynthesis protein C	Coenzyme transport and metabolism
PA1988		Biosynthesis of cofactors, prosthetic groups and carriers	-	-
PA1989		Biosynthesis of cofactors, prosthetic groups and carriers	Predicted Fe-S oxidoreductases	General function prediction only
PA1990		Putative enzymes	Dipeptidyl aminopeptidases/acylaminoacyl-peptidases	Amino acid transport and metabolism
PA1991		Putative enzymes	Alcohol dehydrogenase, class IV	Energy production and conversion
PA1992		Two-component regulatory systems	Signal transduction histidine kinase	Signal transduction mechanisms
PA2011		Carbon compound catabolism	Isopropylmalate/homocitrate/citramalate synthases	Amino acid transport and metabolism
PA2012		Carbon compound catabolism	Acetyl/propionyl-CoA carboxylase, alpha subunit	Lipid transport and metabolism

PA2013		Carbon compound catabolism	Enoyl-CoA hydratase/carnithine racemase	Lipid transport and metabolism
PA2014		Carbon compound catabolism	Acetyl-CoA carboxylase, carboxyltransferase component (subunits alpha and beta)	Lipid transport and metabolism
PA2015		Carbon compound catabolism	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA2078	1	Putative enzymes	Oxylipins biosynthesis	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA2212		Hypothetical, unclassified, unknown	Pyridoxal phosphate biosynthesis protein	Coenzyme transport and metabolism
PA2247		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, alpha subunit	Energy production and conversion
PA2248		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, beta subunit	Energy production and conversion
PA2249		Amino acid biosynthesis and metabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide acyltransferase (E2) component, and related enzymes	Energy production and conversion
PA2268		Hypothetical, unclassified, unknown	CTP synthase (UTP-ammonia lyase)	Nucleotide transport and metabolism
PA2338		Transport of small molecules	ABC-type sugar transport system, periplasmic component	Carbohydrate transport and metabolism
PA2339		Transport of small molecules	ABC-type sugar transport systems, permease components	Carbohydrate transport and metabolism
PA2393		Central intermediary metabolism	Zn-dependent dipeptidase, microsomal dipeptidase homolog	Amino acid transport and metabolism
PA2535		Putative enzymes	Predicted oxidoreductases (related to aryl-alcohol dehydrogenases)	Energy production and conversion
PA2552		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA2553		Putative enzymes	Acetyl-CoA acetyltransferase	Lipid transport and metabolism
PA2554	1	Putative enzymes	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	Lipid transport and metabolism
	2	-	-	Secondary metabolites biosynthesis, transport and catabolism
	3	-	-	General function prediction only
PA2555		Putative enzymes	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	Lipid transport and metabolism
PA2557		Fatty acid and phospholipid metabolism	-	-
PA2634		Putative enzymes	Isocitrate lyase	Energy production and conversion
PA2847		Hypothetical, unclassified, unknown	-	-
PA2862	1	Carbon compound catabolism	Predicted acetyltransferases and hydrolases with the alpha/beta hydrolase fold	General function prediction only
	2	Secreted Factors (toxins, enzymes, alginate)	-	-
PA2863	1	Protein secretion/export apparatus	Lipase chaperone	Posttranslational modification, protein turnover, chaperones
	2	Secreted Factors (toxins, enzymes, alginate)	-	-
PA2880		Hypothetical, unclassified, unknown	Predicted membrane protein	Function unknown
PA3035		Putative enzymes	Glutathione S-transferase	Posttranslational modification, protein turnover, chaperones
PA3036		Hypothetical, unclassified, unknown	Predicted flavin-nucleotide-binding protein structurally related to pyridoxine 5'-phosphate oxidase	General function prediction only
PA3038		Transport of small molecules	-	-
PA3233		Hypothetical, unclassified, unknown	Predicted signal-transduction protein containing cAMP-binding and CBS domains	Signal transduction mechanisms
PA3234	1	Membrane proteins	Predicted symporter	General function prediction only
	2	Transport of small molecules	-	-
PA3235		Membrane proteins	Predicted membrane protein	Function unknown
PA3421		Hypothetical, unclassified, unknown	-	-
PA3518		Hypothetical, unclassified, unknown	-	-
PA3566		Hypothetical, unclassified, unknown	Uncharacterized conserved protein	Function unknown
PA3567	1	Putative enzymes	NADPH:quinone reductase and related Zn-dependent oxidoreductases	Energy production and conversion
	2	-	-	General function prediction only
PA3568		Putative enzymes	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	Lipid transport and metabolism
PA3581		Transport of small molecules	Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family)	Carbohydrate transport and metabolism
PA3709	1	Membrane proteins	Arabinose efflux permease	Carbohydrate transport and metabolism
	2	Transport of small molecules	-	-
PA4147	1	Transcriptional regulators	Transcriptional activator of acetoin/glycerol metabolism	Secondary metabolites biosynthesis, transport and catabolism
	2	-	-	Transcription
PA4149		Hypothetical, unclassified, unknown	Predicted inorganic polyphosphate/ATP-NAD kinase	Function unknown
PA4150		Carbon compound catabolism	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, alpha subunit	Energy production and conversion
PA4469		Hypothetical, unclassified, unknown	-	-
PA4588		Amino acid biosynthesis and metabolism	Glutamate dehydrogenase/leucine dehydrogenase	Amino acid transport and metabolism
PA4590	1	Transport of small molecules	-	-
	2	Carbon compound catabolism	-	-
PA4680		Hypothetical, unclassified, unknown	-	-
PA4813		Fatty acid and phospholipid metabolism	Predicted acetyltransferases and hydrolases with the alpha/beta hydrolase fold	General function prediction only
PA5353	1	Central intermediary metabolism	Fe-S oxidoreductase	Energy production and conversion
	2	Carbon compound catabolism	-	-
PA5354	1	Central intermediary metabolism	FAD/FMN-containing dehydrogenases	Energy production and conversion
	2	Carbon compound catabolism	-	-
PA5355	1	Central intermediary metabolism	FAD/FMN-containing dehydrogenases	Energy production and conversion
	2	Carbon compound catabolism	-	-

Shared genes in oleate-induced and PE-chemotaxis induced transcriptome				
Accession Number		PseudoCAP/functional class	COG Prediction	Category
PA0277		Hypothetical, unclassified, unknown	Zn-dependent protease with chaperone function	Posttranslational modification, protein turnover, chaperones
PA0506		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0508		Putative enzymes	Acyl-CoA dehydrogenases	Lipid transport and metabolism
PA0518 (nirM)	1	Biosynthesis of cofactors, prosthetic groups and carriers	Cytochrome c551/c552	Energy production and conversion
	2	Energy metabolism	-	-
PA0519 (nirS)		Energy metabolism	Cytochrome c, mono- and diheme variants	Energy production and conversion
PA2077	1	Putative enzymes	Oxylipins biosynthesis	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	
PA2078	1	Putative enzymes	Oxylipins biosynthesis	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	
PA2137		Hypothetical, unclassified, unknown	Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains	Signal transduction mechanisms
PA2138 (ligD)		DNA replication, recombination, modification and repair	Predicted eukaryotic-type DNA primase	Replication, recombination and repair
PA2139		Hypothetical, unclassified, unknown	-	-
PA2140		Central intermediary metabolism	-	-
PA2386 (pvdA)		Adaptation, Protection	Lysine/ornithine N-monooxygenase	Secondary metabolites biosynthesis, transport and catabolism
PA2393		Central intermediary metabolism	Zn-dependent dipeptidase, microsomal dipeptidase homolog	Amino acid transport and metabolism
PA2394 (pvdN)		Adaptation, Protection	Selenocysteine lyase/Cysteine desulfurase	Amino acid transport and metabolism
PA2397 (pvdE)	1	Transport of small molecules	ABC-type siderophore export system, fused ATPase and permease components	Secondary metabolites biosynthesis, transport and catabolism
	2	Adaptation, Protection	-	Inorganic ion transport and metabolism
	3	Membrane proteins	-	-
PA3013 (faoB)	1	Amino acid biosynthesis and metabolism	Acetyl-CoA acetyltransferase	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA3014 (faoA)	1	Amino acid biosynthesis and metabolism	3-hydroxyacyl-CoA dehydrogenase	Lipid transport and metabolism
	2	Fatty acid and phospholipid metabolism	-	-
PA4054 (ribB)		Biosynthesis of cofactors, prosthetic groups and carriers	3,4-dihydroxy-2-butanone 4-phosphate synthase	Coenzyme transport and metabolism
PA4437		Hypothetical, unclassified, unknown	-	-
PA4468 (sodM)		Adaptation, Protection	Superoxide dismutase	Inorganic ion transport and metabolism
PA4469		Hypothetical, unclassified, unknown	-	-
PA4470 (fumC1)		Energy metabolism	Fumarase	Energy production and conversion