

**SCREENING METAGENOME FOR NOVEL N-HYDROXYLATING
MONOOXYGENASES (NMOs) USING BIOINFORMATICS AND
BIOTECHNOLOGICAL TOOLS**

BY

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DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exception to the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

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DEDICATION

To God be the Glory. This work is dedicated to my Heavenly Father, who is the source of my life and strength.

To the memory of my sweet mother Mrs Adumila Helen Ireiola, who taught me the way of the Lord.

To all my friends, well-wishers and loved ones as well.

To my Kids, the Next Generation Biotechnologists.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BVMOs	Baeyer-Villiger monooxygenases
DNA	Deoxyribonucleic Acid
ϵ	Extinction coefficient
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FMOs	Flavin containing monooxygenases
FPLC	Fast performance liquid chromatographer
IucD	<i>L</i> -lysine N^6 monooxygenase
IPTG	Isopropyl- β -D-thiogalactopyranoside
k_{cat}	The turnover number, that is the maximum number of substrate molecules converted to product per enzyme molecule per second
K_m	The Michaelis constant
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National center for Biotechnology Information protein database
NMOs	Microbial <i>N</i> -hydroxylating monooxygenases
TU-GENDB	Microbial genetics database of the Bioscience, TU Bergakademie Freiberg, Germany
V_{max}	The velocity of an enzyme at its maximal substrate concentration
YUCCAs	Class B flavoprotein monooxygenases in plants

GENERAL ABSTRACT

Microbial *N*-hydroxylating monooxygenases (NMOs) are useful industrial biocatalysts that perform the regioselective addition of a single oxygen atom to the terminal amino group of their target substrates (primary aliphatic amines and diamines). These NMOs are particularly sought after industrially as they play a major part in the first step of biosynthesis of metal chelating compounds, siderophores/metallophores.

In order to increase available NMOs with novel functional annotations, the microbial metagenome of the TU-GENDB Freiberg, Germany and the National center for Biotechnology Information protein database (NCBI) were screened for novel NMOs with promising and unique functions using some previously known NMOs protein sequences as potential baits. A gene, *gorA*, encoding for GorA, a putative NMO with functional annotation as a diamine monooxygenase has been discovered from the genome of the *Gordonia rubripertincta* CWB2. GorA has been cloned and overexpressed in a suitable microbial host *Escherichia coli* DH5 α and *Escherichia coli* BL21 (DE3) (pLysS) respectively.

GorA has been further characterized for its physiological and biochemical properties including its different substrates scope (eleven different substrates, namely ethanolamine, propylamine, *N*-butylamine; primary chain diamines: 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), 1,6-diaminohexane (hexanemethylenediamine), 1,7-diaminoheptane, and 1,8-diaminooctane; amino acids, *L*-ornithine and *L*-lysine were assessed. Substrate and co-factor specificity, pH conditions, buffer tests, temperature optimal conditions were evaluated using an appropriate amount of the enzyme and measuring the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm on a spectrophotometer using the standard NADPH oxidation assay. The steady state kinetics of GorA with different

concentrations of flavin adenine dinucleotide (FAD) and different concentrations NADPH were assessed spectrophotometrically at 24°C. The kinetic parameters of GorA (1 µM) were assessed spectrophotometrically at 24°C with different concentrations of 1,4-diaminobutane. The hydroxylation assay of GorA (1 µM) were assessed using the Csáky assay at 24°C with different concentrations of 1,4-diaminobutane. The steady state, kinetic parameters, and the hydroxylation assay were fit into the Michealis-Menten constant. The phylogenetic relationship with other known NMOs showed GorA to be grouped with known but yet to be characterized microbial diamine NMO. GorA is thus to the best of our knowledge the first characterized diamine NMO from the genome of the *Gordonia rubripertincta* CWB2.

GorA has an optimal pH range of 7.0-8.0, it is soluble in potassium phosphate buffer, it accepts FAD and NADPH as cofactor and electron donor respectively. GorA is active in the presence of a range of primary diamines, 1,4-diaminobutane > 1,5-diaminopentane > 1,6-diaminohexane > 1,3-diaminopropane > 1,7-diaminoheptane > 1,8-diaminooctane. The activity of GorA in the presence of either of the monoamines or the amino acids was not significant. GorA demonstrated a 100% relative activity in the presence of 1,4-diaminobutane (putrescine) as compared to other substrates tested. The apparent V_{max} , K_m , and k_{cat} of the NADPH oxidation is $310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $361 \pm 0.1 \text{ µM}$ and 0.27 s^{-1} respectively whereas the hydroxylation assay showed GorA with an apparent V_{max} , K_m and k_{cat} of $246 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $737 \pm 0.1 \text{ µM}$, and 0.21 s^{-1} respectively.

NMOs belongs to the class B monooxygenase family, the other members of this family are the Baeyer-Villiger monooxygenases (BVMOs), the flavin-containing monooxygenases (FMOs) and the class B flavoprotein monooxygenases in plants (YUCCAs). The NMOs have been indicated as the least studied members of these class B monooxygenases, hence in Chapter 2, the

known NMOs are reviewed. The most studied members of the class B monooxygenase family are the BVMOs. The NMOs share conserved motif sequence fingerprints with the BVMOs; these allows for their successful identification from the metagenome. The classification and biocatalysis application of BVMOs are reviewed in Chapter 4.

NMOs are also implicated as important in diseases causing ability of pathogenic bacteria and fungi, such as DfoA, a putative *L*-lysine *N*6-monooxygenase that converts cadaverine into 1-amino-5-(*N*-hydroxy)-aminopentane, isolated from the enterobacterium *Erwinia amylovora*, the causative agent of fire blight of pome fruit, and plant growth promoting ability such as PsbA, a pseudobactin A enzyme, that is involved in the first step biosynthesis of pseudobactin from *Pseudomonas* strain B10. A review of these examples is presented in Chapter 5. This shows the significance of NMOs as novel therapeutic drugs and antibiotics target.

Overall, this study has contributed to the available pool of NMOs; particularly, GorA has a biosynthetic role in the production of hydroxamate metallophores for a vibrant and environmentally-safe mobilization of industrially important metals.

CHAPTER 1

1.1 General Introduction

Enzymes are biocatalysts or proteins responsible for the thousands of biochemical reactions that take place in every living organism (Jacobsen et al. 2000). They specifically act with only one reactant (called a substrate) to produce their respective products. Substrates are converted by conventional chemical transformation or biotransformation (Jacobsen et al. 2000). Biotransformation or biocatalysis is essential in bio-industry due to the need to synthesize enantiopure compounds as chiral building blocks for pharmaceutical drugs and agrochemicals (Kamerbeek et al. 2003; Riebel et al. 2013). Enzymes have six main classes. They include transferases, isomerases, ligases, hydrolysis, lysis and oxidoreductases (Kamerbeek et al. 2003; Riebel et al. 2013). The oxidative enzymes also known as oxidoreductases or redox enzymes catalyze the biochemical reactions in which electrons are transferred from one molecule to another (van Berkel et al. 2006; Torres Pazmiño et al. 2010).

In the chemical and pharmaceutical industries, there is an urgent search for biocatalysts and enzymes for development of novel products for agrochemicals and pharmaceuticals. These enzymes have high priority for product development because they are more less toxic, they demand less energy and they are more cost effective than toxic chemicals. More importantly, microbial enzymes have a great advantage over other enzymes from other sources like plants and animals, because they are more active and stable.

Bacteria, fungi and mycobacteria have been known to grow on organic substances and different aromatic pollutants as carbon and energy sources. They are able to degrade such toxic compounds and render them less toxic to the environment. One of the strategies such microorganisms use is to produce metabolites, such as low molecular weight iron chelators,

referred to as siderophores, for the degradation of the anthropogenically toxic wastes as well as for scavenging useful metal resources for their survival in such toxic and metal depleted conditions.

Iron serves as a major metal being scavenged by these microbes due to its inavailability and insufficiency in the host environment for microbial utilization. Iron forms complexes with various functional groups of siderophores such as hydroxamate, catecholates and mixed type siderophores. The hydroxamate siderophores are most abundant in the soil.

Siderophores enables microbes to function as plant growth promoters under iron-stressed conditions, this is the case of pseudobactin isolated from *Pseudomonas* sp. (Ambrosi et al. 2000; Sharma and Johri 2003). Bacterized maize seeds with siderophore-producing pseudomands showed significant increase in germination percentage and plant growth against phytopathogens such as *Colletotrichum dematium*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Sharma and Johri 2003).

A great number of pathogenic bacteria, mycobacteria and fungi also produce such hydroxamate siderophores which they employ in disease-causing in their host organisms, such as plants, animals and humans. A critical study and biochemical characterization of the enzymes involved in the biosynthesis of these siderophores are crucial for drug development and novel antibiotics discovery against these pathogenic microbes.

Examples of such siderophores synthesized by some pathogenic microbes are the well characterized pyoverdine isolated from *Pseudomonas aeruginosa* that enables this pathogen to cause severe lung disease in immune-compromised individuals, the enzyme involved in the first step biosynthesis of pyoverdine is the *Pa* PvdA. *Af* SidA is also well characterized and is involved in the biosynthesis of ferricrocin and other siderophores produced by the pathogenic

fungi *Aspergillus fumigates* causing deadly human diseases. IucD is the bacterial *L*-lysine N^6 -monooxygenase isolated from *Escherichia coli* involved in the synthesis of aerobactin, causing serious intestinal disease in humans, MbtG is bacterial N^6 -acyl *L*-lysine monooxygenase isolated from *Mycobacterium tuberculosis* and involved in the biosynthesis of mycobactin, which the bacteria use in causing human tuberculosis (Macheroux et al. 1993; Ge and Seah 2006; Meneely and Lamb 2007; Mayfield et al. 2010; Huijbers et al. 2014; Robinson et al. 2014).

These siderophores producing enzymes are the microbial *N*-hydroxylating monooxygenases (NMOs) involved in the first step biosynthesis of hydroxamate siderophores. NMOs are flavin and NAD(P)H dependent. They are known as drug target enzymes and are important for a range of eco-friendly biotechnological applications (primarily biosynthesis of microbial hydroxamate siderophores involved in iron-uptake and plant growth promoting ability).

The mechanisms of *Pa* PvdA and *Af* SidA are observed in a reductive and oxidative cycle, this serve as a basis for other NMOs (Fig. 1.1) (Meneely et al. 2009). They are able to stabilize long-lived C4a-hydroperoxyflavin intermediates, which is important in the enzyme coupling of oxygen activation for a successful hydroxylation of the substrate into their respective hydroxylamine product derivatives. In cases of uncoupling, there is release of hydrogen peroxide or other reactive oxygen species (Torres Pazmiño et al. 2008; Meneely et al. 2009; Mayfield et al. 2010).

In the reduction half reaction observed for *Pa* PvdA, the substrate is not required for the reduction of the flavin by NADPH, neither does it accelerate the process, the flavin is reduced at 450 nm absorbance, oxygen binds the flavin at the C4a position producing a long-lived flavin intermediate. There is no initial peroxyflavin intermediate for *Af* SidA as observed for *Pa* PvdA.

In the oxidative half reaction, the hydroperoxyflavin donates the distal oxygen to the substrate, hydroxylating it into the desired hydroxyornithine product and the hydroxyflavin intermediate. The hydroxyflavin intermediate later dehydrates to form the oxidized flavin, while the hydroxyornithine and the NADP^+ dissociate and the cycle continues.

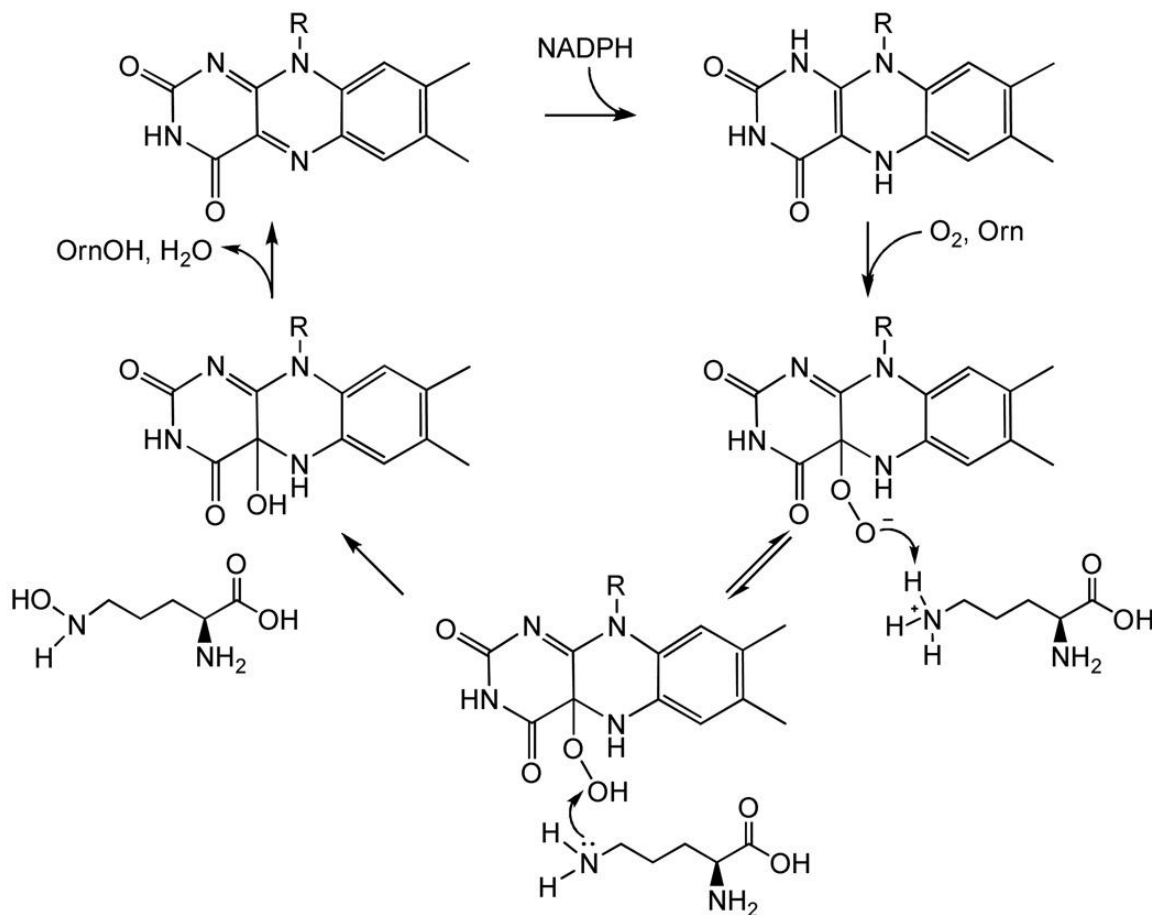


Figure 1.1: The mechanisms of *Pa* PvdA in a reductive and oxidative cycle (Meneely et al. 2009)

However, one of the major problems facing the applicability of these NMOs is their limited availability. Hence, to generate a large collection of NMOs that cover a wide variety of substrate compounds, the natural diversity and the metagenome must be tapped.

Studies on gene knockout to identify functions and link several NMOs to pathogen virulence were done as reported elsewhere (Hissen et al. 2005). The results showed that NMOs are essential for virulence in both bacteria and fungi. In other words, a knockout of these NMO genes from their host microorganisms, leads to the non-virulence and non-diseases causing capability of such microorganism. Despite these results, which clearly showed that NMOs are potential drug targets, only a small number of them have been characterized so far.

There is an urgent need to increase the available pool of NMOs. Hence, the aim of this research is to increase the set of bacterial NMOs. The specific research objectives include:

1. Using bioinformatic tools for genome mining approach to search for novel putative genes that encode bacterial NMOs. Since nowadays the genomes of a wide variety of organisms have been sequenced and are publicly available, this will offer a new and efficient way of retrieving novel genes.
2. Cloning, gene expression and protein purification of the putative NMO. This will allow proving the presumed function of previously made annotation of the gene.
3. To determine the kinetic parameters of the putative NMO protein for insight into the function of this protein.

LIST OF PUBLICATIONS

Chapter 2: Microbial *N*-Hydroxylating Monooxygenases (NMOs): Some Known Members and their Relevance for Siderophore Biosynthesis *This chapter will be submitted in this format for publication in Archives of Biophysics and Biochemistry.*

Authors: Catherine Oluwakemi Esuola, Olubukola Oluranti Babalola, Thomas Heine, Ringo Schwabe, Micheal Schlömann and Dirk Tischler

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Candidate's Contributions: wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results, managed the literature searches, and wrote the first draft of the manuscript.

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Candidate's Contributions: designed the study, managed the literature searches and wrote the first draft of the manuscript.

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Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

CHAPTER 2

Microbial *N*-hydroxylating monooxygenases (NMOs): some known members and their relevance for siderophore biosynthesis

Abstract

Microbial *N*-hydroxylating monooxygenases (NMOs) represent a group of enzymes that are targeted as potential anti-virulence drugs, biofertilizers, and initiating the biosynthesis of metal chelating compounds (hydroxamate-containing siderophores). Despite their usefulness, NMOs have been pointed out as the least studied enzymes among the Class B flavoprotein monooxygenase family. NMOs are particularly substrate specific and they carry out the regio-specific side chain hydroxylation of terminal amine groups of diamino acids, *L*-ornithine (*L*-Orn) and *L*-lysine, and the primary aliphatic diamines such as 1,3-diaminopropane, putrescine and cadaverine. NMOs are now gaining more attention for enzyme research and in industries; some of them are discussed here to emphasis their various names, characteristics, and activities. Research efforts to elucidate their activities and biosynthetic pathways along with their respective gene cluster are important to demonstrate a clear understanding of the biosynthesis of their different siderophores and their industrial applications.

Keywords: Hydroxamate siderophores, Metallophores, Sequence motif, Kinetic parameters, Gene cluster, NADPH oxidation assay, Enzymes

2.1 Introduction

The microbial *N*-hydroxylation monooxygenases (NMOs) [EC.1.14.13] are mostly involved in the biosynthesis of hydroxamate-containing siderophores; these NMOs are attractive targets for medicinally controlling some pathogenic species, biofertilizers, and metallophores

(Thariath et al. 1993; Lamont and Martin 2003; Heemstra et al. 2009; Mayfield et al. 2010; Qi et al. 2012; Romero et al. 2012; Ahmed and Holmström 2014). They belong to the Class B flavoprotein monooxygenases which include three sub-classes, the Baeyer-Villiger monooxygenases (BVMOs), the flavin-containing monooxygenases (FMOs), and the YUCCAs (van Berkel et al. 2006; Olucha et al. 2011; Huijbers et al. 2014). They are involved in the catalyzes of the side chain hydroxylation of the terminal amino group of amines or diamino acids; *L*-Orn and *L*-lysine, and the primary aliphatic diamines; 1,3-diaminopropane, putrescine, and cadaverine (Fig. 2.1) (Ge and Seah 2006; Robinson and Sobrado 2013).

They have been observed to play biosynthetic roles rather than functioning in degradation of their organic substrates (Mei et al. 1993; Visca et al. 1994; Kang et al. 1996; Quadri et al. 1998; Yamada et al. 2003; Pohlmann and Marahiel 2008; Heemstra et al. 2009; Olucha et al. 2011). The microbial NMOs are able to carry out their catalytic functions in response to the need to tap iron from the environment when its supply is very limiting for the survival of the pathogenic microbes (Griffiths 1991; Kang et al. 1996; Meyer et al. 1996; Ambrosi et al. 2000). Iron is an essential element for all life forms. Notwithstanding, iron is only available in the form of insoluble oxyhydroxide polymers at neutral pH under aerobic conditions, making its availability very difficult (Lynch et al. 2001). Thus microbes have evolved mechanisms to sequester iron from their environment by using small molecular weight iron chelators called siderophores/metallophores (Robinson and Sobrado 2013).

Some members of the NMOs family include the bacterial *L*-ornithine monooxygenase *Pa* PvdA, from *Pseudomonas aeruginosa*, the fungal monooxygenase *Af* SidA (OMO) from *Aspergillus fumigatal*; the bacterial *L*-lysine monooxygenase IucD, from *Escherichia coli*; and IucD from *Shigella flexnerii*; the 1,3-diaminopropane monooxygenases, RhbE, from

Sinorhizobium meliloti, the putrescine monooxygenase, AlcA, from *Bordetella bronchiseptica* RB50, the cadaverine monooxygenase, DesB, from *Streptomyces scabies* 87-22 (Fig. 2.1, Table 2.1) (Giardina et al. 1995; Stehr et al. 1999; Wei et al. 2003; Ge and Seah 2006; Bignell et al. 2010; Romero et al. 2012). *Pa* PvdA, *Af* SidA and IucD from *E. coli* are the most studied NMOs (Ge and Seah 2006; Heemstra et al. 2009; Qi et al. 2012). They have all been produced as soluble recombinant and purified proteins (Stehr et al. 1999; Ge and Seah 2006; Qi et al. 2012; Romero et al. 2012). NMOs are able to use flavin adenine dinucleotide (FAD) as flavin co-factor, nicotinamide adenine dinucleotide phosphate (NADPH), and molecular oxygen as co-substrates (Robinson and Sobrado 2013).

Fortunately, *Af* SidA, KtzI, and MbsG accept either NADPH or nicotinamide adenine dinucleotide (NADH) (Robinson and Sobrado 2011; Romero et al. 2012). They have been noted to be very specific for their substrates (Macheroux et al. 1993; Mei et al. 1993; Thariath et al. 1993; Visca et al. 1994; Sokol et al. 1999; Stehr et al. 1999; Yamada et al. 2003; Ge and Seah 2006; Meneely and Lamb 2007; Pohlmann and Marahiel 2008; Watanabe et al. 2008; Heemstra et al. 2009; Mayfield et al. 2010; Olucha et al. 2011; Qi et al. 2012; Romero et al. 2012; Robinson and Sobrado 2013). NMOs have been indicated as the least studied of the Class B monooxygenases (Robinson and Sobrado 2013), consequently this has prompted the discussion here on some of the NMOs studied so far for a better understanding of these enzymes.

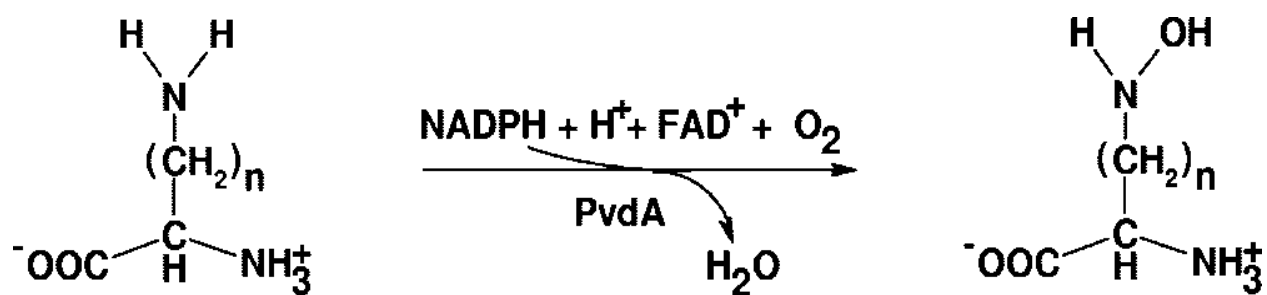


Figure 2.1: General reaction catalyzed by flavin-dependent monooxygenase involved in hydroxamate siderophore biosynthesis. $n = 3$ for *L*-orn, and $n = 4$ for *L*-lysine (Ge and Seah 2006)

Table 2.1: List of microbial NMOs showing their respective accession numbers, amino acid sequence lengths, source microorganisms

Enzyme	Accession	Length	Source microorganism	Siderophore	Substrate	Product	References
Bacterial <i>L</i> -ornithine <i>N</i> ⁵ -monooxygenases							
<i>Pa</i> PvdA	Q51548	443	<i>P. aeruginosa</i>	Pyoverdine	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Ge and Seah 2006)
<i>Bc</i> PvdA	O51940	444	<i>Burkholderia cepacia</i>	Ornibactin	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Sokol et al. 1999)
PsbA	Q9F8X0	445	<i>Pseudomonas</i> sp B10	Pseudobactin ₁₀	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Ambrosi et al. 2000)
KtzI	A8CF85	424	<i>Kutzneria</i> sp. 744	Kutzneride ^a	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Setser et al. 2014)
CchB	A0ACR3	451	<i>S. ambofaciens</i>	Coelichelin	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Barona-Gómez et al. 2006)
Fungal <i>L</i> -ornithine <i>N</i> ⁵ -monooxygenases							
<i>Af</i> SidA	E9QYP0	501	<i>Aspergillus fumigatus</i>	Ferricrocin	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Romero et al. 2012)
SidA	Q7Z8P5	498	<i>Emericella nidulans</i>	Triacetylfulsarine C	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Eisendle et al. 2003)
OMO1	F7VJJ9	563	<i>Magnaporthe grisea</i>	Ferricrocin	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Hof et al. 2007)
Sid1	P56584	649	<i>Ustilago maydis</i>	Ferrichrome	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Mei et al. 1993)
DffA	Q2TZB2	502	<i>Aspergillus oryzae</i>	Deferri-ferrichrysin	<i>L</i> -Orn	<i>L</i> - <i>N</i> ⁵ -OH-Orn	(Yamada et al. 2003)
Bacterial <i>L</i> -ornithine <i>N</i> ⁵ -monooxygenases							
IucD	P11295	425	<i>Escherichia coli</i>	Aerobactin	<i>L</i> -lysine	<i>N</i> ⁶ -OH- <i>L</i> -lysine	(Stehr et al. 1999)
IucD	Q9XCH1	445	<i>Shigella flexneri</i>	Aerobactin	<i>L</i> -lysine	<i>N</i> ⁶ -OH- <i>L</i> -lysine	(Wei et al. 2003)
VbsO	Q2JYJ0	452	<i>Rhizobium etli</i> CFN 42	Vicibactin	<i>L</i> -lysine	<i>N</i> ⁶ -OH- <i>L</i> -lysine	(González et al. 2006)
Bacterial diamine monooxygenases							
RhbE	Q9Z3Q8	454	<i>S. meliloti</i> 1021	Rhizobactin	1,3-diaminopropane	<i>N</i> ⁴ -OH-1-aminopropane	(Lynch et al. 2001)
AlcA	Q44740	461	<i>B. bronchiseptica</i> RB50	Alcaligin	Putrescine	<i>N</i> -OH-putrescine	(Giardina et al. 1995)
DesB	C9Z469	419	<i>S. scabiei</i> 87-22	Desferrioxamine	Cadaverine	<i>N</i> -OH-cadaverine	(Bignell et al. 2010)
Bacterial <i>N</i> ⁶ -acyl <i>L</i> -lysine monooxygenases							
MbtG	P9WKF6	431	<i>M. tuberculosis</i>	Mycobactin	<i>L</i> -lysine	<i>N</i> ⁶ -OH- <i>L</i> -lysine	(Quadri et al. 1998)
MbtG	Q73XY8	428	<i>M. paratuberculosis</i>	Mycobactin	<i>L</i> -lysine	<i>N</i> ⁶ -OH- <i>L</i> -lysine	(Li et al. 2005)

^aA new chemical scaffold with antifungal and antimicrobial properties

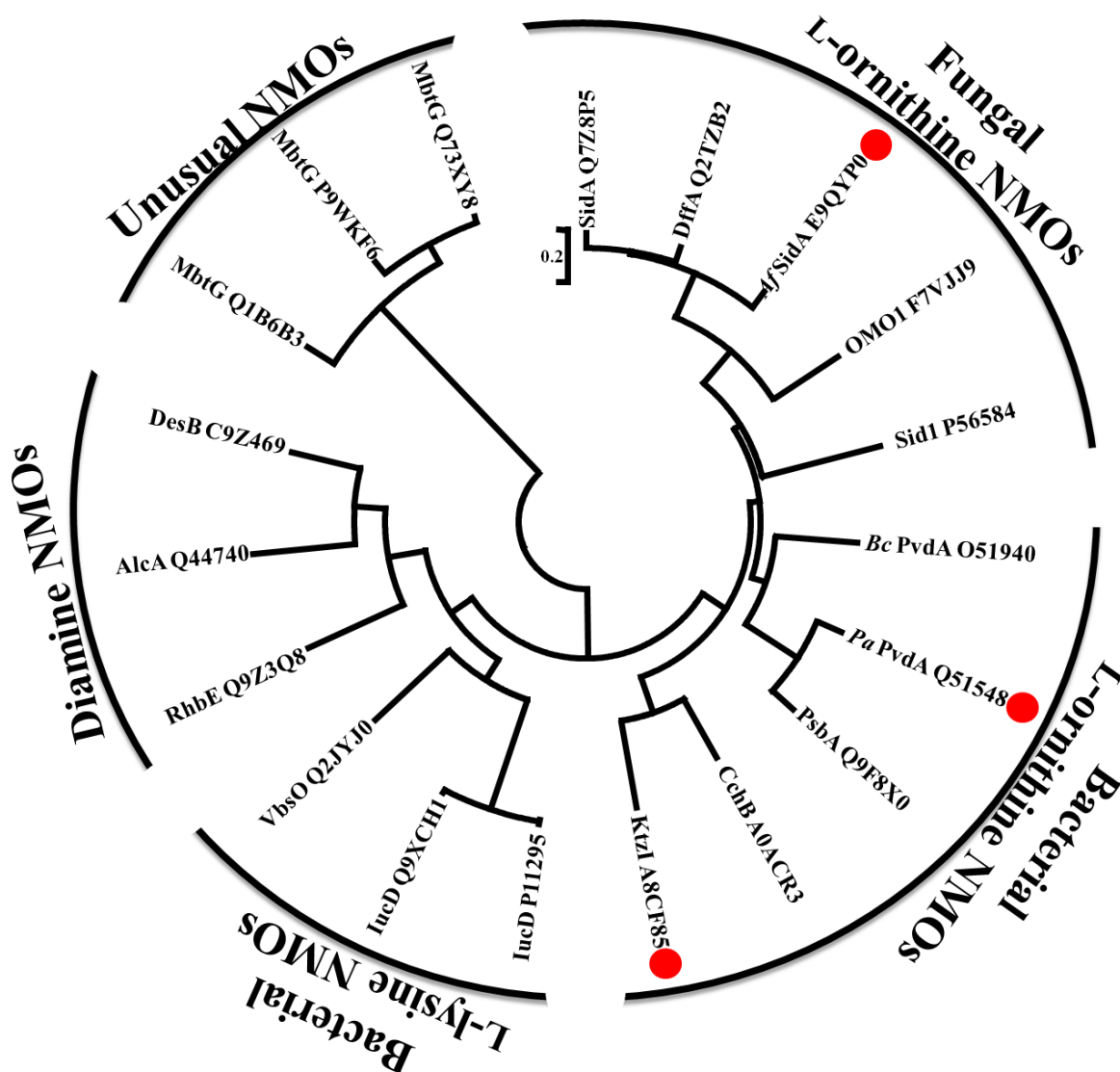


Figure 2.2: Circular phylogenetic relationship of the microbial NMOs

Figure 2.2 shows the NMOs; the bacterial *L*-ornithine monooxygenases, *Pa* PvdA, *Pseudomonas aeruginosa* pyoverdine A (Q51548); *Bc* PvdA, *Burkholderia cepacia* pyoverdine A (O51940); *PsbA*, *Pseudomonas* sp. B10 (Q9F8X0); *KtzI*, *Kutzneria* sp. 744 (A8CF85); *CchB*, *Streptomyces ambofaciens* (A0ACR3) fungal *L*-ornithine N^5 -monooxygenases (EC.1.14.13.196), *Af* SidA (OMO), *Aspergillus fumigatus* siderophore A (E9QYP0); *SidA*, *Emericella nidulans* (Q7Z8P5); *OMO1*, *Magnaporthe grisea* (F7VJJ9); *Sid1*, *U. maydis* (P56584), *DffA*, *Aspergillus oryzae* (Q2TZB2); the bacterial *L*-lysine N^6 -monooxygenases (EC.1.14.13.59) *IucD*, *Escherichia coli* (P11295); and *Shigella flexneri* (Q9XCH1); *VbsO*, *Rhizobium etli* CFN 42 (Q2JYJ0); diamine monooxygenases, *RhbE*, *S. meliloti* 1021 (Q9Z3Q8); *AlcA*, *B. bronchiseptica* RB50 (Q44740); *DesB*, *S. scabiei* 87-22 (C9Z469); bacterial N^6 -acyl-*L*-lysine monooxygenases (EC.1.14.13.59), *MbtG*, *M. tuberculosis* (P9WKF6) and *M. paratuberculosis* (Q73XY8). Red balls indicate NMOs with known structures. Distance trees were created with respect to the software program ClustalW (version 2.0) (Thompson et al. 1994).

2.2 Bacterial *L*-ornithine monooxygenases (EC.1.14.13.195)

2.2.1 *Pa* PvdA

Pa PvdA is the *L*-ornithine N^5 -monooxygenase (*L*-Orn N^5 -monooxygenase) that catalyzes the hydroxylation of *L*-Orn into N^5 -hydroxyl-*L*-ornithine (*L*- N^5 -OH-Orn) in the early biosynthetic step of pyoverdine (PVD1) by human opportunist pathogen *Pseudomonas aeruginosa* during Fe(III) limited growth conditions (Figure 2.1; Figure 2.2; Table 2.1) (Visca et al. 1994; Ge and Seah 2006). *P. aeruginosa* produces pyoverdine, a hydroxamate siderophore, under iron-limiting conditions of the human host. Thus, it causes severe disease in iron-stressed individuals, including patients suffering from cystic fibrosis. Pyoverdine has a high affinity for Fe(III) and it promotes the growth of *P. aeruginosa* in the presence of the human transferrin or serum (Visca et al. 1994).

PVD1 is composed of 6,7-dihydroxyquinoline-containing fluorescent chromophore linked to the N-terminus of a partly cyclic octapeptide (D-Ser-*L*-Arg-D-Ser-*L*- N^5 -OH-Orn-*L*-Lys-*L*- N^5 -OH-Orn-*L*-Thr-*L*-Thr) in *P. aeruginosa* PAO1 (Briskot et al. 1986). It is produced first in the cytoplasm and then is transported into the periplasm before being later secreted into the extracellular medium by a bacterial efflux pump. PVD1 is a water soluble yellow-green fluorescent compound. *Pa* PvdA forms ferripyoverdine complexes and pyoverdines from plant related isolates are called pseudobactins (Visca et al. 1994). *Pa* PvdA is a well studied and characterized NMO. It is the first NMO with an elucidated structure identified through co-crystallization of the protein with FAD, NADPH and *L*-Orn (Ge and Seah 2006). Structures of the oxidized form (1.9 Å resolution) and reduced enzymes (3.03 Å resolution) are available as PDB codes 3S5W and 3S61, respectively (Olucha et al. 2011).

Pa PvdA requires both FAD and NADPH for activity; it was found to be a soluble monomer most active at pH 8.0 (Ge and Seah 2006; Meneely and Lamb 2007). In 2006, *Pa* PvdA was first purified and overexpressed as an active recombinant gene with molecular weight of 51 kDa by Ge and Seah (2006). Its activity in the presence of varying concentrations of *L*-Orn fits the classical Michaelis-Menten kinetic model in an NADPH oxidation assay (Meneely and Lamb 2007). The result of this kinetic model showed an apparent V_{max} , K_m , k_{cat} and K_{cat}/K_m value of $528 \pm 8 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $593 \pm 12 \text{ }\mu\text{M}$, $26.4 \pm 0.4 \text{ min}^{-1}$, and $742 \text{ M}^{-1}\text{s}^{-1}$ respectively (Meneely and Lamb 2007). *Pa* PvdA is highly specific for both substrate and coenzyme.

It catalyzes ornithine and not lysine, as the latter was shown to be a non-substrate effector and a mixed inhibitor of the enzyme (Meneely and Lamb 2007). Chloride has been shown as a mixed inhibitor and a bulky mercurial compound (*para*-chloromercuribenzoate) with respect to ornithine, but a competitive inhibitor with respect to NADPH (Meneely and Lamb 2007). Steady-state experiments indicated that *Pa* PvdA/FAD forms a ternary complex with NADPH and ornithine for catalysis (Meneely and Lamb 2007). In the absence of ornithine, *Pa* PvdA showed a slow substrate-independent flavin reduction by NADPH (Meneely and Lamb 2007). *Pa* PvdA gene cluster involved in the PVD1 biosynthesis include *pvdLIJD*; *pvdHF*, *pvdE* and *pvdQ* (Visca et al. 1994). *P. aeruginosa* PAO1 has been sequenced, which allowed insight into identifying and understanding the functions of *pvdA* gene cluster enzymes involved for pyoverdine biosynthesis (Winsor et al. 2009).

2.2.2 *Bc* PvdA

This is the *L*-Orn N^5 -monooxygenase isolated from *Burkholderia cepacia* (Fig. 2.2) (Sokol et al. 1999). It catalyzes the hydroxylation of the *L*-Orn to produce *L*- N^5 -OH-Orn which is later incorporated in the biosynthesis of ornibactin siderophore (Fig. 2.2; Table 2.1) (Sokol et al.

1999). *B. cepacia* was formerly known as *Pseudomonas cepacia*, a human opportunist pathogen causing respiratory infections in low immunity patients suffering from cystic fibrosis diseases. Ornibactin has been shown to play a major role in the virulence of *B. cepacia*.

Ornibactin is a linear hydroxamate-hydroxycarboxylate siderophore with peptide structures that are related to pyoverdines from fluorescent Pseudomonads, *P. aeruginosa* and *P. fluorescence* (Meyer et al. 1989). Ornibactin is composed of the conserved tetrapeptide $L\text{-Orn}^1(N^{\delta}\text{-OH}, N^{\delta}\text{-acyl})\text{-D-threo-Asp}(\beta\text{-OH})\text{-L-Ser-L-Orn}^4(N^{\delta}\text{-OH}, N^{\delta}\text{-formyl})\text{-1,4-diaminobutane}$ (Sokol et al. 1999). The *pvdA* gene cloned and sequenced from *B. cepacia* K56-2 has amino acid length of 444 and it is approximately 47% identical and 59% similar to *L*-ornithine N^5 -monooxygenase of *P. aeruginosa* (Sokol et al. 1999).

2.2.3 PsbA

This is the *L*-Orn N^5 -monooxygenase, encoded for by *pseudobactin gene A*, *psbA* gene, isolated from the genomic library of *Pseudomonas* strain B10; a plant growth promoting rhizobacterium (Ambrosi et al. 2000). It catalyzes the hydroxylation of the amino side chain of *L*-Orn into *L*- N^5 -OH-Orn in the biogenesis of Pseudobactin_{B10} siderophore which is synonymous to the pyoverdine siderophore from *P. aeruginosa* (O'Sullivan and O'Gara 1992).

Pseudobactin_{B10} is a fluorescent siderophore known to be produced by group I pseudomonads. Pseudobactin_{B10} is proposed to play a crucial role in the biological control of phytopathogenic microbes by depriving them of the available nutrients in the rhizosphere. Pseudobactin_{B10} consists of a linear hexapeptide (*L*-Lys-D-threo- β -OH-Asp-*L*-Ala-D-allo-Thr-*L*-Ala-D- N^5 -OH-Orn) (O'Sullivan and O'Gara 1992). PsbA is predicted as a 49.8 kDa protein and it shows significant homology to other NMOs. It has 76% identity to *Pa* PvdA, 49% identity to *Bc* PvdA, and approximately 40% identity to Sid1 from *Ustilago maydis* (O'Sullivan and O'Gara 1992). PsbA

has less similarity to IucD from *E. coli* and *S. flexneri*, AlcA from *B. bronchiseptica* and RhbE from *S. meliloti* (Ambrosi et al. 2000). The conserved motif of the amino acid sequence of PsbA aligned as expected in comparison with other NMOs (Ambrosi et al. 2000).

2.2.4 KtzI

This is the *L*-ornithine N^5 -monoxygenase identified from the kutzneride gene cluster (Fujimori et al. 2007). The kutznerides are antifungal and antimicrobial cyclic hexadepsipeptides isolated from the soil actinomycete *Kutzneria* sp. 744 (Broberg et al. 2006). KtzI has been structurally characterized and the structures are available on the PDB database, an example is the 4TLX with 2.23 Å resolution (Setser et al. 2014). The amino acid sequence length of KtzI is 424 and it has homology to IucD1, CchB, VbsO, and *Pa* PvdA (Neumann et al. 2012). KtzI has been cloned, overproduced in *E. coli* and purified to homogeneity (Neumann et al. 2012).

The enzymatic activity of KtzI carried out in aerobic incubations with FAD, NAD(P)H and *L*-Orn. The enzymatic assay showed that KtzI converted *L*-Orn to N^5 -OH-Orn in the presence of FAD as flavin cofactor, NADH and NADPH [NAD(P)H] and molecular oxygen as cosubstrates; although the subsequent chemical steps catalyzing N^5 -OH-Orn to piperazate moiety was not clear (Neumann et al. 2012). Flavin mononucleotide (FMN) on the other hand was not accepted as a flavin cofactor, thus the reaction was dependent on FAD as flavin cofactor (Neumann et al. 2012). Ornithine was suggested as its physiological substrate, as the enzyme could not tolerate or convert other derivatives of lysine and ornithine such as D - Lys, L/D - Glu, L/D - Gln to useful products (Neumann et al. 2012). The kinetic parameters of KtzI in the presence of *L*-Orn was found as follows K_m was 0.091 ± 0.008 mM, k_{cat} was found to be 6.96 ± 0.13 min⁻¹, and k_{cat}/K_m was 76.48 min⁻¹ mM⁻¹ (Neumann et al. 2012). This value was found to be in the range reported for other NMOs (Neumann et al. 2012). The kutznerides gene cluster have been studied and they include *ktzABCDEFGHIJKLMNOPS* (Fujimori et al. 2007).

2.2.5 CchB

It is an *L*-ornithine N^5 -monooxygenase involved in the first enzymatic step in the generation of Coelichelin, a novel tetrapeptide siderophore containing iron-chelating hydroxamate groups (Challis and Ravel 2000; Pohlmann and Marahiel 2008). CchB was overproduced as an N-terminal hexahistidine tag fusion protein in *E. coli* BL21 cells and it was purified by Ni-NTA affinity chromatography as a soluble protein with over 95% purity (Challis and Ravel 2000). The activity of CchB was determined in a typical NADPH oxidation assay in the presence of FAD and *L*-Orn. This reaction was monitored by measuring the decrease in NADPH absorption at 340 nm. The optimum pH range for maximal turnover of catalytic activity was between 8.0-9.0 (Challis and Ravel 2000).

The substrate specificity of CchB was evaluated using different substrates such as *L*-Orn, N^5 -Formylornithine, D-ornithine, *L*-lysine and *L*-glutamate. It was only the *L*-Orn that was hydroxylated among all the tested substrates. Tests were also conducted to determine co-substrate specificity of CchB with NADH or NADPH, and CchB was found to be specific for NADPH and *L*-Orn (Challis and Ravel 2000). The kinetic parameters of CchB for *L*-Orn resulted in an apparent K_m , k_{cat} and k_{cat}/K_m value of 3.6 ± 0.58 mM, 17.4 ± 0.87 min⁻¹, and 4.83 ± 0.64 min⁻¹ mM⁻¹ respectively (Challis and Ravel 2000).

2.2.6 VbsO

A *vbsO* gene that encodes for an *L*-ornithine N^5 -monooxygenase (VbsO), involved in the first step biosynthesis of vicibactin was isolated from the *Rhizobium etli* CFN 42 (ATCC51251). It is involved in the hydroxylation of amino side group of *L*-Orn to *L*- N^5 -OH-Orn (Heemstra et al. 2009). Vicibactin is a cyclic trihydroxamate siderophore produced by *Rhizobium* spp. for iron sequestration from their host environment. The *Rhizobium* spp. are nitrogen fixing symbiotic

bacteria and they are very useful as biofertilizers as a result of their symbiotic relationship with leguminous plants (Bloembergen and Lugtenberg 2001).

Heemstra et al. (2009) studied the overproduction and purification of six gene clusters involved in vicibactin biosynthesis (Heemstra et al. 2009). They include *vbsACGOLS* in bacterial host *Escherichia coli*. VbsO is homologous to IucD, CchB, and PvdA. The kinetic parameters of VbsO in the presence of *L*-Orn were assessed using the NADPH oxidation assay in Tris-HCl buffer (pH 8.0) at 25°C on spectrophotometer by measuring the oxidation of NADPH to NADP⁺ (Heemstra et al. 2009). The V_{max} , K_m and k_{cat} were found to be $528 \pm 8 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $305 \pm 0.024 \text{ }\mu\text{M}$ and $108 \pm 2 \text{ min}^{-1}$ respectively (Heemstra et al. 2009). The substrate specificity test with substrates such as D-Orn showed that this VbsO is specific for the *L*-isomer of ornithine (Heemstra et al. 2009). Surprisingly, the NCBI protein database contains many other deposited putative VbsO which are yet to be characterized. In this study, a putative VbsO with accession number Q2JYJO clustered together with the bacterial *L*-lysine monooxygenases (Figure 2.2; Table 2.1) (González et al. 2006).

2.3 Fungal *L*-ornithine *N*⁵-monooxygenases (EC.1.14.13.196)

2.3.1 *Af* SidA (OMO)

This is referred to as the *Aspergillus fumigatus* siderophore A, an *L*-ornithine *N*⁵-monooxygenase enzyme produced by the pathogenic fungus *A. fumigates* (Hissen et al. 2005; Chocklett and Sobrado 2010; Mayfield et al. 2010; Romero et al. 2012). Fortunately, its structure has been resolved by X-ray crystallography at 1.9 Å (PDB 4B63), this provide additional structural insight for NMOs (Franceschini et al. 2012). It catalyzes the first step hydroxylation of *L*-Orn to produce *L*-*N*⁵-OH-Orn, later incorporated for the biosynthesis of ferricrocin, ferrichrome and other siderophores (Mayfield et al. 2010).

Ferricrocin is an hydroxamate siderophore that is necessary for virulence of both *A. fumigatus* and *Aspergillus nidulans* (Eisendle et al. 2003; Hissen et al. 2005). *Af* SidA (OMO) has been recombinantly expressed and purified as a soluble homodimer (Mayfield et al. 2010). It is the first member of the microbial NMOs that was isolated with a bound flavin cofactor containing FAD and NADP⁺ cofactor-binding sites and the FATGY signature of the putative substrate recognition pocket (Mayfield et al. 2010; Romero et al. 2012).

It uses NADPH or NADH as co-substrates to reduce flavin (Mayfield et al. 2010; Romero et al. 2012). The steady state kinetic parameter of *Af* SidA in the presence of L-Orn and NADPH at 25°C showed an apparent K_m , k_{cat} and k_{cat}/K_m value of $5.8 \pm 0.4 \times 10^{-4}$ M, 0.611 s^{-1} , and $1.1 \pm 0.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ respectively (Mayfield et al. 2010).

2.3.2 SidA

This is another L-Orn N^5 -monooxygenase isolated from *A. nidulans* also known as *Emericella nidulans* (Eisendle et al. 2003). It catalyzes the hydroxylation of the amino side chain of L-Orn to form L- N^5 -OH-Orn. This is the first committed step in biosynthesis of triacetylfusarinine C (TAFC) siderophore. *A. nidulans* is a filamentous fungus; a model ascomycetes rather than human pathogen. It uses TAFC extracellularly to obtain iron from its host environment under iron-limiting conditions. TAFC is a cyclic tripeptide consisting of three N^2 -acetyl- N^5 -cis-anhydromevalonyl- N^5 -hydroxyornithine residues (Winkelmann 2003).

SidA has been predicted with a molecular mass of 56.6 kDa and it has 498 amino acid length which is similarly significant to *Pa* PvdA with 34.1% identity and to Sid1 from *U. maydis* with 27.6% identity (Mei et al. 1993; Visca et al. 1994; Eisendle et al. 2003). However SidA has less similarity to IucD from *E.coli* with 19.1% identity (Herrero et al. 1988). It contains FAD motif of GxGxxG at residue 41 which is found for most hydroxylating enzymes. A putative nicotinamide binding domain motif GxGxxG/A at residue 250 and D(X)3(L/F)ATGY(X)4(H/P) at 396 residue

was proposed to be the substrate binding site (Seth et al. 1998). It was found that a disruption of *sidA* gene in a single conidia strain of *A. nidulans* led to non-formation of siderophores on growth media containing 10 μ M FeSO₄ or FeCl₃. This proves further that *A. nidulans* produces siderophores only under iron-limiting conditions (Eisendle et al. 2003).

2.3.3 Sid1

This is the *L*-ornithine *N*⁵-monooxygenase which catalyzes the first committed step of ferrichrome A and ferrichrome biosynthesis in *U. maydis* (Mei et al. 1993). It catalyzes the hydroxylation of the amino side chain of *L*-Orn to produce *L*-*N*⁵-OH-Orn (Mei et al. 1993).

U. maydis is the causative agent of corn smut disease. Ferrichrome A and ferrichrome are cyclic hexapeptides consisting each of three residues of δ -*N*-acyl- δ -*N*-hydroxyornithine. Ferrichrome was first identified in 1952 by Neilands as the first chemically defined cyclic hexapeptide hydroxamate-type siderophore (Neilands 1952). The putative amino acid sequence length of Sid1 is 649 and it shows similarity to the *iucD* gene from *E. coli* with above 74% identity when the conservative amino acid substitutions are added (Mei et al. 1993).

2.3.4 DffA

It is the *L*-ornithine *N*⁵-monooxygenase involved in the conversion of *L*-Orn into *L*-*N*⁵-OH-Orn; in the first committed step in the biosynthesis of Deferriferrichrysin (Fig.2.2; Table 2.1) (Yamada et al. 2003). A *dffA* gene, encoding for DffA, was identified and analysed from among more than 20,000 clones in an *Aspergillus oryzae* expressed sequence tag library (EST) (Yamada et al. 2003). Deferriferrichrysin is a cyclic hexapeptide hydroxamate-type siderophore and it is very important for iron uptake in *A. oryzae* during iron limiting conditions. It exhibits sequence similarity to Sid1 from *Ustilago maydis* with 27.9% identity and to *Pa* PvdA from *Pseudomonas aeruginosa* with 36.0% identity (Yamada et al. 2003).

The *dffA* gene encodes a protein of 502 amino acid sequence length with putative FAD-binding, NADP-binding and substrate, *L*-Orn binding site common to other NMOs (Watanabe et al. 2008). *A. oryzae* is very useful filamentous fungi in commercial enzyme production of various foods such as soy sauce, sake, Japanese Koji etc.. (Watanabe et al. 2008). It is commonly known as koji mold used in brewing and traditional fermentation processes to enhance the taste and smell of the food products (Watanabe et al. 2008). By their work on *dffA* gene disruption, Watanabe and co-workers showed that *A. oryzae* lacking ferrichrysin displayed hypersensitivity to hydrogen peroxide suggesting that DffA plays a major role in protecting *A. oryzae* against the cytotoxicity of intracellular iron (Watanabe et al. 2008).

2.4 Bacterial *L*-lysine N^6 -monooxygenases (EC.1.14.13.59)

2.4.1 IucD

This is the *L*-lysine N^6 -monooxygenases that catalyzes the hydroxylation of the terminal amino group of the *L*-lysine (Macheroux et al. 1993). It catalyzes the first step in the biosynthesis of aerobactin; a dihydroxamate siderophore and a virulence factor for *E. coli* strains and other members of the Enterobacteriaceae (Griffiths 1991). Aerobactin is implicated in the increase in virulence of enteric bacteria. IucD activity involved the use of NADPH, FAD and molecular oxygen (Macheroux et al. 1993; Thariath et al. 1993). Macheroux et al. (1993) studied the physicochemical and catalytic activities of IucD in *E. coli* strain EN 222 (Macheroux et al. 1993). FAD free lysine N^6 -monooxygenases was purified, this suggested that its binding to co-factor was weak with a K_d of approximately 30 μ M at 4°C (Macheroux et al. 1993). This was due to the FAD binding pocket of the enzyme which provided fewer stabilizing interactions between the protein and its co-factor (Macheroux et al. 1993).

The K_m value for *L*-lysine of 105 μ M was observed and it was noted that in the absence of *L*-lysine, NADPH oxidation resulted in production of hydrogen peroxide. However, with increase

in *L*-lysine, there was a decrease in the amount of hydrogen peroxide (Macheroux et al. 1993). In the absence of FAD on the other hand, there was no oxidation of NADPH. A maximum catalytic reaction around pH 8 was observed for this enzyme. Inhibition of enzyme activity by excess binding of the enzyme to the substrate was also reported (Macheroux et al. 1993). In their studies on the activity of IucD with different substrates, Macheroux et al. (1993) showed that there was no enzyme activity when D-isomers of ornithine and lysine were used as substrates respectively (Macheroux et al. 1993). Furthermore, compounds lacking either the α -carboxy- or the α -amino function have little activity when used as substrates, for example 1,5-diaminopentane and 6-aminohexanoic acid. A putative gene *iucD*, isolated from *S. flexneri*, encodes for IucD, a lysine N^6 -monooxygenases with a putative amino sequence length of 445 (Fig.2.2; Table 2.1) (Jin et al. 2002; Wei et al. 2003).

2.5 NMOs involved in hydroxylation of amino side group of aliphatic primary diamines (EC.1.14.13.-)

2.5.1 RhbE

This is a putative diamine monooxygenase involve in the hydroxylation of 1,3-diaminopropane to N^4 -OH-1-aminopropane in the biosynthesis of rhizobactin, a hydroxamate siderophore produced by *Sinorhizobium meliloti* under iron-limiting conditions (Fig.2.2; Table 2.1) (Lynch et al. 2001). Gene cluster involved in rhizobactin biosynthesis are *rhbABCDEF*, *rhtA* and *rhrA*. It is noted that *rhbA-F* function in the biosynthesis of the siderophore. *S. meliloti* is a nitrogen fixing bacteria formerly known as *Rhizobium meliloti*. It has the ability to take up ferrisiderophores, which is made possible by reception on its outer membrane (Lynch et al. 2001).

The siderophore produced by *S. meliloti* 1021 is an endosymbiont of *Medicago sativa*. It has been structurally characterized and its siderophore is named rhizobactin 1021 to differentiate it

from unrelated rhizobactin from *S. meliloti* DM4, an amino polycarboxylic acid siderophore (Lynch et al. 2001). Although rhizobactin 1021 is not essential for symbiosis, it contributes to the efficiency of nitrogen fixation under certain conditions of plant growth. Rhizobactin 1021 is also likely to be a factor that contributes to the competitive ability of free-living *S. meliloti* in non-depleted soils. RhbE is homologous to IucD from *E. coli* and AlcA from *B. bronchiseptica* (Lynch et al. 2001).

2.5.2 AlcA

AlcA is produced by *Bordetella* spp. and it catalyzes conversion of 1,4-diaminobutane (putrescine) into *N*-hydroxy-putrescine in the biosynthesis of alcaligin (Kang et al. 1996). *Bordetella pertussis* is the causative agent of human whooping cough or pertussis (Kang et al. 1996). *Bordetella bronchiseptica*, the causative agent of swine atrophic rhinitis and kennel, produces alcaligin a macrocyclic dihydroxamate siderophore (Brickman et al. 1996). Gene cluster involved in putative alcaligin biosynthesis were identified as *alcABC* (Giardina et al. 1995; Kang et al. 1996).

2.5.3 DesB

This is the 1,5-diaminopentane (cadaverine) monooxygenase involved in the catalyzes of cadaverine to give *N*-hydroxy-cadaverine in the early step biosynthesis of desferrioxamine B siderophore (Barona-Gómez et al. 2006). Desferrioxamines cause interspecies stimulation of *Streptomyces* growth and development (Yamanaka et al. 2005). Gene cluster involved in the assembly of desferrioxamine B are *desA*, *desB*, *desC* and *desD* and a previously unidentified tris-hydroxamate from lysine, succinyl CoA and molecular oxygen (Barona-Gómez et al. 2006). The amino acid sequence of DesB shows similarity of 47% to AlcA produced by *Bordetella bronchiseptica*. A putative DesB from *S. scabies* 87-22, clustered together with bacterial diamine monooxygenases (Fig.2.2; Table 2.1) (Bignell et al. 2010).

2.6 Bacterial N⁶-acyl *L*-lysine monooxygenases (EC.1.14.13.59)

MbtG and MbsG from *Mycobacterium tumefaciens* and *Mycobacterium smegmatis* G respectively are referred to as unusual NMOs (Robinson and Sobrado 2013). They are *L*-lysine monooxygenases involved in the hydroxylation of two acylated residues in *L*-lysine in the biosynthesis of mycobactin siderophore, an important virulence factor produced by the pathogens (Quadri et al. 1998; Li et al. 2005; Madigan et al. 2012). *M. tumefaciens* and *M. smegmatis* are known human pathogen causing tuberculosis (Madigan et al. 2012; Robinson et al. 2014). They differ from other NMOs in that they do not share the mechanistic features of other NMO family (Robinson and Sobrado 2011).

For MbsG, the flavin intermediates that prevent the release of oxygen reactive species are not conserved. Also MbtG has been proposed to be involved in the final enzymatic step in the biosynthesis of mycobactin (Madigan et al. 2012). MbsG is a homolog of MbtG with nearly 75% identity and can use both NADH and NADPH as co-substrates with same catalytic efficiency (Madigan et al. 2012). The mechanistic study of MbsG in the presence of 3 mM (*L*-lysine and NADH) showed an apparent K_m of 7.0 ± 0.2 mM, k_{cat} was found to be 0.90 ± 0.02 min⁻¹ while in the presence of NADPH and *L*-lysine (3 mM) the enzyme showed an apparent K_m of 12 ± 1 mM, and k_{cat} of 0.55 ± 0.02 min⁻¹ (Robinson et al. 2014).

2.7 Conclusions

The phylogenetic analysis of the microbial NMOs depicted in Figure 2.1 on page 28 clearly shows their relationship. Visca and co-workers suggested that NMOs probably evolved from a common ancestor (Visca et al. 1994). NMOs are encoded for by a single gene, they are able to perform their biosynthesis function along with their respective gene cluster, although most of

them are yet to be characterized. Most NMOs have been successfully purified and expressed as recombinant proteins using *E. coli* as the host vector.

Pa PvdA, IucD, and *Af* SidA are the most studied NMOs. They do not stably bind FAD, however, *Af* SidA is an exception, it binds FAD covalently (Macheroux et al. 1993; Chocklett and Sobrado 2010), and FMN is not a cofactor of NMOs studied so far (Macheroux et al. 1993; Stehr et al. 1999; Ge and Seah 2006; Meneely and Lamb 2007; Pohlmann and Marahiel 2008; Heemstra et al. 2009; Mayfield et al. 2010; Olucha et al. 2011; Robinson and Sobrado 2011; Romero et al. 2012). NMOs use NADPH as an electron donating co-substrate; with *Af* SidA, KtzI, and MbsG as exceptions as they accept either NADH or NADPH (Mayfield et al. 2010; Madigan et al. 2012; Neumann et al. 2012; Romero et al. 2012). *Pa* PvdA, *Af* SidA and KtzI are NMOs with known structures (Fig. 2.2) (Olucha et al. 2011; Franceschini et al. 2012; Setser et al. 2014).

The kinetic mechanism and structural analysis of *Pa* PvdA, *Af* SidA, and KtzI provide basic information for other NMOs (Heemstra et al. 2009; Olucha et al. 2011; Franceschini et al. 2012; Setser et al. 2014). Kinetic data for NMOs have been studied using NADPH oxidation assay which fits well into the Michaelis Menten model. Results of the kinetic parameters of the biochemically characterized NMOs so far seem to be in agreement with one another.

Product formation of the specific substrates of NMOs into the corresponding hydroxylamines has been studied using HPLC, hydroxylation assay, or other assays (Challis and Ravel 2000; Meneely and Lamb 2007). A thorough understanding of these NMOs is necessary for their wide applications in human therapeutic drug development, agriculture, food industries and for novel antimicrobial synthesis.

2.8 Acknowledgements

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CHAPTER 3

Identification and characterization of a putrescine *N*-hydroxylase (GorA) from the *Gordonia rubripertincta* CWB2

Abstract

A putrescine *N*-hydroxylase from *Gordonia rubripertincta* CWB2 (GorA), a microbial *N*-hydroxylating monooxygenase (NMO), specific for a range of diamines (putrescine > cadaverine > hexamethylenediamine) was identified using bioinformatic tools. It has been successfully cloned, overexpressed, and purified as a soluble flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) dependent protein using *Escherichia coli* as the cloning host organism and pET16bP as the cloning vector. The NADPH oxidation assay and an hydroxylation assay were used to assess its biochemical properties. This NMO clustered together with some known but yet to be characterized diamine NMOs which are RhbE, from *Sinorhizobium meliloti* 1021; AlcA, from *Bordetella bronchiseptica* RB50, and DesB, from *Streptomyces scabiei* 87-22. It has 438 amino acid sequence length and approximately 51 kDa. The pH optimum is between the range of 7.0-8.0 in a phosphate buffer. GorA demonstrated a 100% relative activity in the presence of 1,4-diaminobutane (putrescine) as compared to other substrates tested. With the NADPH oxidation assay, the kinetic parameters of this enzyme showed an apparent V_{max} , K_m and k_{cat} of $310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$, $361 \pm 0.1 \text{ }\mu\text{M}$ and 0.27 s^{-1} , respectively, whereas the hydroxylation assay showed GorA with an apparent V_{max} , K_m and k_{cat} of $246 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and, $737 \pm 0.1 \text{ }\mu\text{M}$ and 0.21 s^{-1} . Thus this is the first diamine *N*-hydroxylating monooxygenase characterized in detail with a physiological role in siderophore biosynthesis.

Keywords: *Gordonia rubripertincta* CWB2, *N*-hydroxylating monooxygenases (NMOs),

1,4-diaminobutane (putrescine), kinetic parameters, metallophores, niconatimide adenine dinucleotide phosphate (NADPH)

3.1 Introduction

Gordonia spp. are aerobic and gram positive actinomycetes. They are important bacteria involved in the biodegradation of hydrocarbons, bioremediation and siderophore production (Hong et al. 2011). Schwyn and Neilands (1987) identified siderophores as metal-chelating compounds of about 200-2000 Da. Metallophores are employed by bacteria for iron uptake during iron-limiting conditions of their environment. They have been shown as important metal chelators, since they bind not only iron but other important metals like aluminium, copper, gallium and vanadium among others (Baysse et al. 2000; del Olmo et al. 2003; Ahmed and Holmström 2014).

N-hydroxylase or NMOs have been known to be involved in the first step biosynthesis of hydroxamate siderophores; they belong to the Class B Flavoprotein monooxygenases (van Berkel et al. 2006; Schlaich et al. 2007; Huijbers et al. 2014). Studies to find novel NMOs have been based on characterization of gene clusters involved in the biosynthesis of several hydroxamate-containing siderophores. Thus, IucD, from *Escherichia coli* was the first NMO to be characterized (Thariath et al. 1993). Similar studies to identify related NMOs were performed in *Ustilago maydis*, *Pseudomonas aeruginosa*, *Burkholderia cepia*, *Omphalotus olearius*, and *Aspergillus* spp. (Visca et al. 1994; Sokol et al. 1999; Romero et al. 2012).

Studies on gene knockout to identify functions and link several NMOs to pathogen virulence were also done as reported elsewhere (Visca et al. 1994; Sokol et al. 1999). Unfortunately, despite the results that showed NMOs as essential virulence factors in both bacteria and fungi and potential metallophores and drug targets; only a small number of them have been

characterized so far. There is a need to increase the available pool of NMOs. Here, we present the successful overexpression, cloning and kinetic parameters of GorA, a novel putrescine *N*-hydroxylase from *Gordonia rubripertincta* CWB2, which is a potential NMO for environmental metal extraction.

3.2 Materials and Methods

3.2.1 Chemicals and enzymes

Enzymes used for cloning purposes were obtained from MBI Fermentas (St. Leon-Rot, Germany) and Roche Diagnostics. Oligonucleotides and the codon optimized gene *gorA* (KX345394) were synthesized by Eurofins MWG (Ebersberg, Germany). The codon usage had been optimized according to *Acinetobacter* as described earlier (Oelschlägel et al. 2015). A range of eleven substrates were tested with GorA, namely ethanolamine, propylamine, *N*-butylamine; primary chain diamines 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), 1,6-diaminohexane (hexanemethylenediamine), 1,7-diaminoheptane, and 1,8-diaminooctane; two amino acids, *L*-ornithine and *L*-lysine. Respective substrates were obtained from Sigma-Aldrich or TCI Europe at highest purity available.

3.2.2 Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. *Escherichia coli* strains were cultured as described elsewhere (Sambrook and Russell, 2001).

Table 3.1: Strains and plasmids including their relevant characteristics and sources

Strains, Plasmids	Relevant characteristic(s)	Sources
Strains		
<i>G. rubripertincta</i> CWB2	Isolated with styrene as sole source of carbon. Produces siderophores upon iron limitation.	DSM 46758
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>lacZ</i> M15 (<i>lacZYA argF</i>)U169 end A1 <i>recA</i> <i>hsR17</i> (<i>r_k-m_k</i>) <i>supE44</i> Δ ⁻ <i>thi-1 gyrA96 relA1</i>	Gibco-BRL
<i>E. coli</i> BL21 (DE3) (pLysS)	<i>hsdS gal</i> (Δ <i>cl</i> s857 <i>ind1</i> <i>Sam7 nin5 lacUV5-T7</i> gene 1) pLysS (Cm ^r)	Stratagene
Plasmids		
pEX-K4_GorA	pEX vector with additional multiple cloning site comprising the codon optimized <i>gorA</i> gene; Kanamycin	MWG Eurofins
pET16bP	pET16b with additional multicloning site; allows expression of recombinant proteins with terminal N-His ₁₀ tag	U. Wehmeyer ^b
pGorA	<i>gorA</i> of <i>Gordonia rubripertincta</i> CWB2 (1328 bp NdeI/NotI fragment; KX345394) cloned into pET16bP via NdeI/NotI sites	This study

^aPersonal communication

3.2.3 Data mining

The NCBI server was used to retrieve protein sequences of some already known NMOs and to perform BLAST searches (Altschul et al. 1990). The *Gordonia rubripertincta* CWB2 GorA was identified from the respective genome sequence (unpublished, in cooperation with Prof. Dr. J. Kalinowski, CeBiTec, Bielefeld, Germany) using these previously retrieved known microbial NMOs. Its phylogenetic analysis and protein sequence alignment with the close related and

known NMOs were performed using the programs CLUSTALW (version 2.0) (Thompson et al. 1994), GeneDoc (version 2.7.000), MEGA (Tamura et al. 2013), Mutalin (Corpet 1988) and ESript 3.0 (Robert and Gouet 2014).

3.2.4 Cloning of the putative NMO gene

The *gorA* gene was purchased from Eurofins MWG GmbH (Ebersberg, Germany) in a pEX-K4 vector system containing *NdeI* and *NotI* restriction sites (Fig. 3.1). The GC content of the *gorA* was adapted to the genome usage of strain *Acinetobacter* ADP1. Received vectors were double digested by *NdeI* and *NotI* and the purified product was directly transferred to the pET16bP system in order to obtain the expression construct pGorA *E.coli* DH5 α resulting 1328 basepair DH5 α *Nde1* and *Not1*. The expression vector pGorA was transformed into *E. coli* BL21 (DE3) (pLysS) (Stratagene).

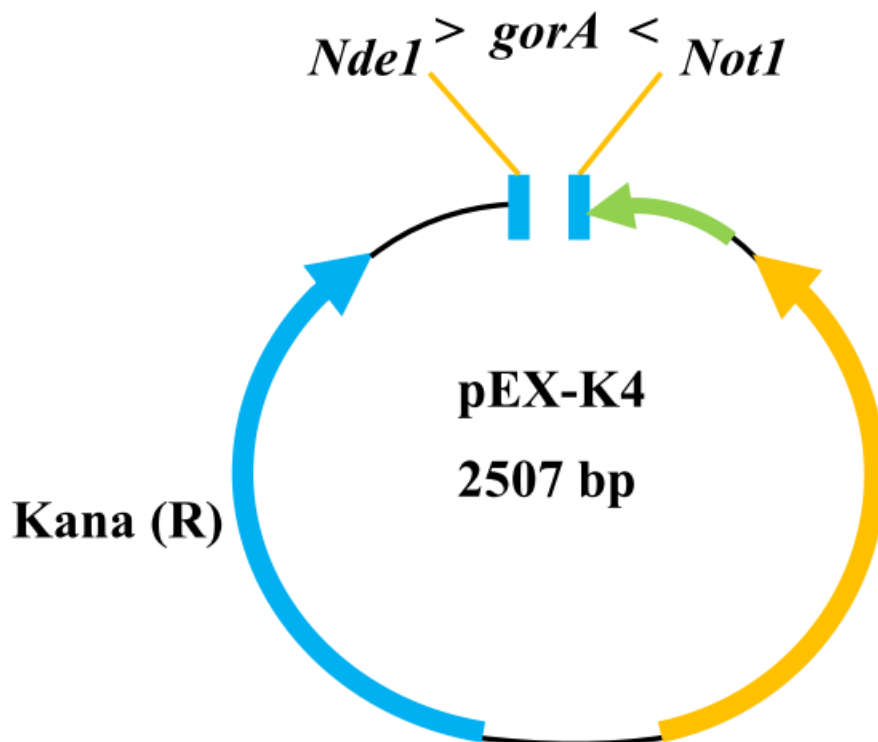


Figure 3.1: The *gorA* gene from Eurofins MWG GmbH (Ebersberg, Germany) in a pEX-K4 vector system (2507 bp) containing *NdeI* and *NotI* restriction sites and Kanamycin resistance gene, Kana (R)

3.2.5 Overexpression and purification of the putative diamine protein

E. coli BL21 (DE3) containing the expression construct pGorA were grown in 3 L shaking flasks (120 rpm, 37°C) containing Luria Bertani (LB) medium supplemented with Ampicillin (100 µg/mL) and Chloramphenicol (50 µg/mL). Cells were grown until an optical density at 600 nm (OD₆₀₀) of ~0.6, after which 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction for 3-4 h. Optimization of the expression condition was carried out by testing bacterial cultures incubated at 20°C on a shaker at 120 rpm overnight, and also sets of bacterial cultures were incubated at 37°C for 4 hours at 120 rpm in 3 L shaking flasks respectively.

Cells were harvested by centrifugation (5.000 x g, 15 min, 4°C) and they were stored at -80°C. The production of GorA was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis as previously described (Sambrook and Russell, 2001).

Crude extract was prepared from the freshly thawed biomass and diluted with a threefold excess of 10 mM phosphate buffer (pH 8.0). Purification of His₁₀-GorA was achieved by Ni chelate chromatography on a 1 mL HisTrap FF column, using an ÄKTA fast-performance liquid chromatographer (both from GE Healthcare). After the application of the crude extract of *E. coli* BL21 (pGorA), nonspecific proteins were removed with binding buffer 10 mM phosphate buffer (pH 8.0), 500 mM NaCl, 1 mM EDTA 25 mM MgCl₂, and 25 mM Sodium Citrate, 500 mM imidazole).

His₁₀-GorA protein was eluted with linear imidazole gradient of 6 column volume of elution buffer. Fractions with NADPH dependent FAD oxidoreductase activity were pooled and concentrated with 10 mM phosphate buffer (pH 8.0) using 30,000 MW Falcon filter tube using an ultra-centrifugation tube. Recombinant proteins were collected by centrifugation at 5,000 rpm for 30 min, washed twice with 10 mM phosphate buffer (pH 8.0), dissolved in storage buffer containing 50% [v/v] glycerol and stored at -20°C. The concentration of the purified GorA was determined using the Bradford method (Bradford, M. M. 1976).

3.2.6 NADPH oxidation assay

The standard NADPH oxidation assay buffer contained 100 mM phosphate buffer pH 8.0, 0.05 mM FAD, 0.15 mM NADPH and [hexamethylenediamine (6 mM) or 1,4-diaminobutane (1 mM)], and an appropriate amount of GorA (1 µM) which was pre-incubated in the 1 mL assay buffer for 10 min at 24°C. The reaction was initiated by the addition of 0.15 mM NADPH. The NADPH oxidation was monitored at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) and measurements were taken after 2 min using a spectrophotometer (Cary 50; Varian).

3.2.7 Determination of enzyme activity at different conditions

The solubility of GorA in different buffers which were 100 mM phosphate buffer, 20 mM Tris-HCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 20 mM 2-(N-morpholino)ethanesulfonic acid (MES); its pH dependence using 100 mM phosphate buffer ranging from pH (5.7-8.0), and 20 mM Tris-HCl ranging from pH (7.0-9.5) were assessed spectrophotometrically using the standard NADPH oxidation assay mixture with hexamethylenediamine (6 mM). The ionic strength of 100 mM phosphate buffer enhanced the solubility and stability of GorA, thus it was used for the subsequent experiments.

The temperature optimum for GorA was assessed from 10°C-50°C, and temperature stability for GorA from 10°C-48°C was also assessed spectrophotometrically using the standard NADPH oxidation assay mixture with hexamethylenediamine (6 mM).

3.2.8 Coenzymes and co-substrates specifically for the putative diamine hydroxylase

The activity of GorA with different coenzymes i.e. 0.05 mM each of FAD and FMN were separately tested with either 0.15 mM (NADPH or NADH) co-substrates spectrophotometrically in a 1 mL buffered standard NADPH oxidation assay after 10 minutes of pre-incubation of GorA (1 µM) at 24°C with 1 mM 1,4-diaminobutane separately and measuring the oxidation of either NADPH or NADH at 340 nm absorption for 2 min; a separate assay with the addition of no substrate serve as the control.

3.2.9 Determination of substrate specificity for the putative diamine hydroxylase

The activity of GorA with different substrates (mentioned above) was assessed spectrophotometrically (Cary 50; Varian) in a 1 mL buffered assay after 10 min pre-incubation of the enzyme (1 µM) at 24°C. Each substrate was tested separately at three different concentrations separately; these include 1 mM, 3 mM, and 6 mM; using 0.05 mM FAD as a

cofactor, and measuring the consumption of NADPH at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) absorption after 2 min. A separate assay without the addition of a substrate served as the control.

3.2.10 Hydroxylation assay

The amount of hydroxylated product formed by GorA (1 μM) in the presence of the different substrates (the same as those used for the NADPH oxidation assay above), at 1 mM each, were assessed separately using the Csáky iodine oxidation reaction (Csáky et al. 1948; Gillam et al. 1981). The standard assay buffer is the same as described for the NADPH oxidation assay with GorA (1 μM). The reaction took place at 24°C and was initiated by the addition of 0.15 mM NADPH at different time intervals of 30 s, 60 s, 90 s, 120 s, 180 s, and 240 s. At each time, interval 104 μl of the assay mixture was withdrawn and added to 52.5 μl of 2 N perchloric acid to terminate the reaction.

For each time point, 47.5 μl of the terminated reaction mixture was transferred into a 96 glass well plate and the reaction mixture was neutralised by the addition of an equal amount of 10% (w/v) sodium acetate solution. To each well, 47.5 μl of 1% (w/v) sulfanilic acid in 25% (v/v) acetic acid, and 19 μl of 0.5% (w/v) iodide in glacial acetic acid were added and the reaction was allowed to incubate at room temperature for 15 min. Excess iodine was removed with 19 μl of 0.1 N sodium thiosulfate and the color was developed by adding 19 μl of 0.6% (w/v) α -naphthylamine in 30% (w/v) acetic acid.

Formation of the hydroxylated products were measured at the absorbance of 562 nm immediately after the addition of all the above mixtures using a plate reader (Molecular Devices Spectra Max e2). A standard curve of the hydroxylamine hydrochloride was used to calculate the amount of hydroxylated product generated.

3.2.11 Steady state kinetics

The NADPH standard oxidation assay was used for the steady state parameters of GorA. The apparent K_m value for FAD with varying concentrations from 0.025 mM-0.1 mM was determined by fixing the concentration of 1,4-diaminobutane at 1 mM and that of NADPH at 0.15 mM. The apparent K_m value for NADPH with varying concentrations from 0.025 mM-0.1 mM was determined by fixing the concentration of 1,4-diaminobutane at 1 mM and that of FAD at 0.05 mM.

3.2.12 Determination of kinetic parameters of GorA with 1,4-diaminobutane

Kinetic parameters of the putative diamine monooxygenase GorA (1 μ M) were assessed at 24°C, using both the standard NADPH oxidation assay, measuring the amount of NADPH consumption at 340 nm absorption for 2 min, and the hydroxylation assay as described above, but measuring the amount of hydroxylated product formed after 2 min. In both assays, 1,4-diaminobutane was used as the preferred substrate at different concentrations of 0 mM-5 mM. Also, the reaction was initiated by the addition of 0.15 mM NADPH. The Michealis-Menten kinetics of GorA of both assays were assayed similarly.

3.3 Data Analysis

All reactions were carried out in triplicate with standard deviations as indicated.

3.4 Results

3.4.1 Phylogenetic analysis

Comparison with data sets of known NMOs showed *gorA* as a putative *L*-lysine N^6 -hydroxylase/*L*-ornithine N^5 -oxygenase family with 438 amino acid sequence length. The percentage showed *gorA* as a putative *L*-lysine N^6 -hydroxylase/*L*-ornithine N^5 -oxygenase family with 438 amino acid sequence length (Fig. 3.2). Its percentage similarity with some known

NMOs are as follows: 39% identity to DesB, 36% identity to RhbE, 34% identity to AlcA, 30% identity to IuD and 29% identity to *Pa* PvdA (Fig. 3.3).

3.4.2 GorA protein production and purification

The *gorA* gene was successfully cloned from the ordered plasmid comprising the optimized gene. The GC content of *gorA* was adapted to the genome usage of strain *Acinetobacter* ADP1. Received vectors were double digested by *NdeI* and *NotI* and the purified product was directly transferred to the pET16bP system, in order to obtain the expression construct pGorA. It was then cloned into *E.coli* DH5 α resulting in the 1328 basepair *Nde1* and *Not1*. The expression vector pGorA was transformed into *E. coli* BL21 (DE3) (pLysS). An overexpression plasmid was generated such that expression in *E. coli* BL21 (DE3) (pLysS) yielded the GorA protein of approximately 51 kDa (Fig. 3.4), including an N-terminal His₁₀tag. About 4.74 mg/mL of GorA at approximately 95% purity per litre of culture with the nickel chelate chromatographer was produced. For the overexpression optimised conditions at different temperatures of 20°C (cultures were kept on shaker overnight) and 37°C (cultures were kept on shaker for 4 h), no difference in the amount of GorA production was observed.

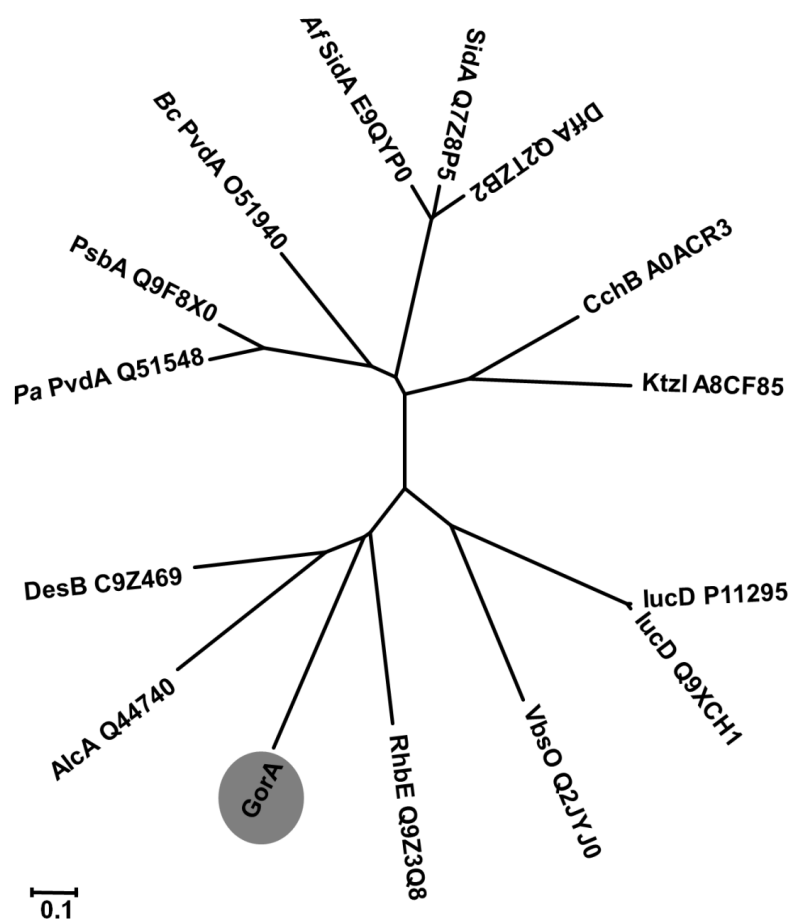


Figure 3.2: Dendograms showing the relationship of GorA to other known NMOs

Figure 3.2 shows the bacterial *L*-ornithine monooxygenases, *Pa* PvdA, *Pseudomonas aeruginosa* pyoverdine A (Q51548); *Bc* PvdA, *Burkholderia cepacia* pyoverdine A (O51940); *PsbA*, *Pseudomonas* sp. B10 (Q9F8X0); *KtzI*, *Kutzneria* sp. 744 (A8CF85); *CchB*, *Streptomyces ambofaciens* (A0ACR3); fungal *L*-ornithine *N*⁵-monooxygenases (EC.1.14.13.196), *Af* SidA (OMO), *Aspergillus fumigatus* siderophore A (E9QYP0); *SidA*, *Emericella nidulans* (Q7Z8P5); *DffA*, *Aspergillus oryzae* (Q2TZB2); the bacterial *L*-lysine *N*⁶-monooxygenases (EC.1.14.13.59), *lucD*, *Escherichia coli*, *VbsO*, *Rhizobium etli* CFN 42 (Q2JYJ0); diamine monooxygenases, *RhlE*, *S. meliloti* 1021 (Q9Z3Q8); *AlcA*, *B. bronchiseptica* RB50 (Q44740); *DesB*, *S. scabies* 87-22 (C9Z469). Distance trees were created with respect to the software programs ClustalW (version 2.0) (Thompson et al., 1994), MEGA 6 (version 2.6.003). *Gordonia rubripertincta* CWB2 (GorA), is shaded

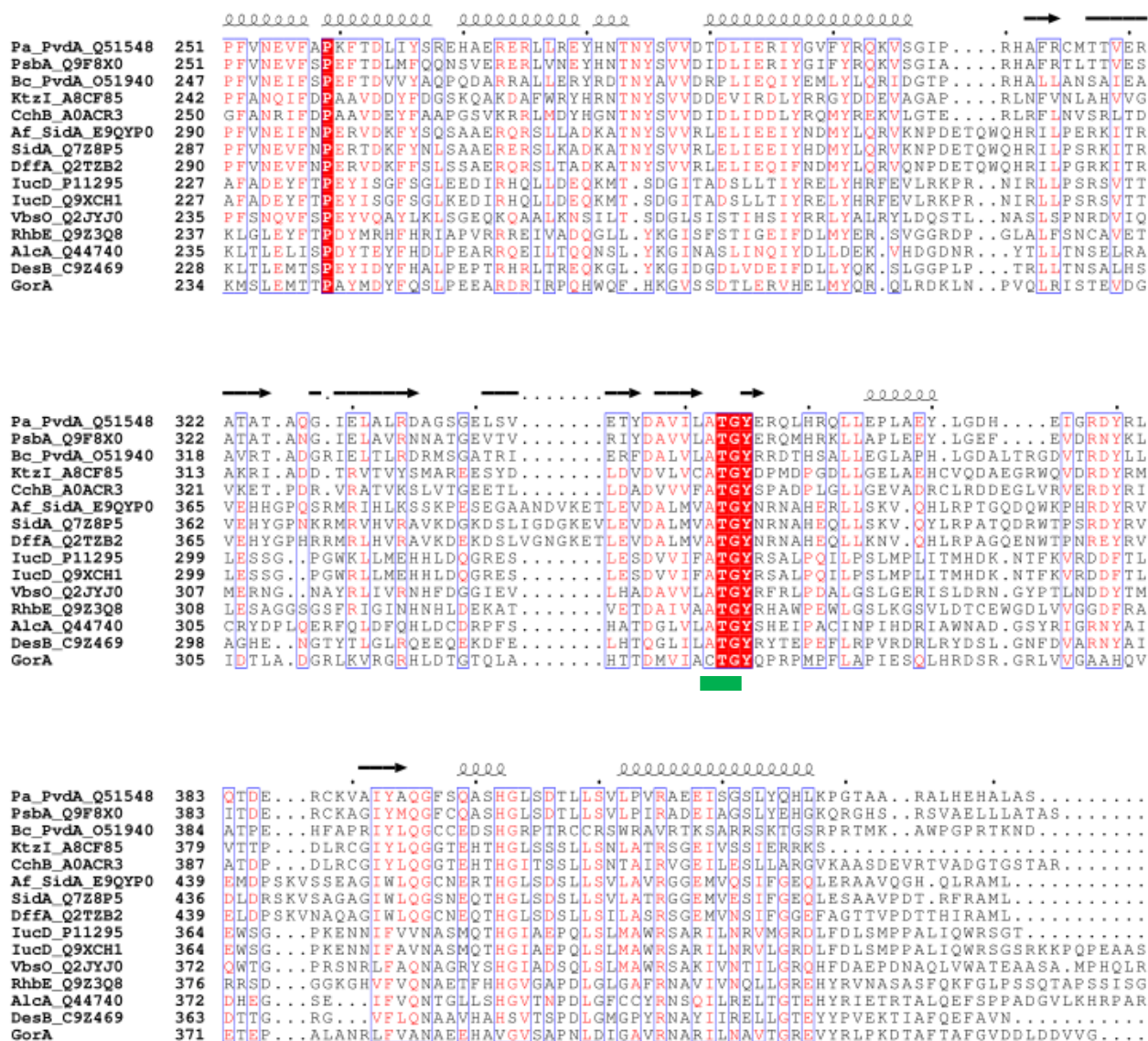


Figure 3.3: Multiple sequence alignment of GorA and some of the known NMOs

Figure 3.3 shows that alignment was carried out using the programs ClustalW (version 2.0) (Thompson et al., 1994), Mutalin (Corpet 1988) and ESript 3.0 (Robert and Gouet 2014). Relevant amino acid sequence motif for an involvement of flavin and nicotinamide adenine dinucleotides are indicated. FAD binding fingerprint GxGxGP, indicated by the yellow bar, were found at the amino acid sequence positions, as well as NADPH binding fingerprint, indicated by the black bar, and substrate binding fingerprint are shown by the green bar. The secondary structure the *L*-Orn monooxygenase, *Pa* PvdA with PDB code 3S5W was used as the standard for the amino acid sequence alignment. The Loops are α -helices, arrows are β -sheets while the spaces are loops.

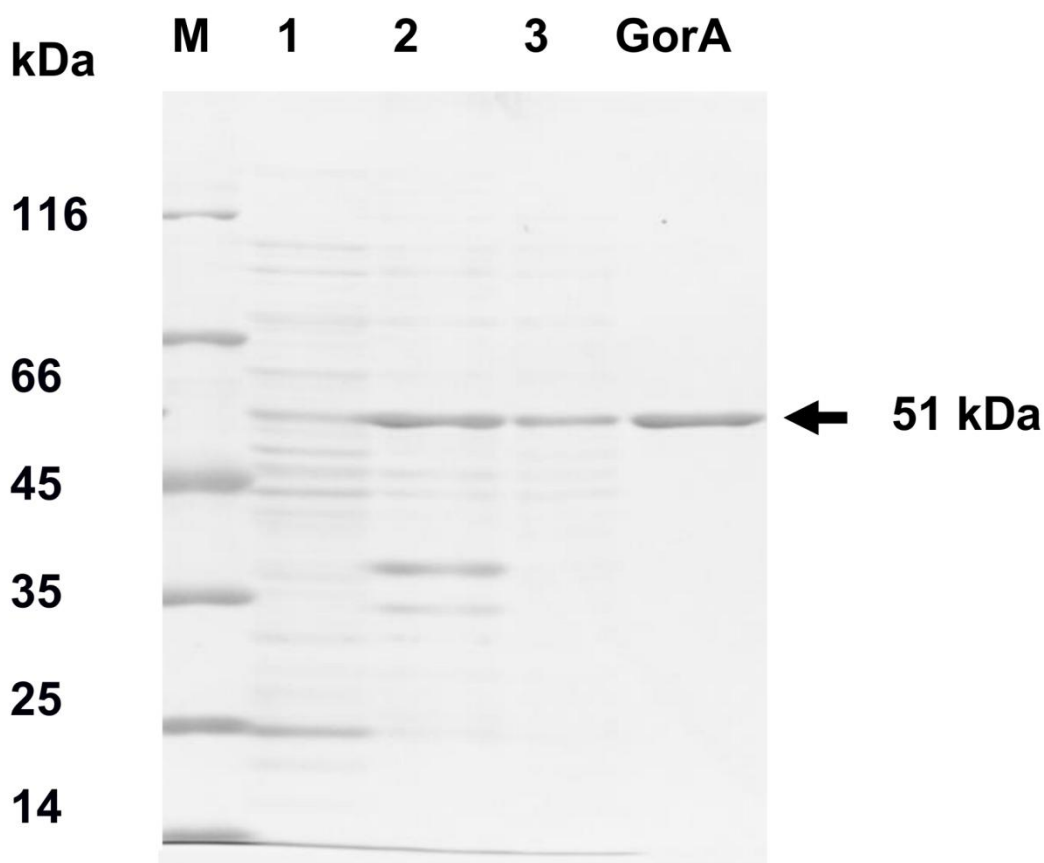


Figure 3.4: Result of the coomassie blue-stained SDS-PAGE of GorA purification

Figure 3.4 shows a 10% polyacrylamide gel was loaded as follows; lane M, Page Ruler 14 - 116 kDa protein ladder (Fermentas); lane 1, crude extract of induced *E. coli* BL21 (DE3) pLysS pGorA; lane 2, first fraction of eluted protein; lane 3, last fraction of eluted protein; GorA, about 2 μ g of His₁₀-GorA after the Ni-chelate chromatography, pooling and concentration by filtration devices (cut-off: 30 kDa). The gel run was performed and stained as described in methods section.

3.4.3 Temperature optimum and pH for catalytic activity

The temperature optimum and stability tests showed that the enzyme was active at 32.5°C but it was unstable at this temperature (Fig. 3.5 top). Thus at higher temperature regimes over the optimum temperature, GorA exhibits, a low activity and stability.

The catalytic activity of GorA using different buffers which include 100 mM phosphate buffer pH 8.0, 20 mM Tris-HCl buffer pH 7.5, 20 mM HEPES buffer pH 8.0, and 20 mM MES buffer pH 6.0 were assessed separately.

The initial velocities for NADPH oxidation by GorA were measured using 100 mM phosphate buffer ranging from pH 5.7-8.0, and 25 mM Tris-HCl ranging from pH 7.0-9.5. The pH maximal turnover was observed at 7.0-8.0 for both the phosphate buffer and the Tris HCl buffer (Fig. 3.5 bottom). Higher activity with GorA at 100 mU/mg was observed when 100 mM phosphate buffer was used, but it was 60 mU/mg mM for Tris-HCl buffer. Thus 100 mM phosphate buffer was used for subsequent assays.

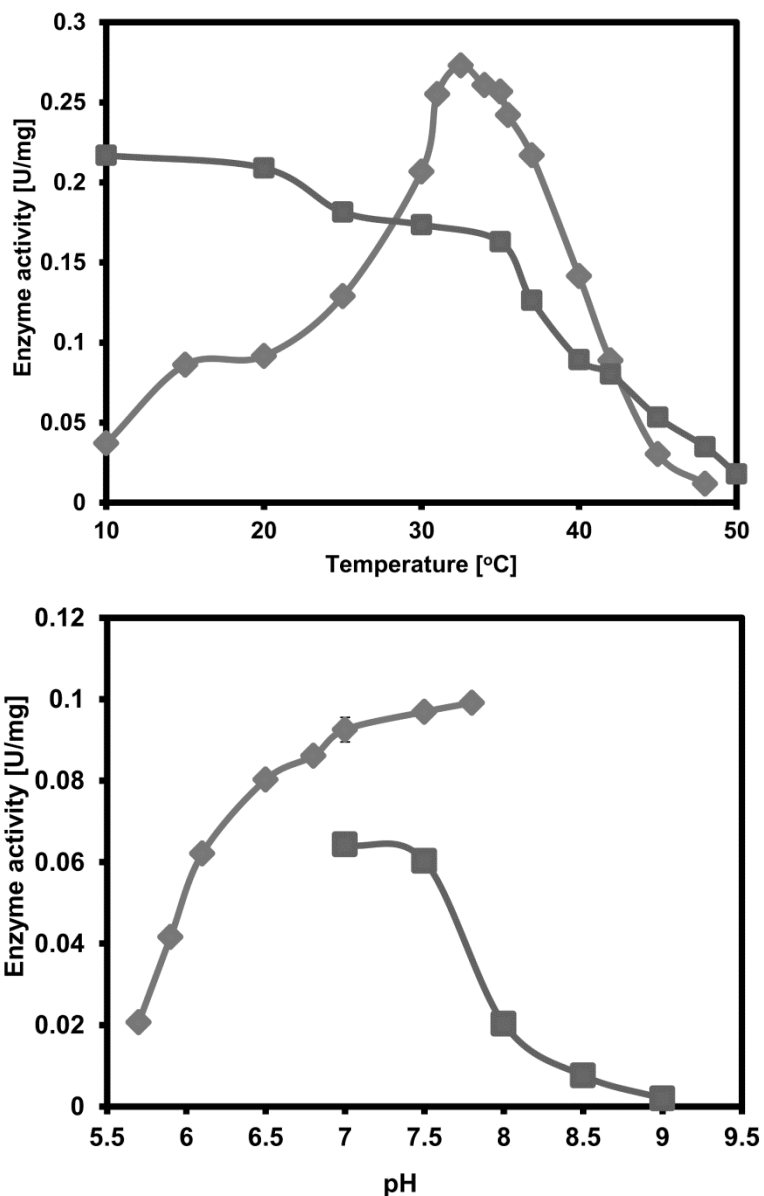


Figure 3.5: Buffer, pH and temperature dependence of GorA activity

Figure 3.5 shows the Buffer, pH and temperature dependence of GorA activity derived from triplicate measurements. The assay mixtures contained NADPH (0.15 mM), FAD (0.05 mM) and hexamethylenediamine (6 mM). Top: Various temperatures regimes of GorA temperature optimum (tilted boxes) and temperature stability (boxes). Bottom: The pH assessment of GorA in 100 mM phosphate buffer (tilted boxes) and in 20 mM Tris-HCl (boxes) at different pH-values was separately determined. Data were obtained from triplicate measurements by means of the NADPH oxidation assay. The standard deviations calculated from these triplicates were less than 1% in case of temperature dependency and 0.3% in case of pH variation.

3.4.4 Coenzyme specificity and cosubstrate specificity

GorA exhibited a relative activity of 100% in the presence of FAD and 1,4-diaminobutane. The NADPH-FAD substrate independent showed a relative activity of about 24%, whereas the relative activity of NADPH-FMN substrate independent and NADPH-FMN substrate dependent were about 6.46% and 8.39% respectively. This showed that FMN is not a coenzyme for GorA (Fig. 3.6A).

In the same vein, the NADH-FAD substrate independent of GorA showed a relative activity of about 20.6%, and of NADH-FAD substrate dependent was about 23%, indicating also that GorA did not accept NADH as cosubstrate, although there was product formation when NADH was used as cosubstrate in the hydroxylation assay.

3.4.5 Substrate specificity

GorA is more active with 1,4-diaminobutane than all the remaining substrates tested, as shown Figure 3.6B. This indicated GorA immediately as a putrescine *N*-hydroxylase. The enzyme showed a relative activity of 100% with 1 mM 1,4-diaminobutane, although an increase of over 5 mM 1,4-diaminobutane resulted in a decrease in the activity of GorA. The same is true for all the other substrates tested, except for ethanolamine that showed a relative activity increase from 8.1% to 17.7% as it was increased from 1 mM to 6 mM.

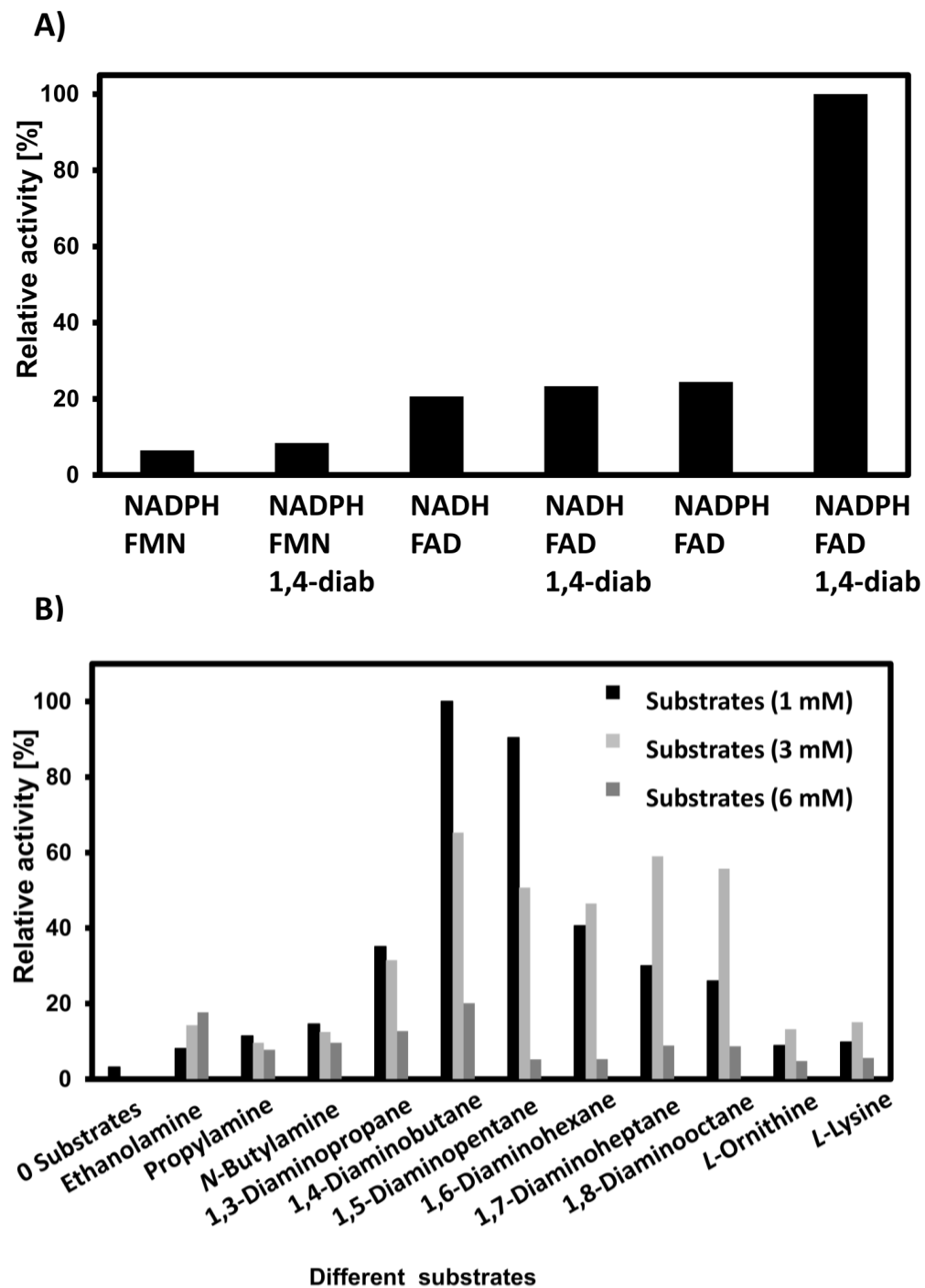


Figure 3.6: Substrates and coenzyme specificity of GorA

Figure 3.6 shows the NADPH-oxidation assay. A) The relative activity of GorA with 0.05 mM (FAD and FMN) as coenzymes; and with either 0.15 mM NADPH or NADH as electron donor in presence or absence of the substrate 1,4-diaminobutane: 1,4-diab. B) The relative activity of different substrates; primary chain amines: ethanolamine, propylamine, *N*-butylamine; primary chain diamines 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), 1,6-diaminohexane (hexanemethylenediamine), 1,7-diaminoheptane (heptanediamine), and 1,8-diaminooctane (octanediamine), as well as for the two amino acids *L*-ornithine and *L*-lysine were assessed at 1 mM, 3 mM, and 6 mM separately. An assay without substrate (0) addition served as the control. Data were obtained from triplicate measurements by means of the NADPH oxidation assay. The standard deviations calculated from these triplicates were in all cases less than 2.75%.

3.4.6 Steady State Kinetics

In the NADPH oxidation assay, GorA showed an apparent V_{max} of $310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$. This was in agreement with its V_{max} from the hydroxylation assay, which was $246 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$ (Fig.3.7A and 3.7B, Table 3.2). However, the apparent K_m value of GorA with different concentrations of 1,4-diaminobutane was $361 \pm 0.1 \text{ }\mu\text{M}$, in the NADPH assay, whereas it was $737 \pm 0.1 \text{ }\mu\text{M}$ in the hydroxylation assay.

The apparent K_m value for NADPH and FAD were determined to be $5.2 \pm 0.6 \text{ }\mu\text{M}$ and $16 \pm 0.01 \text{ }\mu\text{M}$ respectively whereas their V_{max} were $249 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and $301 \pm 0.02 \text{ nmol min}^{-1} \text{ mg}^{-1}$ as shown in Figures 3.7C and 3.7D.

3.4.7 Determination of kinetic parameters

The kinetic parameters for GorA were determined using both NADPH oxidation and hydroxylation assay with 1,4-diaminobutane concentrations ranging from 0 to 5 mM (Fig.3.7A and 3.7B). At higher 1,4-diaminobutane concentration, of over 6 mM, substrate inhibition was observed for the NADPH oxidation assay. The V_{max} and k_{cat} values for the two assays are consistent, except K_m as shown in Table 3.2. At saturating concentrations of NADPH and FAD (0.15 mM and 0.05 mM respectively), the activity of GorA fits a classical Michealis-Menten yielding an apparent V_{max} value of $310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$ and K_m of $361 \pm 0.1 \text{ }\mu\text{M}$.

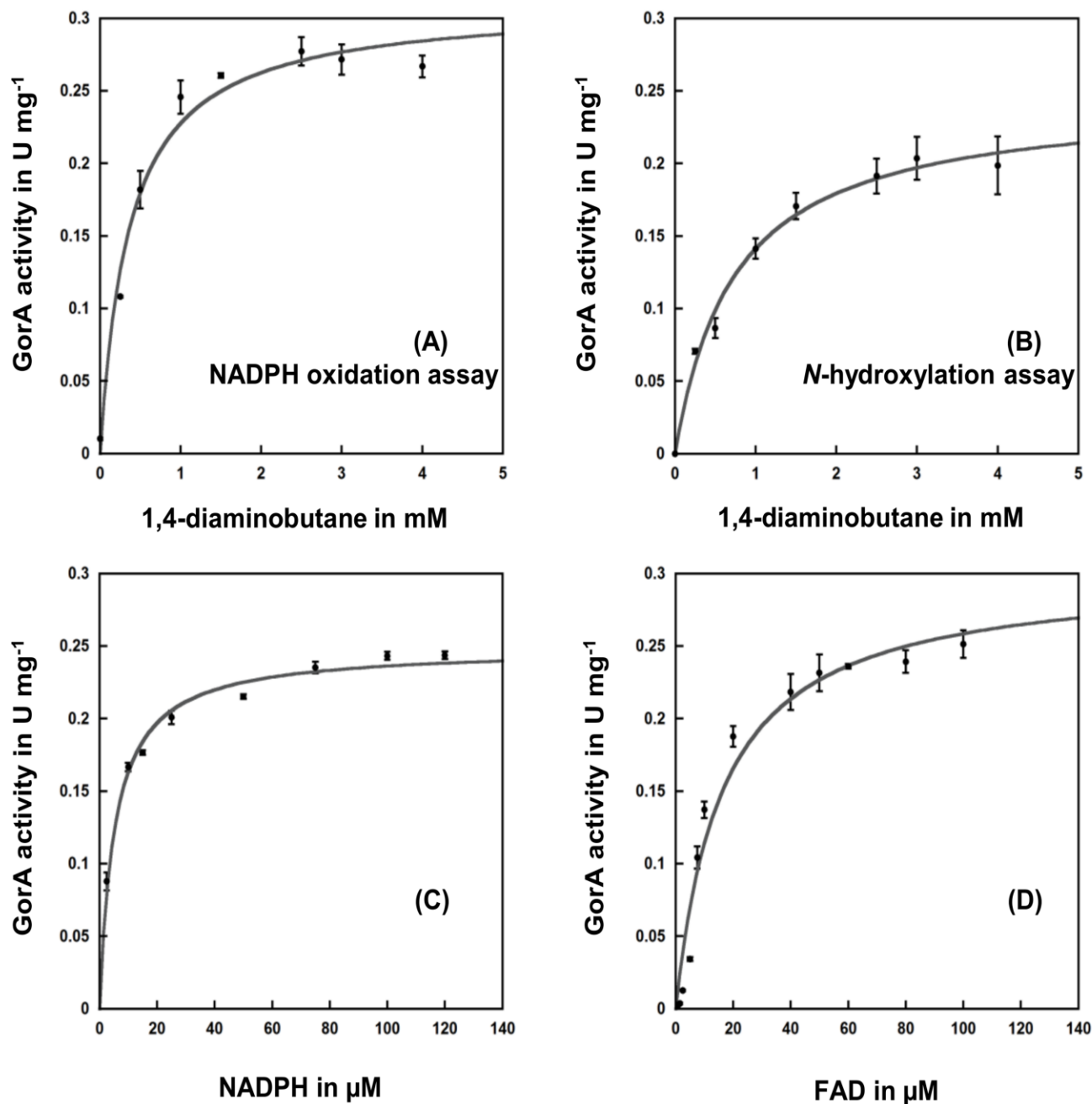


Figure 3.7: Michealis-Menten kinetics parameters of GorA

Figure 3.7 is as follows (A) different concentrations (0 mM - 4 mM) of 1,4-diaminobutane assessed with the NADPH oxidation assay, $V_{\max} = 310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$; $K_m = 361 \pm 0.1 \text{ } \mu\text{M}$ (B) Hydroxylated product formation at different oncentrations of 1,4-diaminobutane (0 mM - 4 mM), $V_{\max} = 246 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$; $K_m = 737 \pm 0.1 \text{ } \mu\text{M}$ (C) NADPH, $V_{\max} = 249 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$; $K_m = 5.2 \pm 0.6 \text{ } \mu\text{M}$ (D) FAD, $V_{\max} = 301 \pm 0.02 \text{ nmol min}^{-1} \text{ mg}^{-1}$; $K_m = 16 \pm 0.01 \text{ } \mu\text{M}$.

Table 3.2: Summary of the GorA kinetic parameters

	NADPH oxidation assay	Hydroxylated product formation assay
V_{max}	$310 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$	$246 \pm 0.01 \text{ nmol min}^{-1} \text{ mg}^{-1}$
K_m	$361 \pm 0.1 \text{ }\mu\text{M}$	$737 \pm 0.1 \text{ }\mu\text{M}$
k_{cat}	0.27 s^{-1}	0.21 s^{-1}
k_{cat}/K_m	$748 \text{ M}^{-1} \text{ s}^{-1}$	$285 \text{ M}^{-1} \text{ s}^{-1}$

Conditions: Reactions were assessed in 100 mM phosphate buffer (pH 8.0) at 24°C

3.5 Discussion

GorA, a *Gordonia rubripertincta* CWB2, *N*-hydroxylating monooxygenase (NMO) or *N*-hydroxylase, specific for a range of diamines (1,4-diamniobutane > cadaverine > hexamethylenediamine) has been identified from the microbial metagenomic sequence database in our group. GorA shares a high sequence similarity with some known but uncharacterised diamine *N*-hydroxylase; RhbE, a putative 1,3-diaminopropane monooxygenase, from *Sinorhizobium meliloti* 1021, AlcA, from *Bordetella bronchiseptica* RB50, and DesB, from *Streptomyces scabiei* 87-22. To the best of our knowledge, these diamine NMOs are yet to be characterized, hence, GorA represents the first characterized diamine NMO from the *G. rubripertincta* CBW2.

GorA shares sequence similarity with the well characterized *Pa* PvdA, an *L*-ornithine hydroxylase from *Pseudomonas aeruginosa* (Ge and Seah 2006). Phylogenetic analysis data has a direct implication for the functional predictions of previously characterized NMOs (Stehr et al. 1998; Meneely and Lamb 2007; Romero et al. 2012), thus GorA predictions from the phylogenetic tree as a putative diamine *N*-hydroxylase were useful, and showed GorA immediately with a functional annotation towards diamines. Additionally, the conserved amino acid sequence motif of NMOs is important for their identification from the metagenome and their substrate specificity predictions. The proline amino acid sequence necessary for the identification of NMOs was identified in GorA (Fig. 3.3).

This proline has been identified as the amino acid sequence conserved in the FAD binding motif for the identification of NMO among the other sub-classes of the Class B flavoprotein monooxygenases (Stehr et al. 1998). Surprisingly, the conserved hydrophobic amino acid sequence Leucine (L), common to the well characterized PvdA and IucD, has been replaced by another hydrophobic amino acid sequence, Alanine (A), in the substrate binding motif of GorA; this might be responsible for its affinity for diamines.

Purification of NMOs as soluble proteins have been possible using the N-terminal Histidine tag, (Seth et al. 1998; Ge and Seah 2006; Meneely and Lamb 2007). This study demonstrated that GorA is an FAD and NADPH dependent soluble protein when purified using the N-terminal Histidine tag. Substrate inhibition is observed for both NADPH oxidation assay and hydroxylation assay at 1,4-diaminobutane concentrations above 5 mM. IucD exhibited substrate inhibition similarly at high concentrations of *L*-lysine of over 5 mM (Macheroux et al. 1993). For PvdA, inhibition at the same *L*-ornithine concentration was only observed in the hydroxylation assay (Meneely and Lamb 2007), with the NADPH assay for PvdA, the initial velocities increased with increasing *L*-ornithine concentrations.

GorA yielded a turnover number of 0.27 s^{-1} with the NADPH assay and 0.21 s^{-1} with the hydroxylation assay at 24°C (Table 3.2), which is faster than what is reported for PvdA, 0.43 s^{-1} (Meneely and Lamb 2007). NMOs hydroxylate only the amino group of the side chain of their specific substrates. GorA seems to be specific for the diamines; diaminopropane, putrescine, cadaverine and hexamethylenediamine. On the other hand, *L*-lysine, *L*-ornithine and other amines were not hydroxylated by GorA. Conversely, 1,4-diaminobutane was not hydroxylated by PvdA (Meneely and Lamb 2007). GorA formed hydroxylated product with NADH as cosubstrate,

although in the NADPH oxidation assay, NADH was not comparable with NADPH as a suitable cosubstrate for GorA.

3.6 Conclusions

GorA seems to appear as the first reported NMO from the *Gordonia* spp. with a wide range acceptance for diamines. More importantly its characterization seems to be the first among the known diamine monooxygenases. NMOs have a broad range of biotechnological applications. They are being tapped as novel drug targets (Romero et al. 2012). Nowadays, NMOs are sought after as metallophore compounds for bio-friendly and safe metal-extraction.

3.7 Acknowledgements

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CHAPTER 4

Baeyer-Villiger Monooxygenases (BVMOs): Classification And Application In Biocatalysis

Abstract

BVMOs are external flavoprotein monooxygenases. They are industrially important due to their specificity and selectivity i.e regio- and enatio- selectivity for biotransformation processes. The Baeyer-Villiger oxidation was first reported in 1899, by the discovery of Adolf von Baeyer and Victor Villiger. They reported the chemical conversion of ketones or cyclic ketones into esters or lactones, respectively. BVMOs represent the biological analog and also perform Baeyer- Villiger oxidation by means of a FAD-peroxy intermediate. They were found among bacteria and fungi but have not been found in higher organisms so far. Through database search, genome mining, gene cloning and expressions studies; a number of novel BVMOs were identified. Interestingly, BVMOs serve as a kind for vehicle of reactive FAD-peroxy species, allowing numerous reactions. Besides Baeyer-Villiger oxidation, BVMOs carry out for example oxygenation of aldehydes to acids and N- or S-atom oxygenations. There is a need to increase the application of these enzymes for the synthesis of fine chemicals. However, a better understanding of the different types of BVMOs currently available, including the scope of substrates and the source microorganisms, is very important to enhance the specific application of BVMOs. Hence, some of the known Type I BVMOs and their biocatalytic potentials are presented.

Keywords: BVMOs, Biocatalysis, Flavoprotein monooxygenases, Organic substrates, Fine chemicals, Biofertilizers, Green chemistry

4.1 Introduction

The Baeyer-Villiger Monooxygenases (BVMOs) are oxidative flavoprotein monooxygenases. They are important biocatalysts for industry and academia due to their specificity and selectivity i.e regio- and enatio- selectivity for biotransformation processes (van Berkel et al. 2006; Secundo et al. 2011; Riebel et al. 2013). They carry out their catalytic processes by the presence of a prosthetic flavin cofactor and oxygen atom transfer, using nicotinamide adenine dinucleotide and/or nicotinamide adenine dinucleotide phosphate (NAD(P)H) as electron donors (van Berkel et al. 2006; Secundo et al. 2011; Riebel et al. 2013). Baeyer-Villiger oxidation was first reported by the discovery of Adolf von Baeyer and Victor Villiger on the chemical conversion of ketones or cyclic ketones into esters (Baeyer and Villiger 1899). The microbial BVMOs degradation of steroid by fungi were indicated by Turfitt (1948) and Fried et al. (1953). Subsequently, the demand for microbial BVMOs for research and industrial applications has been growing. This offers an eco-friendly advantage over chemically synthesized BVMOs (Turfitt 1948). The search for novel microbial BVMOs has been by screening of metagenome or use site directed mutagenesis to modify the available BVMOs (Riebel et al. 2013).

Description of each of the flavoprotein has been discussed elsewhere (van Berkel et al. 2006; Hille et al. 2013). A detailed highlight of the various types of BVMOs with their source microorganisms, as well as the details of the types of organic substrates they catalyze, is necessary for improvement and research work of BVMOs. Thus, this review summarizes the extent and scope of BVMOs.

4.2 Chemical and biological Baeyer-Villiger oxidation

Baeyer-Villiger oxidation for biocatalytical processes involves the use of both a chemical approach and a biological approach (Kamerbeek et al. 2003). The chemical approach involves

the use of chemical reagents and solvents, including peracids such as 3-chloroperoxybenzoic acid and peroxotrifluoroacetic acid. This is a great disadvantage due to the shock sensitivity and explosive character of peracids in large scale reactions and the subsequent increase in the potential risk of accidents; in addition, halogenated reagents and solvents are environmentally unfriendly. The best method that overcomes all these difficulties is the biological approach for biocatalytical conversions of organic substrates (Fig. 4.1). It involves the use of biological enzymes from microbes, plants and animals at relatively mild conditions. Microbial enzymes are easily biodegradable and have been employed over the years for industrial biocatalysis they are preferred to the other sources (Kamerbeek et al. 2003).

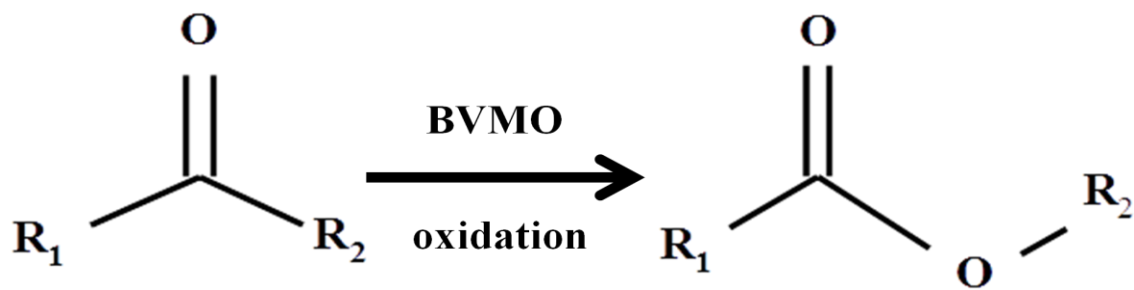


Figure 4.1: Scheme of carbonyl compounds oxidation by BVMOs (de Gonzalo et al. 2010; Ceccoli et al. 2014)

4.3 Classification of flavoprotein monooxygenases and BVMOs

As mentioned earlier, BVMOs are a class of flavin-dependent monooxygenases, which are classified based on the type of their cofactors. Monooxygenases are classified into six different families, namely heme-dependent monooxygenases, flavin-dependent monooxygenases, copper-dependent monooxygenase, non-heme-iron-dependent monooxygenases, pterin-dependent monooxygenases, other cofactor-dependent monooxygenases, and cofactor-independent monooxygenases (van Berkel et al. 2006). The flavin dependent monooxygenases or flavoprotein monooxygenases use flavin as their cofactor and NAD(P)H as electron donor (Riebel et al.

2013). Based on similarity in amino acid sequence and structural homology, six subclasses of flavoprotein monooxygenases were identified (de Gonzalo et al. 2010). They are class A, B, C, D, E and F flavoprotein monooxygenases. All members of flavoprotein monooxygenases use a flavin called FAD, while only class C monooxygenases use FMN (van Berkel et al. 2006).

All of the classes are reported to also use NADH and/or NADPH as electron donor (Torres Pazmiño et al. 2010; Torres Pazmiño et al. 2009; van Berkel et al. 2006). Of all these classes of flavoprotein monooxygenases; Baeyer-Villiger reactions were reported only in class B flavoprotein monooxygenases (van Berkel et al. 2006) and class C flavoprotein monooxygenases, although class A flavoprotein monooxygenases were also discovered to catalyze Baeyer-Villiger oxidation, but little is known about their catalytic potential and mode of action (Gibson et al. 2005).

4.4 Types of BVMOs

BVMOs are classified into two different major groups. They are Type I BVMOs and Type II BVMOs (van Berkel et al. 2006; Riebel et al. 2013). However, other sources of BVMOs without particular groups have been discovered. In class A flavoprotein monooxygenases and some other alternative BVMOs have been reported (van Beilen et al. 2003; van Berkel et al. 2006). The Type I BVMOs are characterized based on their biochemical properties (Kamerbeek et al. 2001; van Berkel et al. 2006). They belong to class B flavoprotein monooxygenases. They are intracellular and use flavin adenine dinucleotide as cofactor. They also use NAD(P)H as source of electrons and consist of identical subunits. They are differentiated by a strictly conserved sequence motif (FXGXXXHXXXW(P/D)). This helps to identify new BVMO sequences from metagenomes and also differentiate them from the other class B flavoprotein monooxygenases i.e flavin containing monooxygenases (FMOs) and *N*-hydroxylating monooxygenases (NMOs)

(Kamerbeek et al. 2001; van Berkel et al. 2006; Riebel et al. 2013). However, very interestingly, with a careful study, Riebel et al. (2013) discovered a new subclass of Class B flavoprotein monooxygenases that utilize both NAD(P)H as electron donors. They denoted this new class as Type II FMOs (Riebel et al. 2013).

Type II BVMOs on the other hand belong to class C flavoprotein monooxygenases. They are typified by flavin mononucleotide (FMN) as cofactor, they use NADH as electron donor and are composed of $\alpha_2\beta$ trimers. Kamerbeek et al. (2001) noted that all characterized BVMOs are of Type I origin and they are part of a superfamily of sequence-related flavin dependent monooxygenase enzymes. They also noted that since no Type II BVMOs sequences are known, it will be very interesting and worthwhile to explore the natural diversity for novel BVMOs (Kamerbeek et al. 2001).

Kamerbeek et al. (2003) indicated the existence of small alternative BVMOs which do not belong to either Type I or Type II BVMOs, nonetheless, they are capable of carrying out Baeyer-Villiger biocatalysis. Examples include pig enzyme, NADH and FMN dependent luciferases from light emitting bacterium *Vibrio fischeri* (Eckstein et al. 1993); these are reported to resemble the few characterized Type II BVMOs (Chen et al. 1988). However, the first Type II BVMOs gene sequence to be obtained is expected to reveal this clearly (Kamerbeek et al. 2003).

4.5 Some BVMOs and their respective organisms

In 1948, the biological BVMOs synthesis was reported (Turfitt 1948). It was the transformation of steroids by fungi. Since then BV oxidation pathway has been found in different organisms. For instance, aflatoxin synthesis in fungi, irridoids and steroids in plants, and toxins in shellfish (Damtoft et al. 1995; Vannelli et al. 2002). Baeyer-Villiger oxidation has also been frequently observed in microbial degradation pathways. BVMOs are abundant in bacteria and

fungi, but are few in eukaryotes (Kamerbeek et al. 2003). Efforts are now geared toward identification of novel BVMOs from metagenomes. Thus, a number of novel BVMOs genes were identified and sequenced (Riebel et al. 2013).

Riebel et al. (2013) reported that all known novel BVMOs till date are Type I. Kamerbeek et al. (2003) reported the use of the BVMO-specific fingerprint to search for novel Type I BVMOs in organisms via the PEDANT database (<http://pedant.gsf.de>). They reported that Type I BVMOs are present in approximately 15% of all sequenced microbial genomes. They discovered that there is no occurrence of Type I BVMOs in *Escherichia coli* and *Saccharomyces cerevisiae*. This in turn has made both organisms interesting as host for protein expression of BVMOs. Also, no Type I BVMO has been reported in Archaea genome sequences. This was due to the fact that Archaea live in extreme anaerobic environments (Kamerbeek et al. 2003).

In addition, no BVMOs are reported in plant or human genomes (Kamerbeek et al. 2003). Mycobacterium has been reported to catalyze a variety of Baeyer-Villiger reactions (Van Beilen et al. 2003). In eukaryotes, the fungus *Aspergillus parasiticus* has been observed for biosynthesis of aflatoxin. Kamerbeek et al. (2003) reported that a set of 68 putative microbial BVMO sequences were obtained from PHI-BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>). These consist of uncharacterized putative BVMO genes, thus indicating that a large pool of unexplored BVMOs are still very much available for biocatalytic explorations. Riebel et al. (2013) noted that the bacterium *Rhodococcus jostii* RHA1 is of particularly interesting genome, with a lot of novel BVMOs found in its genome.

4.6 Range of some BVMOs and their organic substrates

As mentioned earlier, Adolf von Baeyer and Victor Villiger first discovered the conversion of ketones into esters or cyclic ketones into lactones using toxic chemicals mentioned above (Baeyer and Villiger 1899). Thus BVMOs were reported as interesting tools for the synthesis of lactones and esters. Interestingly, in 1948, the microbial conversion of steroids by steroid monooxygenases (SMOs) from fungi was reported (Turfitt 1948).

The second generation of self-sufficient BVMOs include other SMOs, phenylacetone monooxygenase (PAMO) (Fraaije et al. 2005), cyclohexanone monooxygenase (CHMO) (Donoghue et al. 1976), cyclopentanone monooxygenases (CPMO) (Turfitt 1948; Iwaki et al. 2002; Jensen et al. 2012), cyclododecanone monooxygenases (CDMO), 4-Hydroxyacetophenone monooxygenases (HAPMO) (Kamerbeek et al. 2001), and ethionamide monooxygenases (EtaA) (Prado et al. 1999; Vannelli et al. 2002; Gibson et al. 2005). These already known BVMOs are used as probes to find new BVMOs (Kamerbeek et al. 2003). Twenty SMOs performing BV reactions from *Cylindrocarpon radiocicola* and *Rhodococcus rhodochrous* were purified and characterized (Itagaki 1986; Miyamoto et al. 1995). It is worthy to note again that CHMO from *Acinetobacter* is the most studied BVMO but is very unstable (Kamerbeek et al. 2003). Although PAMO from *Thermobifida fusca* is thermostable and for a long time it was the only BVMO with a known crystal structure. However its substrate scope is limited to small aromatic substances (Fraaije et al. 2004; Secundo et al. 2011).

Substrate profiling allows determination of the functional role of novel BVMOs. Model substrates like phenylacetone and bicyclo [3.2.0]hept-2-en-6-one are used to validate the results obtained from substrate profiling (Torres Pazmiño et al. 2009). Promiscuity in substrate specificity has also been observed. This is the case where BVMOs evolve to catalyze compounds

unrelated to their physiological substrate (Kamerbeek et al. 2003). Riebel et al. (2013) tested 39 different substrates to validate the biocatalytic potential of rhodococcal BVMOs.

The substrates they used were grouped into linear aliphatic ketones, cyclic aliphatic ketones, aromatic ketones, aromatic amine and aromatic sulfides; BVMO number 3 and number 8 were found to be acting as oxygenase or NADPH oxidases, a dual functional role in *Rhodococcus jostii* RHA1. They also reported that FMO E, FMO F, and FMO G catalyzed the conversion of phenylacetone and bicyclo [3.2.0]hept-2-en-6-one. Phenylacetone was converted into ester and benzylacetate. Jensen et al. (2012) discovered a novel FMO X which performs sulfoxidation and BV oxidation. Type I BVMO activity was identified in the genome of *Aspergillus fumigatus* Af293 (Mascotti et al. 2013). The BVMO activity of BVMO_{Af1} was confirmed using a model substrate rac-bicyclo (3.2.0)hept-2-en-6-one and other possible substrates, which included linear, cyclic, bicyclic and aromatic ketones, steroids, aromatic prochiral sulfides and diketones (Mascotti et al. 2013). Thus, the fungus is very interesting model to understand the genetic and molecular basis of the diversity of these enzymes within eukaryotic genomes (Mascotti et al. 2013).

4.7 Conclusions

The Baeyer-Villiger Monooxygenases (BVMOs) are important for various biocatalytic applications (Mihovilovic et al. 2002; Mihovilovic et al. 2006; van Berkel et al. 2006; de Gonzalo et al. 2010; Leisch et al. 2011; Jensen et al. 2012; Riebel et al. 2013). Microorganisms have been found to use BVMOs in order to grow on toxic organic compounds. These include aliphatic methyl ketones, alicyclic hydrocarbons, aromatic compounds and terpenes. To overcome limitations in BVMO based biocatalysis, enzyme discovery and engineering studies to generate a large collection of BVMOs that show good operational stability while covering a wide

varieties of organic compound is necessary (Riebel et al. 2013). Thus to increase the number of available and distinct BVMOs the natural diversity can be tapped. A detailed awareness of the available BVMOs types including their various source microorganisms and biocatalytic potentials is necessary for better use of this notable enzyme.

Chapter 5

Horticultural Crops Development: the Importance of Fine Chemicals

Production from Microbial Enzymes

Abstract

Economically important horticultural crops worldwide are being faced with the challenges of diseases, pests and changing climatic conditions. Research efforts to solve these problems are focused on plant breeding and genetic engineering to create new hybrids, and pest management through the use of synthetic agrochemicals. Horticultural crops development using fine chemicals produced from microbial enzymes for production of non-toxic agrochemicals and other bioproducts need also to be considered as an alternative to synthetic agrochemicals. To this end, some of the microbial enzymes presently used for the production of fine chemicals that are very useful as building blocks for agroactive products are discussed here. Thus, this paper aims to create awareness of the need to extend the application of these microbial fine chemicals to horticulture for safe and environmentally friendly bioprocesses and crop development.

Keywords: Microbial biopesticides, fluorophenols, 4-hydroxyacetophenone monooxygenase (HAPMO), desferrioxamine E siderophore, vegetables

5.1 Introduction

Microbes (bacteria, fungi, archaea, viruses etc.) play important functions in all forms of life. Microbes have been known to show a beneficial relationship with plants, and not all microbes cause plant diseases. They assist in the biodegradation of toxic compounds and disease suppression (Ali Siddiqui and Ehteshamul-Haque, 2001). They are also involved in providing important nutrients such as fixing of atmospheric nitrogen and providing phosphorus, potassium, sulfur, iron and trace elements, all of which are necessary for plant growth. More interestingly,

some microbes are particularly important for biocatalysis to produce fine chemicals that are very useful for horticultural crops development. This is possible through the use of their enzymes as biocatalysts for the production of environmentally friendly agroactive products. Thus, microbes play a significant role in agricultural development.

Horticultural crops including vegetables, fruits and ornamentals supply nutrients and provide income for people. Remarkable progress in research has been made in the stable introduction of foreign genes into plants through genetic engineering. Since the introduction of the first transgenic antibiotic resistant tobacco, horticultural crops such as tomato, lettuce, carrot, alfalfa, cucumber, papaya, and banana have been engineered against pests, diseases, insects, and for herbicide resistance (Dunwell, 2000).

More importantly, biosafety issues are being considered for acceptance of genetically modified foods, especially for countries where malnutrition is prevalent. Plant breeding studies for new varieties of crops with desired traits are also being done. Pesticides and fertilizers have been of great help to horticultural crop production and reduction of food losses (Tanaka and Omura, 1993).

Despite the intense concern in public health on the safe use of pesticides and fertilizers especially for growing vegetables, there is also the dilemma of how to tackle crop pests and diseases. Some of the synthetic insecticides like organophosphorus, carbamates as well as fungicides like acylanines, triazoles, benzimidazoles, and dicarboximides are showing decreased efficacies. Thus in order to improve crop productivity, there is a need to pay attention to the production of biopesticides, biofertilisers and other useful novel agroactive products through the use of microbial enzymes.

5.2 Microbial fine chemicals

These are low molecular weight compounds that are building blocks for larger, often complex compounds. They include antibiotics, vaccines, bioflavors, biopigments, vitamins, amino acids, nucleotides, steroids, alkaloids, targeted toxins, enzymes, enzyme inhibitors, bio-plastics, biopolysaccharides etc. (Gavrilescu and Chisti, 2005). Fine chemicals are produced either by chemical means using substrates from petroleum, or they are produced by biological means (biocatalysis). Fine chemicals are referred to as microbial fine chemicals when they are of microbial origin, since microbes produce both primary and secondary metabolites that are useful in the food, agrochemical and pharmaceutical industries (Vandamme, 1994).

Microbial fine chemicals are now being utilized as potential biopesticides, biofertilizers and biocontrol agents. They are better suited to the environment than chemical synthetic pesticides and fertilizers. Biopesticides, biofertilizers and biocontrol agents are expected to overcome the problems of environmental pollution caused by synthetic products. Unfortunately, a very few compounds from microbial metabolites have been screened and used for the synthesis of agroactive products (Vandamme, 1994; Tanaka and Omura, 1993). This shows the importance to harness research to discover new fine chemicals for the agricultural industry. Further, if such compounds and producing microorganisms are identified, the production needs to be optimized to have valuable mass/time yields.

5.3 Biopesticides, biofertilizers and biocontrol agents

Microbial pesticides are expanding to include newly developed biopesticides such as avermectin, milbemycin and bialaphos (Tanaka and Omura 1993). Earlier reported biopesticides include bastacidin S, polyoxin, kasugamycin, and validamycin. Avermectin is used as an acaricide in horticulture and as an anti-arthropod agent for both crop and non-crop applications

(Lasota and Dybas 1991). A number of these pesticides and their mode of action has been reviewed (Tanaka and Omura 1993). They have been discovered by fermentation, screening techniques and chemical modification of commercial microbial metabolites (Tsuzuki et al. 1991). *Streptomyces* spp. represent a major microbial source for biopesticides (Tanaka and Omura 1993). *Bacillus popilliae* and *Bacillus thuringensis* (Bt) have long been used as biopesticides (Gavrilescu and Chisti, 2005; Tanaka and Omura, 1993). Other microbial sources include rare Actinomycetes like *Kitasatospora*, Basidiomycetes and filamentous fungi such as *Oudemansiella radicata*, *Aleurodiscus mirabilis*, *Strobilrus* sp. etc..

Biofertilizers and soil inoculants are safe and inexpensive alternatives to chemical fertilizers. The nitrogen fixing rhizobial bacteria found naturally in the root nodules of leguminous crops are the first generation of biofertilizers. Other microbes that are used as biofertilizers include *Mycorrhizae*, *Azospirillum* sp., *Penicillium bilaii*, cyanobacteria etc. (Gavrilescu and Chisti, 2005). Various biocontrol agents of economic importance have been reviewed. They include *Ampelomyces quisqualis* against powdery mildew in crops such as curcubits, grapes, ornamentals, strawberries, tomatoes; and *Trichoderma harzianum* against wilt and root rot pathogens of fruits, vegetables, flowers and turf (Gavrilescu and Chisti, 2005). Widespread fungal diseases of horticultural importance like grape downy mildew caused by *Plasmopara viticola*, apple scab caused by *Venturia inaequalis* and *Erwinia amylovora*, tomato bacterial wilt caused by *Pseudomonas solanacerum* and many virulent horticultural crop pathogens need to be urgently addressed.

5.4 Novel microbial enzyme for fine chemicals production

5.4.1 Importance of 4-hydroxyacetophenone monooxygenase (HAPMO) for the production of fluorophenol in an environmentally friendly manner

Fluorophenols are important pesticides used in agriculture. The chemical synthesis of fluorophenols involved the use of expensive toxic compounds like peracids ($\text{ROO}^\cdot$) [Fig. 5.1A]. The bioprocess of this pesticide using HAPMO isolated from *Pseudomonas fluorescens* ACB is the best alternative (Fig. 5.1B) (Kamerbeek et al. 2001). HAPMOs are Baeyer-Villiger monooxygenases (BVMOs), members of the flavoprotein monooxygenase family, which are frequently found in some Actinomycetes and filamentous fungi. BVMOs enable their respective microbes to catalyze a variety of organic substances to yield fine chemicals that are building blocks for industries.

HAPMO involves a regioselective biotransformation of fluorobenzaldehydes to yield fluorophenyl formates which undergo a spontaneous hydrolysis to fluorophenols in an environmentally friendly manner. This enzymatic approach gives a high yield and it is selective towards the sustainable production of fluorophenols. The purification, characterization and heterologous expression of HAPMO from *Pseudomonas fluorescens* ACB has been reported (Kamerbeek et al. 2001). This helps to understand the catalytic potentials of HAPMO and its industrial uses.

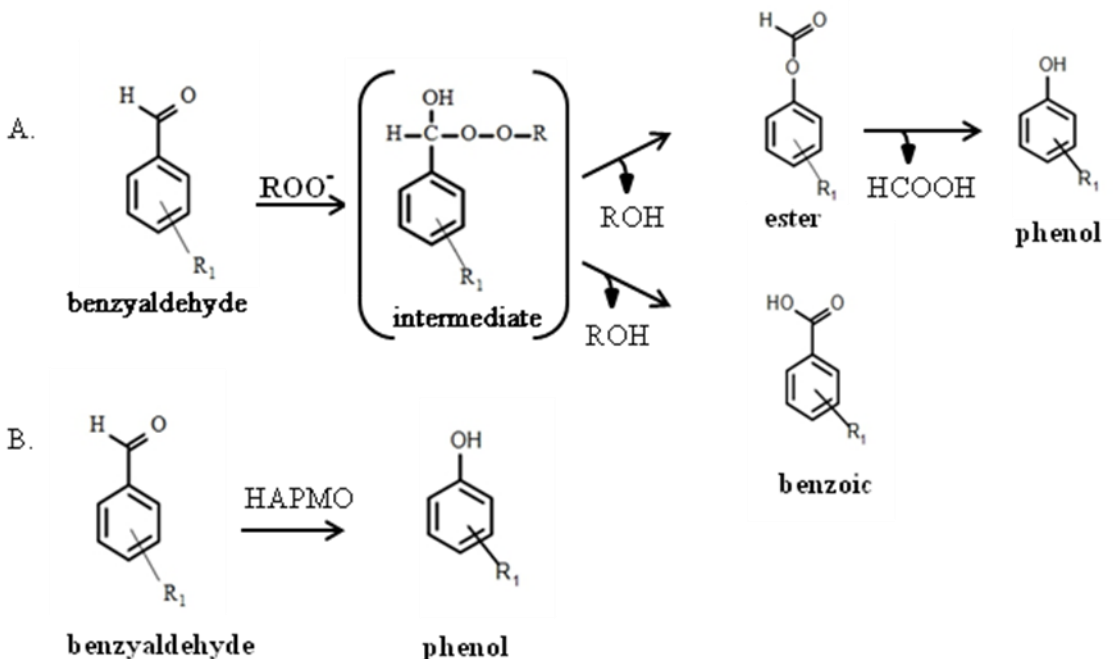


Figure 5.1: Schematic diagram showing Baeyer-Villiger oxidation of benzaldehyde. A. chemical oxidation and B. Enzymatic Baeyer-Villiger oxidation using HAPMO as the biocatalyst

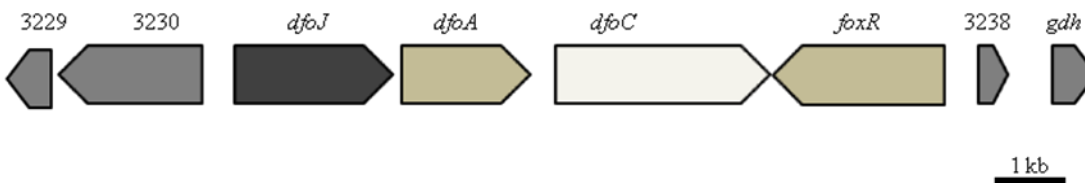


Figure 5.2: A schematic diagram for the proposed biosynthetic cluster genes of desferrioxamine E (DFO-E), the virulence factor produced by *Erwinia amylovora* CFBP 1430, for causing fire blight disease in apple, pear, and other rosaceous plants. The three cluster genes *dfoJ*, *dfoA*, and *dfoC* are involved in the biosynthesis of DFO-E, while *foxR* is a putative Ton-dependent ferrioxamine receptor

5.5 Importance of enzyme inhibitors

Some phytopathogens produce iron chelators called siderophores that act as virulent factors and enable the pathogens to cause diseases in their host plants. Enzymes involved in siderophore biosynthesis are potential drug targets. An example of such phytopathogens is the enterobacterium *Erwinia amylovora* the causative agent of fire blight, a devastating necrotic

disease in pome fruit i.e. apple, pear and quince; and a number of ornamental, landscape and forest *Rosaceae* species (Smits and Duffy 2011). The pathogen has been reported to play an important economic role as a major global threat to the sustainable production, ecological damage, and trade of these important horticultural crops (Smits et al. 2010; Smits and Duffy 2011).

E. amylovora produces Desferrioxamine E hydroxamate siderophore (DFO-E) and its biosynthetic pathway had been proposed (Fig. 5.3) (Feistner 1995; Kachadourian et al. 1996). The genes involved in the biosynthesis of DFO-E in *E. amylovora* CFBP 1430 are clustered as follows *dfoJ*, *dfoA* and *dfoC* (Fig. 5.2) (Smits et al. 2010). DfoJ is the putative lysine/ornithine decarboxylase that converts *L*-lysine into cadaverine, DfoA is the putative *L*-lysine N^6 -monooxygenase that converts cadaverine into 1-amino-5-(*N*-hydroxy)-aminopentane, while DfoC enzyme catalyzes the last step in the conversion of 1-amino-5-(*N*-hydroxy)-aminopentane and other substrates to form DFO-E (Fig. 5.3) (Smits and Duffy 2011).

Feistner (1995) also proposed a theoretical alternative biosynthetic pathway for DFO-E, here *dfoA* gene was proposed as the first key enzyme in the biosynthesis of DFO-E. It catalyzes the hydroxylation of the terminal amino group of *L*-lysine into 5-(*N*-hydroxy)-lysine, as shown in Figure 5.3.

Dellagi et al. (1998) and co-workers studied the role of a gene knockout of this *dfoA* in *E. amylovora* CFBP 1430. Their results revealed a low level of virulence of the pathogen in causing disease in apple trees, as the pathogen could not produce DFO-E. Hence, this strongly indicates that research efforts to develop inhibitors of the *dfoA* gene are important for controlling the pathogen. Interestingly, *dfoA* gene encodes for a *N*-hydroxylating flavoprotein which belongs to the same group of flavoproteins as HAPMO (Huijbers et al. 2014; Smits and Duffy 2011).

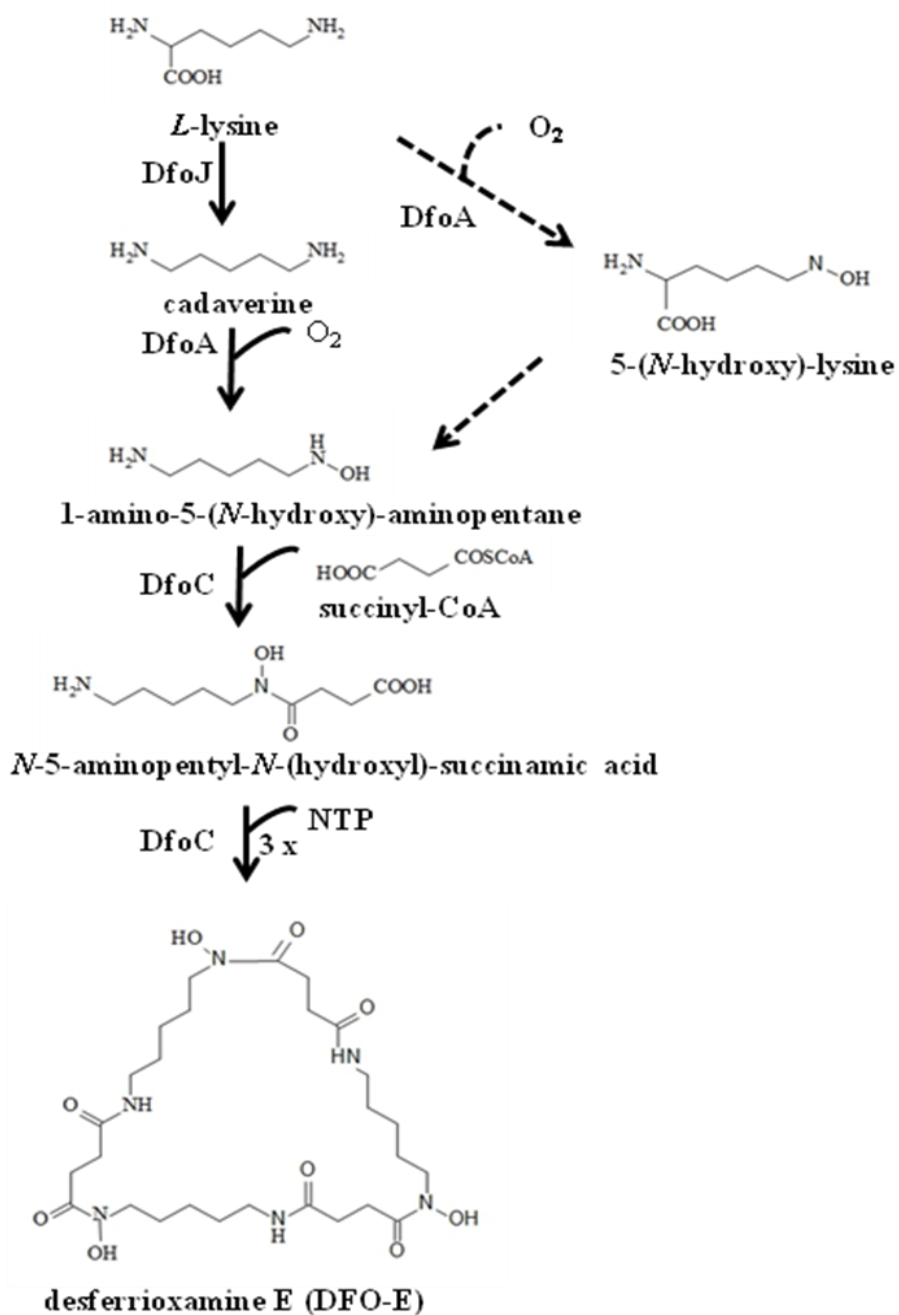


Figure 5.3: A schematic diagram of the proposed biosynthetic pathway for desferrioxamine E, the virulence factor produced by *E. amylovora* CFBP 1430, for causing fire blight disease in apple, pear and other rosaceous plants (left) and the theoretical alternative pathway (right)

5.6 Conclusions

The clear realizations that most chemical products are toxic, environmentally unsafe, wasteful, and non-degradeable are making chemical industries change their current working practices in favor of greener alternatives (Gavrilescu and Chisti 2005). Discovery of new fine chemicals have been made possible in the past through improvement in fermentation and screening techniques. More importantly nowadays is the wealth of novel products that are generated through microbial functional genomics studies, although the results of most of these studies have been used for the production of important pharmaceutical and material compounds. Research efforts to increase the application of microbial fine chemicals for the horticultural industry are very crucial. This will enlarge the sets of novel enzymes especially from the microbial metagenomes for production of fine chemicals for the horticultural and other agro-allied industries.

Horticultural crops, especially vegetables and fruits, are mostly eaten raw. They provide sufficient nutrients, protection, and they maintain the human immune system. It has often been said that “food is drug”. People need to be encouraged to eat more vegetables and fruits which are important to keep one healthy. On the contrary, there is a great worry of eating toxic chemicals along with these foods. Hence, it is important to produce horticultural crops with little or no synthetic chemicals. There is also an urgent concern to have enough food to feed the ever growing world population.

The alternative is to increase the set of microbial fine chemicals for horticultural industries. Bioprocesses involved in the production of pesticides using novel microbial enzymes such as HAPMO are cost-effective, energy saving and environmentally friendly. The genetic determination of targets of pest causing pathways, as in the case of some siderophore producing plant pathogens, becomes important. Research efforts to search for more of these novel enzymes will go a long way to complement the effort of plant breeding, genetic engineering and crop management for the sustainability and development of horticultural crops. Conclusively, we showed two possible flavoproteins important for agro-industries. HAPMO as a productive one and DfoA as a drug target.

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CHAPTER 6

6.1 General discussion

The microbial metagenome represents a wealthy resource for mining novel gene sequences with yet undiscovered functional annotations and potential roles for industrial, pharmaceutical, agricultural, and research studies. Enzymes such as NMOs are greatly sought after because they are useful for catalyzing the majority of their target substrates in a regioselective manner which is impossible or difficult when chemicals are employed.

Recently, enzymes discovery have been possible through genome mining, gene cloning and bioinformatics search using known protein sequences as baits (Giardina et al. 1995; Kang et al. 1996; Iwaki et al. 2002; Jin et al. 2002; Lamont and Martin 2003; Van Beilen et al. 2003; Ge and Seah 2006; Mascotti et al. 2013; Riebel et al. 2013; Riebel et al. 2014). It is possible to search for novel NMOs using the known NMO sequences.

The NCBI server is also useful for BLAST searches and the retrieval of available known protein sequences, other servers available are the UNIPROT, www.uniprot.org; EXPASSY, www.expasy.org; BRENDA, www.brenda-enzyme.org. The protein sequences have been deposited with their respective accession numbers, these allow for their easy identification from the various protein database servers.

The search for novel NMOs has been carried out using the known sequences of different NMOs as baits. These NMOs are grouped into their substrates specificities, the bacterial *L*-ornithine N^5 -monooxygenase *Pa* PvdA isolated from *Pseudomonas aeruginosa*, *Bc* PvdA isolated

from *Burkholderia cepacia*, PsbA isolated from *Pseudomonas* sp. B10, KtzI isolated from *Kutzneria* sp. 744, CchB isolated from *Streptomyces ambofaciens*, the fungal *L*-ornithine N^5 -monooxygenase *Af* SidA (OMO) isolated from *Aspergillus fumigatus*, SidA isolated from *Emericella nidulans*, Sid1 isolated from *Ustilago maydis*, DffA isolated from *Aspergillus oryzae*; the bacterial *L*-lysine N^6 -monooxygenases IucD isolated from *Escherichia coli* and *Shigella flexneri*; VbsO isolated from *Rhizobium etli* CFN 42; RhbE, a putative diamine monooxygenase isolated from *Sinorhizobium meliloti* 1021, AlcA, a putative diamine monooxygenase isolated from *Bordetella bronchiseptica* RB50, DesB, a putative diamine monooxygenase *Streptomyces scabies* 87-22 (Stephan et al. 1993; Visca et al. 1994; Giardina et al. 1995; Sokol et al. 1999; Ambrosi et al. 2000; Challis and Ravel 2000; Hissen et al. 2005; Ge and Seah 2006; Heemstra et al. 2009; Romero et al. 2012).

Therefore, *gorA*, a gene encoding the protein GorA, a diamine monooxygenase has been identified from the genome of the *Gordonia rubripertincta* CWB2. It was successfully identified using the aforementioned known protein sequences of the NMOs. The phylogenetic relationship of GorA to these known NMOs revealed that it clustered along side with the known putative diamine monooxygenases.

The phylogenetic genetic relationship has been used to identify functional annotation of NMOs. For instance, CchB has been proved to show high sequence similarities to previously known NMOs such as PvdA and IucD. The fungal *L*-ornithine N^5 -monooxygenase, *Af* SidA (OMO), also shows sequence similarities to the other known NMOs. GorA appears to be involved in the biosynthesis of a novel hydroxamate siderophore, since it clusters together with known NMOs, RhbE, AlcA, and DesB that are involved in the first step biosynthesis of such hydroxamate group of siderophores as rhizobactin, alcaligin, and desferrioxamine respectively.

The known NMOs have been successfully cloned and over expressed in *E. coli* as the suitable host. They have also been produced easily as active soluble proteins using the N-terminal histidine tag. Most of them have been purified without flavin co-factor and they do not bind flavin tightly, except for *Af* SidA (OMO), the first NMO that has been reported to have been purified with a tightly bound FAD.

From this study, GorA has been cloned and overexpressed using *E-coli* as the suitable host and with N-terminal histidine tag. The FAD-binding motif GXGXGP found in other NMOs is also found in GorA. NMOs are FAD and NADPH dependent. They are substrate specific, for instance, PvdA will hydroxylate only the terminal amino group of its preferred substrate which is *L*-ornithine, it will not hydroxylate any other substrate, the same is true for other NMOs like CchB and KtzI.

However, this study has proved GorA has a preference for a range of diamines. It is able to hydroxylate a range of diamines 1,4-diaminobutane, cadaverine, and hexamethylenediamine into their respective products. This may be due to its preference for the straight chain aliphatic diamines, because it will not catalyze aliphatic diamino acids or monoamines, and this may be due to the formation of hydrogen peroxide instead of hydroxylated product. The same is true for other NMOs, as they form hydrogen peroxide in the presence of their non-preferred substrates. The highest NADPH oxidation activity and hydroxylation activity of GorA is observed with 1,4-diaminobutane.

Similar to the IucD which is inhibited at higher concentrations of its preferred substrate, *L*-lysine, due to the excess binding of the substrate, higher concentrations of 1,4-diaminobutane, cadaverine, and hexamethylenediamine inhibit NADPH-oxidation activity of GorA. The kinetic parameters of GorA are comparable to other characterized NMOs such as IucD, PvdA, CchB,

KtzI etc. The physiological parameters of GorA are similar to those found in the other characterized NMOs, except that unlike most of the other NMOs, GorA is soluble in phosphate buffer.

Bacteria, mycobacteria, and fungi are able to grow on organic substances and aromatic pollutants as carbon sources. They are also able to degrade such toxic compounds and render them less toxic to the environment. This is possible because they are able to synthesize various metabolites as siderophores which are enzymatically directed as well as for scavenging toxic metals from the environment. It is thus pertinent to study the biochemical properties and pathways involved in various siderophores biosynthesis.

Different genes encoding the protein involved in the biosynthesis and transport of different siderophores are being studied and characterized to give attention and insight to the biosynthetic pathways of the siderophores (Visca et al. 1992; Visca et al. 1994). siderophores that enabled bacteria to function as plant growth promoters as well as iron-chelating agents around the plant roots, thereby making such metals unavailable to phytopathogens include pseudobactin produced by *Pseudomonas* sp.

A great number of pathogenic bacteria, fungi, and mycobacteria produce siderophores that are involved in causing diseases in plants, animals, and humans. A critical study and biochemical characterization of the enzymes involved in the initial steps in the synthesis of such siderophores are crucial to new drug target discovery and development to combat the virulent nature of the producing pathogenic bacteria and fungi.

Examples of such siderophores mentioned above include pyoverdine produced by *P. aeruginosa*, an opportunistic human pathogen; pyoverdine contributes to the virulence of this *P. aeruginosa*, aerobactin is produced by *E. coli*, ornibactin from *B cepacia*, mycobactin is from

M. tuberculosis and so on. PvdA deletion mutants are pyoverdine-deficient (Visca et al. 1992; Visca et al. 1994). PvdA is functionally related to IucD, para-hydroxybenzoate hydroxylase (PHBH) isolated from *Pseudomonas fluorescence*, important in the biodegradation of wood-lignin, and flavin-containing monooxygenases (FMOs) involved in xenobiotic detoxification.

All of these siderophores are structurally diverse compounds. Pyoverdine includes derivatives of ornithine in the peptide backbone that serve as direct iron chelators i.e. hydroxyornithine and formyl-hydroxyornithine (Meneely and Lamb 2007). PvdA performs the first enzymatic step in the preparation of these derivatives. The derivatives are nonproteinogenic amino acids involved in iron assembly (Pohlmann and Marahiel 2008).

PvdA is the most studied NMO which requires both FAD and NADPH for its activity. It is a soluble monomer with greater activity at pH 8.0, this is the same situation found in GorA. For the NADPH oxidation assay, the activity of PvdA was not inhibited at higher substrate concentrations, but this was the opposite case found in GorA, its activity was lowered at higher substrate concentrations of all the tested substrates. The activity of PvdA was reported to decrease with increase in *L*-ornithine above 5 mM concentration using the hydroxylation assay, the same is true also for GorA with a lowered activity in the presence of 1,4-diaminobutane.

PvdA has been shown to be substrate specific, the same is true for other studied NMOs, which are generally specific for the substrate they catalyze. The active site of PvdA is specific for its substrate, the peptide groups and the side chain amine are required for catalysis and presumably involved in the binding and positioning of the substrate. NADPH oxidation activity of CchB is enhanced only in the presence of *L*-ornithine; it however forms hydrogen peroxide with *L*-lysine. A possible explanation is that the amino group of the latter substrate is not optimally positioned in

the active site for subsequent hydroxylation by CchB due to the additional carbon atom of its side chain. IucD on the other hand showed a reverse situation: it accepts *L*-lysine and not ornithine, or homolysine (Macheroux et al. 1993). GorA showed substrate specificity to three diamines (1,4-diaminobutane, cadaverine, and hexamethylenediamine) in both NADPH oxidation assay and hydroxylation assay for hydroxylamine product formation. Other NMOs demonstrated substrate specificity only towards a particular substrate and not a range of substrates as observed for GorA.

GorA was unable to convert other tested substrates to their corresponding hydroxylamines but showed a NADPH oxidation with these substrates, indicating the promotion of uncoupling of the reaction of GorA with these unconverted substrates. Uncoupling of *Pa* PvdA was observed in the presence of *L*-lysine and other uncatalyzed substrates, there was production of hydrogen peroxide (Ge and Seah 2006). *L*-lysine acted as a non-substrate effector for *Pa* PvdA (Meneely and Lamb 2007). Non-substrate effectors of IucD and PHPB were also reported (Howell and Massey 1970; Macheroux et al. 1993).

Meneely and Lamb (2007) hypothesized that there was closing of the active site of the PvdA in the presence of *L*-lysine by an induced fit mechanism which allowed for the electron transfer from NADPH to FAD. In other words, *L*-lysine is so different in size that it was difficult for it to be efficiently hydroxylated, leading to uncoupling and hydrogen peroxide formation instead of the expected hydroxylamine product, i.e N^5 hydroxyl-*L*-lysine; this phenomenon was also described for non-substrate effectors of IucD and PHPB.

Steady state analysis activity assays for NMOs are assessed by the quantification of hydroxylated product formation using the Csáky iodine oxidation assay. MbsG, an NMO isolated from *Mycobacterium segmatis*, was assessed for its activity in the presence of *L*-lysine at

100 mM phosphate pH 7.2 at 25°C (Robinson et al. 2014).

Comparative genomics has been used in concluding that biosynthesis of the desferrioxamine E (DFO-E), a hydroxamate siderophore, is ancestral within the genera *Erwinia* and *Pantoea* (Smits and Duffy 2011).

A phylogenetic analysis of gene cluster sequences of the pathogenic *Erwinia* spp compared to those from the *Pantoea* spp. revealed a pairwise distance above 75% for all gene cluster sequences which indicated a common origin for the DFO-E.

6.2 Conclusions

In the face of the ever increasing virulent diseases in the world caused by resistance of different bacteria and other pathogenic microbes to available drugs in the market, pharmaceutical industries are relentlessly searching for novel compounds for drug development to face these challenges. The mining industries are also constantly seeking methods for safe and cost effective biomining of important metals from the environment. Fortunately, microbes produce such metabolites as siderophores which are tools for modern industrial developments.

Iron remains an essential nutritional factor for every living organism. It is an indispensable nutritional factor for sustenance and life survival. In addition, iron is a cofactor for numerous enzymes and proteins, unfortunately the bioavailability of iron is fairly low under physiological conditions. Microbes thus solubilize and acquire iron from their environment by excreting low molecular weight molecules (200-2000 Da) referred to as siderophores to increase the solubility of iron and thus sequestrate this important micronutrient from their host environment.

Microbial *N*-hydroxylating monooxygenases (NMOs) are hydroxamate siderophore producing enzymes. These enzymes belong to the Class B flavoprotein monooxygenases, they are involved in the side chain hydroxylation of their specific substrates such as diamino acids, *L*-ornithine

(*L*-Orn) and *L*-lysine, and the primary aliphatic diamines such as 1,3-diaminopropane, putrescine and into their expected hydroxylamine products. These products are later formylated or acylated, and afterwards incorporated into the siderophore/metallophore moiety complex.

Hence, the siderophore metal complexes are transported back into the microbial cells for later useful purposes. The structures and mechanisms of the *Pa* PvdA, *Af* SidA (OMO), KtzI and MbtG are available, these will allow a better insight to understanding of other NMOs.

GorA, a diamine monooxygenase from *Gordonia rubripertincta* CWB2, specific for a range of diamines (1,4-diamniobutane > cadaverine > hexamethylenediamine) has been successfully cloned, overexpressed and biochemically characterized. Its kinetic parameters and physiological state is similar to other known NMOs. It is able to hydroxylate putrescine to *N*-OH-putrescine, cadaverine to 1-amino-5-(*N*-hydroxy)-aminopentane, and hexamethylenediamine into *N*-OH-hexamethylenediamine.

GorA showed some biochemical properties similar to *Pa* PvdA, IucD, and other NMOs. These include its substrates inhibition at higher substrates concentrations, uncoupling by non-substrate effectors, and its substrate specificity for a range of diamines.

GorA is an important enzyme playing a major role in the biosynthesis of such siderophores/metallophores for industrial mining of metal in a cost effective and environmentally friendly manner.

REFERENCES

- Ahmed E, Holmström SJM (2014) Siderophores in environmental research: roles and applications. *Microbial Biotechnology* 7:196-208 doi:10.1111/1751-7915.12117
- Ali Siddiqui I, Ehteshamul-Haque S (2001) *Plant and Soil* 237:81-89 doi:10.1023/A:1013313103032
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215:403-410 doi:10.1016/s0022-2836(05)80360-2
- Ambrosi C, Leoni L, Putignani L, Orsi N, Visca P (2000) Pseudobactin biogenesis in the plant growth-promoting rhizobacterium *Pseudomonas* strain B10: identification and functional analysis of the L-ornithine N(5)-oxygenase (psbA) gene. *Journal of Bacteriology* 182:6233-6238 doi:10.1128/jb.182.21.6233-6238.2000
- Baeyer A, Villiger V (1899) Einwirkung des caro'schen reagens auf ketone. *Berichte der Deutschen Chemischen Gesellschaft* 32:3625-3633 doi:10.1002/cber.189903203151
- Barona-Gómez F, Lautru S, Francou F-X, Leblond P, Pernodet JL, Challis GL (2006) Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambifaciens* ATCC 23877. *Microbiology* 152:3355-3366 doi:10.1099/mic.0.29161-0
- Baysse C, Vos D de, Naudet Y, Vandermonde A, Ochsner U, Meyer JM, Budzikiewicz H, Schafer M, Fuchs R, Cornelis P (2000) Vanadium interferes with siderophore-mediated iron uptake in *Pseudomonas aeruginosa*. *Microbiology* 146:2425-2434 doi:10.1099/00221287-146-10-2425
- Bignell DRD, Seipke RF, Huguet-Tapia JC, Chambers AH, Parry RJ, Loria R (2010) *Streptomyces scabies* 87-22 contains a coronafacic acid-like biosynthetic cluster that

- contributes to plant-microbe interactions. *Molecular Plant Microbe Interactions* 23:161-175
doi:10.1094/mpmi-23-2-0161
- Bloemberg GV, Lugtenberg BJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current Opinion in Plant Biology* 4:343-350
- Brickman TJ, Hansel JG, Miller MJ, Armstrong SK (1996) Purification, spectroscopic analysis and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. *Biometals* 9:191-203
- Briskot G, Taraz K, Budzikiewicz H (1986) Pyoverdine-type siderophores from *Pseudomonas aeruginosa*. *Zeitschrift für Naturforschung C* 41:497-506
- Broberg A, Menkis A, Vasilaitis R (2006) Kutznerides 1-4, depsipeptides from the actinomycete *Kutzneria* sp. 744 inhabiting mycorrhizal roots of *Picea abies* seedlings. *Journal of Natural Products* 69:97-102 doi:10.1021/nj050378g
- Ceccoli RD, Bianchi DA, Rial DV (2014) Flavoprotein monooxygenases for oxidative biocatalysis: recombinant expression in microbial hosts and applications. *Frontiers in Microbiology* 5:25 doi:10.3389/fmicb.2014.00025
- Challis GL, Ravel J (2000) Coelichelin, a new peptide siderophore encoded by the *Streptomyces coelicolor* genome: structure prediction from the sequence of its non-ribosomal peptide synthetase. *FEMS Microbiology Letters* 187:111-114
- Chen YC, Peoples OP, Walsh CT (1988) *Acinetobacter* cyclohexanone monooxygenase: gene cloning and sequence determination. *Journal of Bacteriology* 170:781-789
- Chocklett SW, Sobrado P (2010) *Aspergillus fumigatus* SidA is a highly specific ornithine hydroxylase with bound flavin cofactor. *Biochemistry* 49:6777-6783 doi:10.1021/bi100291n

- Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Research* 16:10881-10890
- Csáky TZ, Hassel O, Rosenberg T, Turunen E, Tuhkanen A (1948) On the estimation of bound hydroxylamine in biological materials. *Acta Chemica Scandinavica* 2:450-454 doi:10.3891/acta.chem.scand.02-0450
- Damtoft S, Franzyk H, Jensen SR (1995) Biosynthesis of iridoids in *Syringa* and *Fraxinus*: secoiridoid precursors. *Phytochemistry* 40:773-784 doi:http://dx.doi.org/10.1016/0031-9422(95)00211-O
- de Gonzalo G, Mihovilovic MD, Fraaije MW (2010) Recent developments in the application of Baeyer-Villiger monooxygenases as biocatalysts. *ChemBioChem* 11:2208-2231 doi:10.1002/cbic.201000395
- del Olmo A, Caramelo C, SanJose C (2003) Fluorescent complex of pyoverdine with aluminum. *Journal of Inorganic Biochemistry* 97:384-387
- Dellagi A, Brisset MN, Paulin JP, Expert D (1998) Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. *Molecular Plant Microbe Interactions* 11:734-742 doi:10.1094/mpmi.1998.11.8.734
- Donoghue NA, Norris DB, Trudgill PW (1976) The purification and properties of cyclohexanone oxygenase from *Nocardia globerula* CL1 and *Acinetobacter* NCIB 9871. *European Journal of Biochemistry* 63:175-192
- Dunwell JM (2000) Transgenic approaches to crop improvement. *Journal of Experimental Botany* 51:487-496. doi:10.1093/jexbot/51.suppl_1.487

- Eckstein JW, Hastings JW, Ghisla S (1993) Mechanism of bacterial bioluminescence: 4a,5-dihydroflavin analogs as models for luciferase hydroperoxide intermediates and the effect of substituents at the 8-position of flavin on luciferase kinetics. *Biochemistry* 32:404-411
- Eisendle M, Oberegger H, Zadra I, Haas H (2003) The siderophore system is essential for viability of *Aspergillus nidulans*: functional analysis of two genes encoding l-ornithine N 5-monooxygenase (sidA) and a non-ribosomal peptide synthetase (sidC). *Molecular Microbiology* 49:359-375 doi:10.1046/j.1365-2958.2003.03586.x
- Feistner G (1995) Proferrioxamine synthesis in *Erwinia amylovora* in response to precursor or hydroxylysine feeding: Metabolic profiling with liquid chromatography-electrospray mass spectrometry. *Biometals* 8:318-327 doi:10.1007/BF00141605
- Fraaije MW, Kamerbeek NM, Heidekamp AJ, Fortin R, Janssen DB (2004) The prodrug activator EtaA from *Mycobacterium tuberculosis* is a Baeyer-Villiger monooxygenase. *The Journal of Biological Chemistry* 279:3354-3360 doi:10.1074/jbc.M307770200
- Fraaije MW, Wu J, Heuts DP, van Hellemond EW, Spelberg JH, Janssen DB (2005) Discovery of a thermostable Baeyer-Villiger monooxygenase by genome mining. *Applied Microbiology and Biotechnology* 66:393-400 doi:10.1007/s00253-004-1749-5
- Franceschini S, Fedkenheuer M, Vogelaar NJ, Robinson HH, Sobrado P, Mattevi A (2012) Structural insight into the mechanism of oxygen activation and substrate selectivity of flavin-dependent N-hydroxylating monooxygenases. *Biochemistry* 51:7043-7045 doi:10.1021/bi301072w
- Fried J, Thoma RW, Klingsberg A (1953) Oxidation of steroids by micro organisms. III. side chain degradation, ring d-cleavage and dehydrogenation in ring A. *Journal of the American Chemical Society* 75:5764-5765

- Fujimori DG, Hrvatin S, Neumann CS, Strieker M, Marahiel MA, Walsh CT (2007) Cloning and characterization of the biosynthetic gene cluster for kutznerides. *Proceedings of the National Academy of Sciences of the United States of America* 104:16498-16503 doi:10.1073/pnas.0708242104
- Gavrilescu M, Chisti Y (2005) Biotechnology-a sustainable alternative for chemical industry. *Biotechnology Advances* 23:471-499 doi:10.1016/j.biotechadv.2005.03.004
- Ge L, Seah SY (2006) Heterologous expression, purification, and characterization of an L-ornithine N(5)-hydroxylase involved in pyoverdine siderophore biosynthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 188:7205-7210 doi:10.1128/JB.00949-06
- Giardina PC, Foster LA, Toth SI, Roe BA, Dyer DW (1995) Identification of *alcA*, a *Bordetella bronchiseptica* gene necessary for alcaligin production. *Gene* 167:133-136 doi:10.1016/0378-1119(95)00659-1
- Gibson M, Nur-e-alam M, Lipata F, Oliveira MA, Rohr J (2005) Characterization of kinetics and products of the Baeyer-Villiger oxygenase MtmOIV, the key enzyme of the biosynthetic pathway toward the natural product anticancer drug mithramycin from *Streptomyces argillaceus*. *Journal of the American Chemical Society* 127:17594-17595 doi:10.1021/ja055750t
- Gillam AH, Lewis AG, Andersen RJ (1981) Quantitative determination of hydroxamic acids. *Analytical Chemistry* 53:841-844 doi:10.1021/ac00229a023
- González V, Santamaria RI, Bustos P, Hernández-González I, Medrano-Soto A, Moreno-Hagelsieb G, Janga SC, Ramirez MA, Jimenez-Jacinto V, Collado-Vides J, Davila G (2006) The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven

- interacting replicons. Proceedings of the National Academy of Sciences of the United States of America 103:3834-3839 doi:10.1073/pnas.0508502103
- Griffiths E (1991) Iron and bacterial virulence- a brief overview. Biology of metals 4:7-13
- Heemstra JR, Jr., Walsh CT, Sattely ES (2009) Enzymatic tailoring of ornithine in the biosynthesis of the Rhizobium cyclic trihydroxamate siderophore vicibactin. Journal of the American Chemical Society 131:15317-15329 doi:10.1021/ja9056008
- Herrero M, de Lorenzo V, Neilands JB (1988) Nucleotide sequence of the iucD gene of the pColV-K30 aerobactin operon and topology of its product studied with phoA and lacZ gene fusions. Journal of Bacteriology 170:56-64
- Hille R, Miller S, Palfey B (2013) Handbook of flavoproteins volume 2 complex flavoproteins, dehydrogenases and physical methods. De Gruyter Berlin pp 436
- Hissen AHT, Wan ANC, Warwas ML, Pinto LJ, Moore MM (2005) The *Aspergillus fumigatus* siderophore biosynthetic gene sidA, encoding L-ornithine N5-oxygenase, is required for virulence. Infection and Immunity 73:5493-5503 doi:10.1128/iai.73.9.5493-5503.2005
- Hof C, Eisfeld K, Welzel K, Antelo L, Foster AJ, Anke H (2007) Ferricrocin synthesis in *Magnaporthe grisea* and its role in pathogenicity in rice. Molecular Plant Pathology 8:163-172 doi:10.1111/j.1364-3703.2007.00380.x
- Hong S, Ryu H, Kim J, Cho K-S (2011) Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp. S2RP-17. Biodegradation 22:593-601 doi:10.1007/s10532-010-9432-2
- Howell LG, Massey V (1970) A non-substrate effector of p-hydroxybenzoate hydroxylase. Biochemical and Biophysical Research Communications 40:887-893

- Huijbers MME, Montersino S, Westphal AH, Tischler D, van Berkel WJH (2014) Flavin dependent monooxygenases. *Archives of Biochemistry and Biophysics* 544:2-17
doi:10.1016/j.abb.2013.12.005
- Itagaki E (1986) Studies on steroid monooxygenase from *Cylindrocarpon radicicola* ATCC 11011. Purification and characterization. *Journal of Biochemistry* 99:815-824
- Iwaki H, Hasegawa Y, Wang S, Kayser MM, Lau PC (2002) Cloning and characterization of a gene cluster involved in cyclopentanol metabolism in *Comamonas* sp. strain NCIMB 9872 and biotransformations effected by *Escherichia coli* expressed cyclopentanone 1,2-monooxygenase. *Applied Environmental Microbiology* 68:5671-5684
- Jacobsen EE, Hoff BH, Anthonsen T (2000) Enantiopure derivatives of 1,2-alkanediols: substrate requirements of lipase B from *Candida antarctica*. *Chirality* 12:654-659
doi:10.1002/1520-636X(2000)12:9<654::AID-CHIR2>3.0.CO;2-3
- Jensen CN, Cartwright J, Ward J, Hart S, Turkenburg JP, Ali ST, Allen MJ, Grogan G (2012) A flavoprotein monooxygenase that catalyses a Baeyer-Villiger reaction and thioether oxidation using NADH as the nicotinamide cofactor. *ChemBioChem* 13:872-878.
doi:10.1002/cbic.201200006
- Jin Q, Yuan Z, Xu J, Wang Y, Shen Y, Lu W, Wang J, Liu H, Yang J, Yang F, Zhang X, Zhang J, Yang G, Wu H, Di Qu, Dong J, Sun L, Xue Y, Zhao A, Gao Y, Zhu J, Kan B, Ding K, Chen S, Cheng H, Yao Z, He B, Chen R, Ma D, Qiang B, Wen Y, Hou Y, Yu J (2002) Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. *Nucleic Acids Research* 30:4432-4441

- Kachadourian R, Dellagi A, Laurent J, Bricard L, Kunesch G, Expert D (1996) Desferrioxamine-dependent iron transport in *Erwinia amylovora* CFBP1430: cloning of the gene encoding the ferrioxamine receptor FoxR. *Biometals* 9:143-150
- Kamerbeek NM, Janssen DB, van Berkel WJH, Fraaije MW (2003) Baeyer-Villiger monooxygenases, an emerging family of flavin-dependent biocatalysts. *Advanced Synthesis and Catalysis* 345:667-678 doi:10.1002/adsc.200303014
- Kamerbeek NM, Moonen MJ, Van Der Ven JG, Van Berkel WJ, Fraaije MW, Janssen DB (2001) 4-Hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB. A novel flavoprotein catalyzing Baeyer-Villiger oxidation of aromatic compounds. *European Journal of Biochemistry* 268:2547-2557
- Kang HY, Brickman TJ, Beaumont FC, Armstrong SK (1996) Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. *Journal of Bacteriology* 178:4877-4884
- Lamont IL, Martin LW (2003) Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*. *Microbiology* 149:833-842 doi:10.1099/mic.0.26085-0
- Lasota JA, Dybas RA (1991) Avermectins, a novel class of compounds: implications for use in arthropod pest control. *Annual Review of Entomology* 36:91-117 doi:10.1146/annurev.en.36.010191.000515
- Leisch H, Morley K, Lau PCK (2011) Baeyer-villiger monooxygenases: more than just green chemistry. *Chemical Reviews* 111:4165-4222 doi:10.1021/cr1003437
- Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, Banerji N, Kanjilal S, Kapur V (2005) The complete genome sequence of *Mycobacterium avium* subspecies paratuberculosis.

Proceedings of the National Academy of Sciences of the United States of America
102:12344-12349 doi:10.1073/pnas.0505662102

Lynch D, O'Brien J, Welch T, Clarke P, Cuiv PO, Crosa JH, O'Connell M (2001) Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. *Journal of Bacteriology* 183:2576-2585 doi:10.1128/JB.183.8.2576-2585.2001

Macheroux P, Plattner HJ, Romaguera A, Diekmann H (1993) FAD and substrate analogs as probes for lysine N6-hydroxylase from *Escherichia coli* EN 222. *European Journal of Biochemistry* 213:995-1002 doi:10.1111/j.1432-1033.1993.tb17846.x

Madigan CA, Cheng T-Y, Layre E, Young DC, McConnell MJ, Debono CA, Murry JP, Wei J-R, Barry CE3, Rodriguez GM, Matsunaga I, Rubin EJ, Moody DB (2012) Lipidomic discovery of deoxysiderophores reveals a revised mycobactin biosynthesis pathway in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* 109:1257-1262 doi:10.1073/pnas.1109958109

Mascotti ML, Juri Ayub M, Dudek H, Sanz MK, Fraaije MW (2013) Cloning, overexpression and biocatalytic exploration of a novel Baeyer-Villiger monooxygenase from *Aspergillus fumigatus* Af293. *AMB Express* 3:33 doi:10.1186/2191-0855-3-33

Mayfield JA, Frederick RE, Streit BR, Wencewicz TA, Ballou DP, DuBois JL (2010) Comprehensive spectroscopic, steady state, and transient kinetic studies of a representative siderophore-associated flavin monooxygenase. *The Journal of Biological Chemistry* 285:30375-30388 doi:10.1074/jbc.m110.157578

Mei B, Budde AD, Leong SA (1993) sid1, a gene initiating siderophore biosynthesis in *Ustilago maydis*: molecular characterization, regulation by iron, and role in phytopathogenicity.

- Proceedings of the National Academy of Sciences of the United States of America 90:903-907 doi:10.1073/pnas.90.3.903
- Meneely KM, Barr EW, Bollinger JM, Jr., Lamb AL (2009) Kinetic mechanism of ornithine hydroxylase (PvdA) from *Pseudomonas aeruginosa*: substrate triggering of O₂ addition but not flavin reduction. *Biochemistry* 48:4371-4376 doi:10.1021/bi900442z
- Meneely KM, Lamb AL (2007) Biochemical characterization of an FAD-dependent monooxygenase, the ornithine hydroxylase from *Pseudomonas aeruginosa*, suggests a novel reaction mechanism. *Biochemistry* 46:11930-11937 doi:10.1021/bi700932q
- Meyer JM, Hohnadel D, Hallé F (1989) Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. *Journal of General Microbiology* 135:1479-1487
- Meyer JM, Neely A, Stintzi A, Georges C, Holder IA (1996) Pyoverdinin is essential for virulence of *Pseudomonas aeruginosa*. *Infection and Immunity* 64:518-523
- Mihovilovic MD, Müller B, Stanetty P (2002) Monooxygenase-mediated Baeyer-Villiger oxidations. *European Journal of Organic Chemistry* 2002:3711-3730 doi:10.1002/1099-0690(200211)2002:22<3711:AID-EJOC3711>3.0.CO;2-5
- Mihovilovic MD, Rudroff F, Winninger A, Schneider T, Schulz F, Reetz MT (2006) Microbial Baeyer-Villiger oxidation: stereopreference and substrate acceptance of cyclohexanone monooxygenase mutants prepared by directed evolution. *Organic Letters* 8:1221-1224 doi:10.1021/ol0601040
- Miyamoto M, Matsumoto J, Iwaya T, Itagaki E (1995) Bacterial steroid monooxygenase catalyzing the Baeyer-Villiger oxidation of C₂₁-ketosteroids from *Rhodococcus rhodochrous*: the isolation and characterization. *Biochimica et Biophysica Acta (BBA)* 1251:115-124 doi:10.1016/0167-4838(95)00090-H

- Neilands JB (1952) A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*)¹. Journal of the American Chemical Society 74:4846-4847 doi:10.1021/ja01139a033
- Neumann CS, Jiang W, Heemstra JR, Gontang EA, Kolter R, Walsh CT (2012) Biosynthesis of piperazic acid via N5-hydroxy-ornithine in *Kutzneria* spp. 744. ChemBioChem 13:972-976 doi:10.1002/cbic.201200054
- Oelschlägel M, Heiland C, Schlömann M, Tischler D (2015) Production of a recombinant membrane protein in an *Escherichia coli* strain for the whole cell biosynthesis of phenylacetic acids. Biotechnology Reports 7:38-43 doi:http://dx.doi.org/10.1016/j.btre.2015.05.002
- Olucha J, Meneely KM, Chilton AS, Lamb AL (2011) Two structures of an N-hydroxylating flavoprotein monooxygenase: ornithine hydroxylase from *Pseudomonas aeruginosa*. The Journal of Biological Chemistry 286:31789-31798 doi:10.1074/jbc.m111.265876
- O'Sullivan DJ, O'Gara F (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews 56:662-676
- Pohlmann V, Marahiel MA (2008) Delta-amino group hydroxylation of L-ornithine during coelichelin biosynthesis. Organic and Biomolecular Chemistry 6:1843-1848 doi:10.1039/b801016a
- Prado L, Fernandez E, Weissbach U, Blanco G, Quiros LM, Brana AF, Mendez C, Rohr J, Salas JA (1999) Oxidative cleavage of premithramycin B is one of the last steps in the biosynthesis of the antitumor drug mithramycin. Chemistry and Biology 6:19-30
- Qi J, Kizjakina K, Robinson R, Tolani K, Sobrado P (2012) A fluorescence polarization binding assay to identify inhibitors of flavin-dependent monooxygenases. Analytical Biochemistry 425:80-87 doi:10.1016/j.ab.2012.03.002

- Quadri LE, Sello J, Keating TA, Weinreb PH, Walsh CT (1998) Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chemistry and Biology* 5:631-645
- Riebel A, de Gonzalo G, Fraaije MW (2013) Expanding the biocatalytic toolbox of flavoprotein monooxygenases from *Rhodococcus jostii* RHA1. *Journal of Molecular Catalysis B: Enzymatic* 88:20-25 doi:<http://dx.doi.org/10.1016/j.molcatb.2012.11.009>
- Riebel A, Fink MJ, Mihovilovic MD, Fraaije MW (2014) Type II flavin-containing monooxygenases: a new class of biocatalysts that harbors Baeyer-Villiger monooxygenases with a relaxed coenzyme specificity. *ChemCatChem* 6:1112-1117 doi:10.1002/cctc.201300550
- Robert X, Gouet P (2014) Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Research* 42:W320-W324 doi:10.1093/nar/gku316
- Robinson R, Sobrado P (2011) Substrate binding modulates the activity of *Mycobacterium smegmatis* G, a flavin-dependent monooxygenase involved in the biosynthesis of hydroxamate-containing siderophores. *Biochemistry* 50:8489-8496 doi:10.1021/bi200933h
- Robinson RM, Rodriguez PJ, Sobrado P (2014) Mechanistic studies on the flavin-dependent N⁶-lysine monooxygenase MbsG reveal an unusual control for catalysis. *Archives of Biochemistry and Biophysics* 550-551:58-66 doi:10.1016/j.abb.2014.04.006
- Robinson RM, Sobrado P (2013) Flavin-dependent monooxygenases in siderophore biosynthesis. In: *Handbook of flavoproteins volume 2 complex flavoproteins, dehydrogenases and physical methods* De Gruyter Berlin (Hille R, Miller S, Palfey B Eds) pp 29-50

- Romero E, Fedkenheuer M, Chocklett SW, Qi J, Oppenheimer M, Sobrado P (2012) Dual role of NADP(H) in the reaction of a flavin dependent N-hydroxylating monooxygenase. *Biochimica et Biophysica Acta (BBA)* 1824:850-857 doi:http://dx.doi.org/10.1016/j.bbapap.2012.03.004
- Sambrook J, Russell DW (2001) *Molecular cloning. A laboratory manual*. 3rd edn. Cold Spring Harbor NY Cold Spring Harbor Laboratory Press
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* 160:47-56
- Secundo F, Fiala S, Fraaije MW, de Gonzalo G, Meli M, Zambianchi F, Ottolina G (2011) Effects of water miscible organic solvents on the activity and conformation of the Baeyer-Villiger monooxygenases from *Thermobifida fusca* and *Acinetobacter calcoaceticus*: a comparative study. *Biotechnology and Bioengineering* 108:491-499 doi:10.1002/bit.22963
- Seth O, Smau L, Welte W, Ghisla S, Stehr M, Diekmann H, Macheroux P (1998) A reply to Dick et al. *Trends in Biochemical Sciences* 23:414-415 doi:10.1016/S0968-0004(98)01306-1
- Setser JW, Heemstra JR, Walsh CT, Drennan CL (2014) Crystallographic evidence of drastic conformational changes in the active site of a flavin-dependent N-hydroxylase. *Biochemistry* 53:6063-6077 doi:10.1021/bi500655q
- Sharma A, Johri BN (2003) Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. *Microbiological Research* 158:243-248 doi:10.1078/0944-5013-00197
- Smits TH, Duffy B (2011) Genomics of iron acquisition in the plant pathogen *Erwinia amylovora*: insights in the biosynthetic pathway of the siderophore desferrioxamine E. *Archives of Microbiology* 193:693-699 doi:10.1007/s00203-011-0739-0

- Smits THM, Rezzonico F, Kamber T, Blom J, Goesmann A, Frey JE, Duffy B (2010) Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Molecular Plant Microbe Interactions* 23:384-393 doi:10.1094/mpmi-23-4-0384
- Sokol PA, Darling P, Woods DE, Mahenthiralingam E, Kooi C (1999) Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of pvdA, the gene encoding L-ornithine N(5)-oxygenase. *Infection and Immunity* 67:4443-4455
- Stehr M, Diekmann H, Smau L, Seth O, Ghisla S, Singh M, Macheroux P (1998) A hydrophobic sequence motif common to N-hydroxylating enzymes. *Trends in Biochemical Sciences* 23:56-57 doi:10.1016/S0968-0004(97)01166-3
- Stehr M, Smau L, Singh M, Seth O, Macheroux P, Ghisla S, Diekmann H (1999) Studies with lysine N6-hydroxylase. Effect of a mutation in the assumed FAD binding site on coenzyme affinities and on lysine hydroxylating activity. *Biological Chemistry* 380:47-54 doi:10.1515/bc.1999.006
- Stephan H, Freund S, Beck W, Jung G, Meyer JM, Winkelmann G (1993) Ornibactins- a new family of siderophores from *Pseudomonas*. *Biometals* 6:93-100
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30:2725-2729 doi:10.1093/molbev/mst197
- Tanaka Y, Omura S (1993) Agroactive compounds of microbial origin. *Annual Review of Microbiology* 47:57-87 doi:10.1146/annurev.mi.47.100193.000421
- Thariath A, Socha D, Valvano MA, Viswanatha T (1993) Construction and biochemical characterization of recombinant cytoplasmic forms of the IucD protein (lysine:N6-

- hydroxylase) encoded by the pColV-K30 aerobactin gene cluster. *Journal of Bacteriology* 175:589-596
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673-4680
- Torres Pazmiño DE, Dudek HM, Fraaije MW (2010) Baeyer-Villiger monooxygenases: recent advances and future challenges. *Current Opinion in Chemical Biology* 14:138-144 doi:<http://dx.doi.org/10.1016/j.cbpa.2009.11.017>
- Torres Pazmiño DE, Riebel A, de Lange J, Rudroff F, Mihovilovic MD, Fraaije MW (2009) Efficient biooxidations catalyzed by a new generation of self-sufficient Baeyer-Villiger monooxygenases. *ChemBioChem* 10:2595-2598 doi:10.1002/cbic.200900480
- Torres Pazmiño DE, Snajdrova R, Baas BJ, Ghobrial M, Mihovilovic MD, Fraaije MW (2008) *Angewandte Chemie International Edition* 47:2275
- Tsuzuki K, Yan FS, Otoguro K, Omura S (1991) Synthesis and nematocidal activities of jietacin A and its analogs. *Journal of Antibiotics* 44:774-784
- Turfitt GE (1948) The microbiological degradation of steroids: fission of the steroid molecule. *Biochemical Journal* 42:376-383
- van Beilen JB, Mourlane F, Seeger MA, Kovac J, Li Z, Smits THM, Fritsche U, Witholt B (2003) Cloning of Baeyer-Villiger monooxygenases from *Comamonas*, *Xanthobacter* and *Rhodococcus* using polymerase chain reaction with highly degenerate primers. *Environmental Microbiology* 5:174-182

- van Berkel WJ, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *Journal of Biotechnology* 124:670-689 doi:10.1016/j.jbiotec.2006.03.044
- Vandamme EJ (1994) The search for novel microbial fine chemicals, agrochemicals and biopharmaceuticals. *Journal of Biotechnology* 37:89-108
- Vannelli TA, Dykman A, Ortiz de Montellano PR (2002) The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase. *The Journal of Biological Chemistry* 277:12824-12829 doi:10.1074/jbc.M110751200
- Visca P, Ciervo A, Orsi N (1994) Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdine biosynthetic enzyme L-ornithine N5-oxygenase in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 176:1128-1140
- Visca P, Serino L, Orsi N (1992) Isolation and characterization of *Pseudomonas aeruginosa* mutants blocked in the synthesis of pyoverdine. *Journal of Bacteriology* 174:5727-5731
- Watanabe H, Hatakeyama M, Sakurai H, Uchimiya H, Sato T (2008) Isolation of industrial strains of *Aspergillus oryzae* lacking ferrichrysin by disruption of the *dffA* gene. *Journal of Bioscience and Bioengineering* 106:488-492 doi:http://dx.doi.org/10.1263/jbb.106.488
- Wei J, Goldberg MB, Burland V, Venkatesan MM, Deng W, Fournier G, Mayhew GF, Plunkett G, Rose DJ, Darling A, Mau B, Perna NT, Payne SM, Runyen-Janecky LJ, Zhou S, Schwartz DC, Blattner FR (2003) Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a Strain 2457T. *Infection and Immunity* 71:2775-2786. doi:10.1128/iai.71.5.2775-2786.2003
- Winkelmann G (2003) Siderophore transport in fungi. In: *Microbial transport systems* Wiley-VCH Weinheim Cambridge Winkelmann G (ed) pp 463-480

- Winsor GL, van Rossum T, Lo R, Khaira B, Whiteside MD, Hancock REW, Brinkman FSL (2009) Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Research* 37:483-488 doi:10.1093/nar/gkn861
- Yamada O, Nan SNA, Akao T, Tominaga M, Watanabe H, Satoh T, Enei H, Akita O (2003) dffA gene from *Aspergillus oryzae* encodes L-ornithine N5-oxygenase and is indispensable for deferriferrichrysin biosynthesis. *Journal of Bioscience and Bioengineering* 95:82-88 doi:10.1263/jbb.95.82
- Yamanaka K, Oikawa H, Ogawa H-o, Hosono K, Shinmachi F, Takano H, Sakuda S, Beppu T, Ueda K (2005) Desferrioxamine E produced by *Streptomyces griseus* stimulates growth and development of *Streptomyces tanashiensis*. *Microbiology* 151:2899-2905 doi:10.1099/mic.0.28139-0